Jihočeská univerzita v Českých Budějovicích

Přírodovědecká fakulta

Habilitační práce

Stories of two life stages: reproduction modes, genome size, diversity and interactions among gametophytes and among sporophytes in ferns

Příběhy dvou životních fází: způsoby rozmnožování, velikost genomu, diverzita a interakce mezi gametofyty a mezi sporofyty u kapradin

RNDr. Libor Ekrt, Ph.D.

České Budějovice 2023

Acknowledgements

First of all, my thanks go to all my students who have contributed with their theses to the research on ferns and to the topics that I present in this habilitation thesis. Their partial investigations of reproduction, genome size have deepened the knowledge of the diversity and mechanisms of different parts of the life cycle of this group of vascular plants. The biggest thanks go to Ondřej Hornych, my first PhD student, with whom we started more detailed research on fern gametophytes and whose approaches inspired me in many ways and pushed me further.

Overall, I would also like to thank my colleagues from the Department of Botany, Faculty of Science, for their collegial and friendly approach to my research on ferns, for the financial support from the department's sources, thanks to which I was able to collect important fern specimens not only in Central Europe, and for the purchase of a key cultivation box, thanks to which I was able to study fern gametophytes in detail. A major boost to my fern research was also the award of GAČR grant 19-17379S, for which I am very grateful to the grant agency.

I would also like to thank my wife for her support, who suggested that the many ferns grown in our home garden are nice after all. To my three children, Sára, Tobík and Markéta, I thank them for having a lot of patience with me when I was sometimes unable to play games with them because of my fern research, and I also apologize to them for focusing my attention not only on them but also on my time-consuming fern research.

Content

Appendix 1: Papers included to the habilitation

Paper 1: Hornych O. & Ekrt L. (2017): Spore abortion index (SAI) as a perspective tool of evaluation of spore fitness in ferns: An insight into sexual and apomictic species. – Plant Systematics and Evolution 303(4): 497–507.

Paper 2: Ekrt L. & Koutecký P. (2016): Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (Dryopteridaceae). – Annals of Botany 117: 97–106.

Paper 3: Hornych O., Férová A., Hori K., Košnar J. & Ekrt L. (2022): Apomictic fern fathers: An experimental approach to the reproductive characteristics of sexual, apomict and hybrid fern gametophytes. – American Journal of Botany 109(4): 628–644.

Paper 4: Hornych O., Černochová L., Lisner A., Ekrt L. (2022): An experimental assessment of competitive interactions between sexual and apomictic fern gametophytes using Easy Leaf Area. – Applications in Plant Sciences 10: e11466.

Paper 5: Hornych O., Testo W., Sessa E., Watkins J., Campany C., Pittermann J. & Ekrt L. (2021): Insights into the evolutionary history and widespread occurrence of antheridiogen systems in ferns. – New Phytologist 229: 607–619.

Paper 6: Hornych O., Černochová L., Košnar J. & Ekrt L. (2022): Biotic interactions between the gametophytes of wall rue (*Asplenium ruta-muraria*) and other fern species. – International Journal of Plant Sciences 183(1): 10–17.

Paper 7: Liu H-M., Ekrt L., Koutecký P., Pellicer J., Hidalgo O., Marquardt J., Pustahija F., Ebihara A., Siljak-Yakovlev S., Gibby M., Leitch I. & Schneider H. (2019): Polyploidy does not control all: lineage-specific average chromosome length constraints genome size evolution in ferns. – Journal of Systematics and Evolution 57: 418–430.

Paper 8: Ekrt L., Holubová R., Trávníček P. & Suda J. (2010): Species boundaries and frequency of hybridization in the *Dryopteris carthusiana* (Dryopteridaceae) complex: A taxonomic puzzle resolved using genome size data. – American Journal of Botany 97: 1208–1219.

Paper 9: Kaplan Z., Danihelka J., Štěpánková J., Ekrt L., Chrtek J. Jr., Zázvorka J., Grulich V., Řepka R., Prančl J., Ducháček M., Kúr P., Šumberová K. & Brůna J. (2016): Distributions of vascular plants in the Czech Republic. Part 2. – Preslia 88: 229–322.

Paper 10: Hornych O., Ekrt L., Riedel F., Koutecký P. & Košnar J. (2019): Asymmetric hybridization in the Central European populations of the *Dryopteris carthusiana* group. – American Journal of Botany 106(11): 1477–1486.

Paper 11: Ekrt L., Podroužek J., Hornych O., Košnar J. & Koutecký P. (2021): Cytotypes of bracken (*Pteridium aquilinum*) in Europe: widespread diploids and scattered triploids of likely multiple origin. – Flora 274: 151725.

Paper 12: Hanušová K., Čertner M., Urfus T., Koutecký P., Košnar J., Rothfels C. J, Jarolímová V., Ptáček J., Ekrt L. (2019): Widespread co-occurrence of multiple ploidy levels in fragile ferns (*Cystopteris fragilis* complex; Cystopteridaceae) likely stems from similar ecology of cytotypes, their efficient dispersal and inter-ploidy hybridization. – Annals of Botany 123: 845–855.

Paper 13: Ekrt L., Košnar J., Rothfels C., Hanušová K., Hornych O. & Urfus T. (2022). Cytogenetic, geographical, spore type and plastid haplotype data reveal cryptic patterns of species diversity in the cosmopolitan *Cystopteris fragilis* complex (Polypodiopsida: Cystopteridaceae). – Botanical Journal of Linnean Society 199: 728–739.

Appendix 2: Professional Curriculum vitae

Annotation

Ferns are primitive spore-bearing plants, which alongside lycophytes, represent the oldest lineage of vascular plants on Earth. They are the sister group to seed plants and currently the second most diversified group of vascular plants. Ferns used the diversification waves of angiosperms during late Cretaceous that formed terrestrial ecosystems on Earth to diversify themselves. In contrast to other land plants, the life cycle of ferns (and lycophytes) is unique. It requires two completely spatially and nutritionally independent generations of plants to complete itself. The leafy diploid sporophyte stage (frond) is bearing sporangia and the sexual haploid gametophyte stage, usually resembling a heart-shaped plantlet (prothallus). The alternation of those two generations greatly influences these plants. Ferns are the subject of this habilitation thesis. The research of this group focused on many aspects of their biology such as modes of reproduction, genome size, diversity, with special interest in interactions among gametophytes and among sporophytes. The thesis presents several thematic storylines. The first story focuses on the evaluation of fern spores formed by sexual, apomictic species and their hybrids (apo-sex hybrids). The special detailed view on the reproductive mechanisms of apo-sex hybrids surprisingly showed us evidence of both apomictic (unreduced spore and apogamous sporophyte formation) and sexual (regular spore formation) reproduction strategies present in one taxon. Furthermore, until now, it was believed, that apogamous sporophytes are generally considered to form earlier than sporophytes originating from the sexual process. Our comprehensive study proved that the apomictic reproduction was not necessarily leading to an earlier sporophyte formation and that the apomictic gametophytes are smaller in size than the sexual species. The apo-sex hybrids also tend to behave more like their apomictic parents but suffer from an early disadvantage in the form of lower spore germination rates. The second story explores the evolutionary significance of fern pheromones called antheridiogens. It was found that a pheromone system is widespread among ferns with several recognized different types. Surprisingly, apomictic species also respond to the pheromone system, despite its original function being the regulation of sexual reproduction. In addition to pheromones, ferns appear to produce exudates that may have suppressive or facilitative effects on younger gametophytes of various fern species. The final storyline reveals the many biosystematic adventures resulting from the distribution and interaction among sporophytes discovered using innovative methods in flow-cytometry. Through the application of genome size measurements, we were able to reveal genome multiplication, hybridization and evolution in target groups. This approach has been used to study aspects ranging from genome size evolution (*Asplenium*), species delimitation, population cytotype screening, detection of hybrids (*Dryopteris*, *Pteridium*) to large-scale cytogeographical studies (*Cystopteris*).

1 Introduction

1.1 Ferns – phylogeny, age and position in a plant tree of life

Ferns are a very diverse plant group growing in many habitats (e.g., terrestrial, arborescent, aquatic, epiphytic, epilithic), and, alongside lycophytes, represent the oldest lineages of vascular plants on Earth. Recent morphological and molecular phylogenetic analyses indicate that the extant vascular plant lineages have a basal dichotomy separating the lycophytes from euphyllophytes – representing ferns and seed plants. Therefore, ferns (referred to as monilophytes) are the sister group of seed plants (Kenrick & Crane 1997, Pryer et al. 2001, 2004). Fossil records date ferns back to the middle Devonian within 383–393 million years ago (mya) (Taylor et al. 2009). Recent divergence time estimates suggest they may be even older, possibly having first evolved as far back as 430 mya (Testo & Sundue 2016). However, despite the considerable age of the group as a whole, most of the earliest ferns have since gone extinct. The majority of living ferns arose from a much later diversification event occurring as recently as ca 70 mya. Molecular data and a reassessment of the fossil records show that polypod ferns (Polypodiales representing more than 80% of living fern species) diversified in the Cretaceous, after angiosperms. The diversification of polypod ferns was apparently caused by ecological responses to the previous diversification waves of angiosperms that formed terrestrial ecosystems on Earth (Schneider et al. 2004). Simply put, recent ferns (monilophytes) diversified in the shadow of angiosperms.

The extant ferns comprise four extant lineages: horsetails, a group composed of whisk ferns and ophioglossoid ferns, marattioid ferns and the most modern leptosporangiate ferns (Barker & Wolf 2010). Today, the group of leptosporangiate ferns with around 9000 living species is the second most diverse group of vascular plants on Earth, outnumbered only by angiosperms (Schuettpelz & Pryer 2007, PPG I 2016).

1.2 The fern life cycle as unique system of two independent life stages

In contrast to other land plants (mosses, seed plants) the life cycle of ferns and lycophytes is unique. It requires two completely spatially vicarious and nutritionally independent generations of plants to complete itself. The leafy stage (frond) bearing sporangia represents the diploid sporophyte and sexual generation, usually resembling a heart-shaped plantlet (prothallus), represents the haploid gametophyte.

In most ferns, the diploid frond (sporophyte) produces haploid spores via meiosis. Spore formation (sporogenesis) occurs in sporangia, usually on the underside of the frond. Each haploid spore grows into a tiny aboveground photosynthetic prothallus (all leptosporangiate ferns, several groups of eusporangiate ferns) or a underground heterotrophic prothallus (lycophytes, whisk ferns, ophioglossopsids). Each haploid prothallus can produce male gametes (spermatozoids) in antheridia and female gametes (eggs) in archegonia. Both free-swimming spermatozoids and sessile eggs may be produced on the same plantlet, possibly leading to self-fertilization. Such event is likely rare in nature, possibly due to the resultant complete homozygosity (Soltis & Soltis 1992, Sessa et al. 2016).

Fern sex can change over time and is environmentally determined, influenced by factors such as pheromones, nutrients, temperature, etc. A particularly interesting sex determinant is a

pheromone called antheridiogen. The main function of antheridiogen is to promote a precocious formation of antheridia, and, to an extent, enforce male unisexuality in younger gametophytes. This in turn promotes outcrossing by increasing the number of free-swimming spermatozoids around older (female) gametophytes. The young gametophyte retains its sensitivity to antheridiogens only until the onset of the meristic phase (the phase associated with archegonia formation) after which the gametophyte may be unable to form antheridia (Döpp 1950, Raghavan 1989). When water is present, sperm use their flagella to swim alongside a pheromonal trail to an egg and fertilize it. The fertilized egg is a diploid zygote formed by the combination of chromosome sets from the egg and sperm. The zygote grows via mitosis into the diploid sporophyte, completing the life cycle (Raghavan 1989).

1.3 Mechanisms of diversification and speciation in ferns

Three different speciation modes are recognized in extant ferns and lycophytes – primary, secondary and tertiary (Ranker & Haufler 2008).

The establishment of isolated populations, by means of rising geological barriers or dispersal events, followed by population evolutionary processes (drift, inbreeding, gene flow, metapopulation concepts etc.) is considered as primary speciation and is us a common pattern of diversification in ferns (Kato 1993). These diversification trends are similar to the vast majority of other organisms.

In ferns, as in the majority of land plants, whole genome duplication (polyploidization) is a major speciation force (Lavania 2020, Van de Peer et al. 2021). This phenomenon is usually connected with hybridization and sometimes called secondary speciation (Ranker & Haufler 2008).

Within land plants, polyploidization appears to be low or absent in liverworts, hornworts, cycads, and conifers, but is remarkably frequent in lycophytes, ferns (monilophytes), and angiosperms. Wood et al. (2009) estimated that of speciation events, 15% for flowering plants and 31% for ferns are associated with polyploidy. In ferns, systematic research of polyploidization was started by Manton (1950), who first reported on the intricacy of fern polyploid groups.

There are three types of genome multiplication rearrangement in ferns (autopolyploidy, allohomoploidy, allopolyploidy) (Ranker & Haufler 2008). Autopolyploidy involves the multiplication of one basic set of chromosomes. Autopolyploidy is in many ferns often associated with distinct reproductive strategies and increases the likelihood of reproductive barriers occuring, thus creating the potential for the development of spatial structure (Lovis 1964, Trewick et al. 2002). In some fern groups, it may serve as a frequent, rapid and important speciation mechanism, especially when accompanied by apomixis (Windham & Yatskievych 2003). During autopolyploidy, there is no mismatch between genetically different chromosome sets , so offspring fertility is high (Soltis et al. 2014, Jighly et al. 2018).

A likely infrequent and poorly studied mechanism of secondary speciation is allohomoploidy (Conant & Cooper-Driver 1980, Sigel 2016). This process comes up in diploid species which may be ecologically isolated, but not always reproductively isolated. Hybrid swarms may arise. Examples of allohomoploidy were found in the genus *Polystichum* in western North America

(Mullenniex et al. 1999) and tree ferns from the family Cyatheaceae (Conant & Cooper-Driver 1980).

The most widely studied speciation process in ferns is likely allopolyploidy. In this case, progenitors of hybrid-derived species are not interfertile and form sterile offspring when they cross (Manton 1950). Pteridophytes are generally prone to the formation of allopolyploid complexes (Haufler 2002, Fujiwara & Watano 2020).

The origin and persistence of polyploid ferns is enhanced by the empty niche space and greater tolerance to disturbances of polyploid ferns related to larger spore size is likely a selective advantage on islands, consistent with the number of high polyploids encountered among ferns on oceanic islands. Though recently originated polyploid ferns usually have identifiable progenitors with which they are partially sympatric, the rate of expansion of these ferns beyond the range of their progenitors varies (Barrington 2020).

Allopolyploidy is preceded by hybridization – the process of crossbreeding between genetically dissimilar parents to produce a hybrid (see below). It seems that barriers to development of hybrid zygotes are weak and field studies have demonstrated high frequency of vigorous but sterile hybrids in some complexes (Reichstein 1981, Petit et al. 1999). Primary interspecific hybrids are usually sterile due to unbalanced meiosis: pairing chromosomes (each coming from distinctly different genome) are not actually homologous, resulting in aborted spores. The polyploidization event restores the possibility of homologous chromosome pairing and proper spore formation. Possessing redundant copies of genes, gametophytes of polyploid species are more tolerant to selfing than their diploid progenitors. Thus, viable sporophytes may develop. However, even though allopolyploidy is generally accepted as a frequent mode of speciation in ferns, there are still many open questions about the so-called species complexes that involve allopolyploids (Ranker & Haufler 2008). Relatively high basal chromosome numbers and large genome sizes of extant ferns have probably been derived from lower ones by ancient (paleo)polyploidy (Walker 1979). Therefore, species recently representing diploids are likely these ancient polyploids whose ancestors became extinct. Although it was initially thought that paleopolyploidy is restricted to ferns only, it has recently been recognized in numerous families of angiosperms as well (Soltis et al. 2009).

Tertiary speciation, in other words polyploid genome reorganization is a major discovery of the past two decades. It results in a rapid reorganization of fern polyploid genomes (Soltis et al. 2003). This process could occur in genetically isolated populations whose separation is maintained by reciprocal gene silencing (Werth & Windham 1991). If the genome is highly polyploid, there can be a risks of aneuploidy caused by changes in cell structure and mistakes during meiosis (Comai 2005). During the following gene silencing, parts of the genome can be inactivated, even active alleles (Martínez de Alba et al. 2013).

1.4 Hybridization – interaction between the species

Despite the fact that a single gametophyte can form male antheridia and female archegonia at the same time and is capable of self-fertilization, most ferns seem to prefer outcrossing. In sexual fern species, if two different species meet, they are very often capable of crossing – hybridization. Fern hybrids (that arose from hybridization of sexual species) are mostly incapable of creating of functional spores (Wagner & Chen 1965).

The phenomenon of hybridization is well-known in ferns, new hybrids are constantly being described (Barrington et al. 1989, Sigel 2016). Many more species are allopolyploids, i.e., hybrids, that restored their fertility by genome duplication. Hybridization has profound positive and negative consequences for fern evolution.

In ferns, researchers presume that hybridization is frequent and the hybridization barriers are weak (Barrington et al. 1989, Sigel 2016). But, with a few exceptions (Testo et al. 2015), the nature of these barriers was not properly studied. Furthermore, there was no research quantitatively measuring the frequency of hybridization, hybridization rates, in natural populations. The much more complicated hybridization system could arise, when one species involved in hybridization is apomictic. The hybridization between fern sexuals and apomictic of different ploidy is one story discussed in this habilitation.

1.5 When it takes one to tango: aspects and consequences of apomixis in ferns

Apomixis in ferns is broadly defined simply as asexual reproduction (apo=without, mixis = mixing/sex). Apomixis encompasses both vegetative reproduction and asexual reproduction through the alternation of generations, including apospory, apogamy, and parthenogenesis (Grusz 2016, Albertini et al. 2019). However, apomixis is mostly used as a term describing an alteration of the typical fern sexual life cycle by a combination of two processes, diplospory (formation of diploid spores) and apogamy (formation of sporophytes without the merging of gametes). Apomictic ferns, by contrast to sexual ones, follow one of two alternative spore-generating pathways to yield chromosomally unreduced diplospores: either premeiotic endomitosis – PE; formerly known as Döpp-Manton sporogenesis (Döpp 1932, Manton 1950, Manton & Walker 1954) or meiotic first division restitution – MFDR; formerly known as Braithwaite sporogenesis (Braithwaite 1964). These diplospores, each having one full chromosome complement, then germinate. The resulting prothalli are capable of generating new sporophytes from somatic cells, which are usually located near the spot archegonia would be produced by sexually reproducing ferns. Rarely archegonia are observed in apomicts as well, but they are likely always abortive. In contrast, apomictic gametophytes have been known to make functional antheridia, but the viability of spermatozoids contained therein is largely dependent upon the pathway to diplospory undertaken in the parent, only PE yields viable spermatozoids. These processes are described in more details by Raghavan (1989) or Grusz (2016).

In ferns, apomixis has mostly been confined to the largest leptosporangiate families Pteridaceae, Dryopteridaceae, Polypodiaceae and Aspleniaceae (Chao et al. 2010, Liu et al. 2012, Dyer et al. 2012, Guo & Liu 2013). It is estimated that about 10% of ferns are without capability of sexual reproduction (Walker 1979), but, apomixis was confirmed in about 3% of extant fern species (Liu et al. 2012). This is rather high number compared to less than 1% of angiosperms (Whitton et al. 2008).

2 Context of habilitation

2.1 The adventurous stories of fern spores and their intricate journey towards forming a green thalloid gametophyte

Fern spores are reproductive propagules capable of long-distance dispersal. Except for the small number of heterosporous species represented by water ferns, homosporous ferns usually reproduce by these tiny oval propagules tens of micrometers in size. Ferns produce a large number of spores ranging from several millions to hundreds of millions every year of its seasonal fertility (Moran 2004). Even homosporous ferns tend to vary in spore size, mainly because of polyploidy. Polyploid species tend to have larger spores compared to their diploid relatives (Barrington et al. 1989, Quintanilla & Escudero 2006, Barrington 2020). Spores are usually typical in their color and this also plays a crucial role in biology, distribution, germination and ecology of fern species. The majority of ferns have non-green (achlorophyllous), usually of brown or yellow spore color. Those spores usually germinate after a longer time (several weeks) and are much more resistant to environmental hazards. The taxa with green (chlorophyllous) spores are in a minority but this trait evolved multiple times and is spread throughout the fern phylogeny tree (Sundue et al. 2011). The presence of chlorophyll in the spore indicates its active metabolism and fundamentally changes spore characteristics. Green spores germinate within a couple of days and tend to result in faster growing gametophytes but have short life spans and die after average 48 days, compared to the tens of years achlorophyllous spores may remain viable for (Lloyd & Klekowski 1970). Sundue et al. (2011), first described so-called cryptochlorophyllous spores with olive color, both green and brown. They are protected by well-developed perispores but carry chlorophyll inside. The division of spore color should probably be viewed as a gradient rather than a dichotomy.

The shape of spores is mostly formed by the type of sporogenesis. As spores are the product of meiosis, four are produced at a time in a tetrad. Based on how the spores are oriented during sporogenesis in the tetrad, a scar is created at the point of contact of all four sister spores. The scar is either trilete (Y shaped), leading to a tetrahedral spore, or monolete (I shaped), resulting in an oval spore. The character of scar shape is highly conservative and rarely varies within family (Tryon & Lugardon, 1991).

Spores may also be of spherical shape with indistinct scar. This is caused by either 4 incomplete meiosis or cytokinesis cycles leading to polyploid (diplo)spores (Morzenti 1962). These aberrant spores can germinate and may serve as an important driver in the evolution of polyploidy in ferns.

Meiosis, through which spores generally form, is an important check point of genetic integrity as chromosomes have to form pairs. In contrast to angiosperms, in which some hybrids may successfully sexually reproduce (Rieseberg & Carney 1998), fern hybrids are unable to properly pair chromosomes during spore formation and their spores are mostly inviable, aborted. Aborted spores vary greatly in size, and are often very dark, shriveled, with a collapsed exospore. This may be very practical for researchers using aborted spores as an identification character (Wagner & Chen 1965). Fern hybrids are sterile and potentially reproductive dead-ends. Apo-sex hybrids (one parent apomictic, one sexual) are a peculiar exception to hybrid sterility. These apo-sex hybrids are generally considered to form 80–95% spores aborted

(Fraser-Jenkins 2007; **Paper 1, Paper 2**). A comprehensive comparison of sexual and apomictic taxa using extensive spore fitness data has been published (**Paper 1**). Based on a representative data set of 109 plants from 23 fern taxa, we accomplished the first robust analysis of spore fitness by examining spore abortion, calculating the ratio of aborted to all examined spores. We compared this trait for different fern reproductive types (sexual/apomicts/hybrids) and ploidy levels (diploid versus polyploid). The results confirmed the general assumption that shows higher spore abortion for apomictic taxa (18%) when compared to sexual taxa (3%). Furthermore, hybrids are characterized by having almost all spores aborted (99.8%) with the rare exception of an apo-sex hybrids. We found no significant difference in spore abortion between sexual taxa of various ploidy levels or between sexual taxa of target genera *Dryopteris* and *Asplenium*.

Fern apo-sex hybrids are also capable of producing viable reduced and unreduced spores, a characteristic rare among ferns (Windham 1983, Sigel et al. 2011, **Paper 2**). Like their apomictic parents, fern apo-sex hybrids can produce gametangia, which may be dysfunctional, and apogamously formed sporophytes (Walker 1962, Regalado Gabancho et al. 2010). Although the information is limited, our findings indicate a possible involvement of sexual as well as apomictic reproduction in apo-sex hybrids. A detailed study concerning the apo-sex pentaploid hybrid of *Dryopteris ×critica*, a hybrid of triploid apomictic *D. borreri* and tetraploid sexual *D. filix-mas* (**Paper 2**) was recently published. This partly fertile hybrid (both well-developed and aborted spores are present) surprisingly shows unstable sporogenesis with sexual and apomictic reproduction combined. While a standard number of spores per sporangium in sexual leptosporangiate ferns is 64 and in apomictic 32 (Regalado Gabancho et al. 2010, Dyer et al. 2012), the number of spores in this hybrid varied from ca 31 to 64. Within a single sporangium it was possible to detect the formation of either only aborted spores or various mixtures of aborted and well-developed reduced spores and unreduced diplospores. The spores germinated into viable gametophytes with two ploidy levels: pentaploid (5x, from unreduced spores) and half of that (ca 2.5x, from reduced spores). Moreover, 2–15 % of gametophytes (both 2.5x and 5x) formed a viable sporophyte of the same ploidy level due to apogamy. The existence of reduced viable spores and the occurrence of both types on one plant and even in one sporangium together is unexpected and novel. Moreover, both spore types are capable of viable next-generation sporophyte (F2) production, which has not been observed previously. In conclusion, the pentaploid hybrid is capable of autonomous reproduction (**Paper 2**).

The interaction and possible hybridization between sexual and apomictic are very challenging and still not fully explained topics in general. Our recent study (**Paper 3**) presents the most thorough comparison of gametangial development in sexual and apomictic ferns to date. We cultivated the spores of 43 apomicts, 7 apo-sex hybrids, and 16 sexuals, and measured their development (germination, lateral meristem formation, sexual expression, production of sporophytes) in vitro over 16 weeks. All three examined groups (sexuals / apo-sex hybrids / apomicts) formed antheridia but differed in overall gametophyte development. Sexuals created archegonia (86% of viable samples), but no sporophytes. Apomicts occasionally created nonfunctional archegonia (8%) but usually produced apogamous sporophytes (75%). Until now, it was believed that apogamous sporophytes are generally considered to form earlier than

sporophytes originating from the sexual process (Whittier 1968, Huang et al. 2006, Regalado Gabancho et al. 2010, Haufler et al. 2016). Only a single previous paper indicated the opposite trend (Laird & Sheffield 1986). So, despite expectations, in our study, the apomictic reproduction was not leading to the earlier sporophyte formation (**Paper 3**). Apo-sex hybrids in this study had lower germination rates than their parental species, indicating a genomic imbalance (chromosomal incompatibilities during meiosis) caused by the hybridization event and leading to post-zygotic hybridization barriers. This effect is multiplied by their considerably higher spore abortion rate (**Paper 1, Paper 2**). Thus, of the ca 10% apparently viable spores, about a half fail to germinate. All germinated apo-sex hybrids produced antheridia (archegonia were absent) and abundant apogamous sporophytes. This is supported by other studies (Walker 1962, Regalado Gabancho et al. 2010). Finally, we can say, that apo-sex hybrids tend to behave more like their apomictic parents than the sexual ones but suffer from an early disadvantage in the form of lower germination rates (**Paper 3**).

We also looked at the problem from an alternative viewpoint. The presence of fern gametophytes has been demonstrated to affect the size and sexual expression of other gametophytes in populations based on the resulting gametophyte density and the release of pheromones into the habitat. When sown at high densities, fern gametophytes tend to be small and male, or may even completely lack gametangia, while at lower densities, gametophytes are larger and female or hermaphroditic (Huang et al. 2004, DeSoto et al. 2008). However, at very low densities or in single-spore cultures, gametophyte growth may be retarded or abnormal (Dyer 1979). Very little is known about the interactions between the gametophytes of multiple species at the same stage/age occurring at densities permitting the formation of both types of gametangia. There are several possible outcomes of such interactions. First, the gametophytes in mixed-species populations may grow at the same rate as in monoculture. Second, overyielding may occur, leading to gametophytes of at least one species growing faster in the presence of another species, as has been observed in angiosperm sporophytes (Turnbull et al. 2013, Wright et al. 2017). Third, one or more species may underyield (grow smaller) due to competition for resources or chemical allelopathy (Rünk et al. 2004, Testo & Watkins 2013, Cheng & Cheng 2015). To address this lack of information, we cultivated three fern species of the *Dryopteris filix-mas* complex (Dryopteridaceae). *Dryopteris filix-mas* is a sexually reproducing tetraploid with diploid gametophytes, while *D. affinis* and *D. borreri* are apomictic and gametophytes have the same ploidy level as sporophytes (**Paper 4**). The gametophytes of the three species in our study differed significantly in their growth capabilities, as expressed by their total cover area in monocultures. The diploid gametophytes of the sexual tetraploid *D. filix-mas* grew largest. In comparison, the apomictic gametophytes were smaller, ordered by ploidy level. Interestingly, the apomicts were smaller than the sexual species overall, which seems to go against the generally presumed faster growth of apomictic gametophytes (Walker 1962, Regalado Gabancho et al. 2010) and also support this trend in our previous study (**Paper 3**). In general, the performance of the tested species in mixtures was dependent on the competitor species identity, indicating the importance of competition between gametophytes. Our innovative method based on software Easy Leaf Area, which evaluate the numbers of red and green pixels that are automatically compared to calculate the green area in a defined region,

can be used for a rapid assessment of fern gametophyte cover in large gametophyte populations (**Paper 4**).

It is well known that most ferns seem to use special pheromones – antheridiogens (Schneller 2008). There is an ongoing discussion about whether antheridiogens primarily slow down growth, which prompts antheridial formation, or vice versa (Näf, 1956; Korpelainen, 1994; Quintanilla et al., 2007). Nevertheless, antheridiogens mediate interactions between gametophytes of different developmental stages or ages. But this is another story presented in the next chapter.

2.2 The secret life of fern gametophytes: An exploration of fern pheromones and their evolutionary significance

Gametophytes of homosporous ferns can be bisexual and are theoretically capable of gametophytic selfing, the fusion of two gametes originating from a single gametophyte via mitosis (Haufler et al. 2016). As the gametophyte grows from a spore originating via a single meiotic event, the sporophyte arising from such event is completely homozygous (Klekowski & Lloyd 1968). Thus, the recent homosporous lineages maintain their genetic diversity by mechanisms that reduce the rate of gametophytic selfing. Fern gametophytes often use a dynamic system controlling sex expression via pheromones called antheridiogens (Schneller 2008). Antheridiogens (AG) were discovered and subsequently described in 1950 during experiments with in vitro gametophyte cultivation (Döpp 1950, Näf et al. 1975). Antheridiogen production and response vary considerably among fern taxa and the system involves complex inter- and intraspecific interactions. Moreover, later studies indicated that various types of AGs occur across fern phylogeny (Schneller et al. 1990). The primary function of AGs is the stimulation of precocious formation of antheridia. When a gametophyte of an AG-responsive species grows in the absence of this pheromone, it first develops archegonia. However, right before the gametophyte reaches the archegoniate phase, it begins exuding AGs into its environment. At the same time, the gametophyte loses the ability to respond to the pheromone. Younger or slow-growing asexual gametophytes in the immediate surroundings of the first gametophyte respond to the AGs by halting growth and forming antheridia (i.e., becoming male). The population ends up composed of a few larger female gametophytes and many smaller male gametophytes (Döpp 1950, Näf et al. 1975, Tryon & Vitale 1977). As fern sperm are flagellated and need to swim through water to reach archegonia, a greater abundance of sperm due to the AG system may help overcome the limitations of dry environments. Likewise, AG leads to a greater number of unisexual gametophytes, therefore limiting self-fertilization and facilitating outcrossing, the exchange of gametes between gametophytes, thus maintaining heterozygosity in fern populations (Schedlbauer & Klekowski 1972). Through the AG system, homosporous ferns gain these advantages, which are usually afforded to heterosporous plants because of their pre-determined sexes and the consequent inability to undergo the extreme form of selfing found in homosporous plants. Additionally, larger gametophytes may be able to pheromonally suppress sporophyte formation in smaller gametophytes, leading to reduced competition. On the other hand, smaller gametophytes may use the system to contribute to the next generation despite being unable to form archegonia or support young sporophytes due to unfavorable conditions (Willson 1981).

Despite the apparently widespread occurrence of AG systems in ferns and their potentially large evolutionary significance via their effects on population structure and mating behavior, our understanding of their evolution and distribution across the fern phylogeny was limited until recently. The meta-analysis of 88 published papers on 208 fern species in total together with new data from cultivation experiments was presented (**Paper 5**). Using this large dataset, we showed that the AG system is widespread among ferns. About two-thirds (64.5%) of all tested species responded positively to AGs. Several AG types are well-established by now, but others still require thorough testing to determine their scope, distinctness, and features. The majority (66.7%) of apomictic species surveyed to date respond to AG. The consequences of this may play an important role in survival and competition among fern gametophytes in nature as well as interactions between apomictic and sexually reproducing taxa. This study also suggests that there is no difference between diploids (67.0%) and polyploids (68.1%) in response to AGs, so the pheromonal system may be advantageous even to species which are predominantly selfers (**Paper 5**).

Apart from the precocious formation of antheridia, AGs also enable spores to germinate in darkness, bypassing the usual requirement of light (Weinberg & Voeller 1969, Haufler & Welling 1994). Such germinated spores grow into small belowground gametophytes with a few antheridia (Schneller et al. 1990). The sperm released from these antheridia then navigate toward open archegonia (Lopez-Smith & Renzaglia 2008) and may fertilize the antheridiogen-releasing gametophytes aboveground. Thus, AGs effectively allow ferns to mine the otherwise dormant spore bank for genetic diversity (Schneller 1988).

There is still a large potential for studying new types of AGs systems and their interactions with biotic or abiotic factors. One of these still-unresolved potential AG types was reported in a small calcicole fern *Asplenium ruta-muraria*. It was initially described as a unique AG system in *A. ruta-muraria* due to the species' lack of response to gibberellins (Schneller 1995), which are similar to Schizaeales-type antheridiogens. Within our study (**Paper 6**) we cultivated the spores of multiple fern species, including representatives of three established antheridiogen types and various related and unrelated members of Aspleniaceae. Specifically, the effect of older meristic gametophytes on the sexual expression of younger gametophytes was observed. Our tested individuals of *A. ruta-muraria* decidedly did not employ any system resembling antheridiogens as observed in Schizaeales and Polypodiales. The exudates of female gametophytes did not preclude the formation of archegonia among younger gametophytes or stimulate germination in darkness. Surprisingly, the exudates of *A. ruta-muraria* may have suppressive or facilitative effects on younger gametophytes of various fern species. The mechanics and properties of these effects have been poorly explored until now and may have evolutionary significance in the subsequent development of sporophytes.

2.3 The story of how fern sporophytes are affected by genome size: delimitation, distribution, diversification and hybridization in the shadow of genome multiplication

As mentioned in the introduction, one of the most important mechanism of plant diversification is genome multiplication – polyploidy (Haufler 2002). The quickest and most powerful tool used to evaluate and determine the plant genome size or estimate the DNA ploidy level is flow-

cytometry (FCM). The evolution of plant genomes is dynamic, encompassing a range of genomic processes including multiple rounds of whole genome duplication (WGD), polyploidization, chromosomal rearrangements and the turnover and evolution of repetitive DNA (Wood et al. 2009). This is mirrored in the tremendous variation in nuclear genome sizes across land plants (ca 11,850-fold). This has crucial implications for flow cytometric applications both with respect to technical issues, e.g., a series of internal standards of different genome size is required (Loureiro et al. 2021, Čertner et al. 2022). In recent years, an increasing degree of attention has focused on the contrasting genomic profiles and evolutionary processes underlying different lineages of land plants, and the consequences of these differences on their diversification and mechanisms driving genome size evolution (Leitch & Leitch 2012, Landis et al. 2018, Fang et al. 2022, Fujiwara et al. 2023).

Although ferns and flowering plants could share some of the pathways involved in diploidizing the genome, they differ in their response to the additional chromosomes and DNA arising from WGD (Clark et al. 2016). The distribution of genome sizes in angiosperms is biased towards small genomes (median = 1.7 pg/1C) but is more normally distributed with medium-sized genomes in ferns (median = 11.4 pg/1C) (Leitch & Leitch 2013, Suda et al. 2015, Pellicer et al. 2018). Ferns are also typically characterized by higher chromosome numbers (mean 2n = 121.0; 2n range = 18–1440) compared to angiosperms (mean 2n = 15.99; 2n range = 4–640) and genome size and chromosome number (2n) are often correlated in ferns but not in angiosperms (Barker 2009, Haufler 2014, Clark et al. 2016). Such differences at the molecular level could also lead to the higher contribution of hybrid speciation to extant fern diversity (Wood et al. 2009, Mayrose et al. 2011, Soltis et al. 2015) and evolutionary potential (Vanneste et al. 2015, Clark et al. 2016) compared to other land plant lineages. This suggests that genome size evolution in ferns is not only shaped by repeated cycles of polyploidy but also by other mechanisms. In our study (**Paper 7**), we aimed to explore whether the evolution of fern genome sizes is not only shaped by chromosome number changes arising from polyploidy but also by constraints on the average amount of DNA per chromosome. To test these relationships, we used 147 accessions belonging to 54 taxa of genus *Asplenium*. This genus is suitable for such analysis because of a highly conserved base chromosome number and a high frequency of polyploidy. We surprisingly revealed that genome size varied substantially between diploid species, resulting in overlapping genome sizes among diploid and tetraploid species of genus *Asplenium*. The observed additive pattern indicates the absence of genome downsizing following polyploidy. The genome size of diploids varied non-randomly and we found evidence for clade-specific trends towards larger or smaller genomes. The 578-fold range of fern genome sizes have arisen not only from repeated cycles of polyploidy but also through clade-specific constraints governing the accumulation and/or elimination of DNA (**Paper 7**).

The application of FCM is very helpful for the development of plant biosystematics. Resolving genome size/DNA ploidy level of many intricate and cryptic groups of plants, including ferns, was a significant step forward (Bureš et al. 2003, Ebihara et al. 2005, Ekrt et al. 2009, Dyer et al. 2013, Henry et al. 2014, Dauphin et al. 2016). We applied flow-cytometry with a combination of multivariate morphometrics to one of the most taxonomically intricate fern group of Central Europe – *Dryopteris carthusiana* group (**Paper 8**). This conspicuous and very common component of temperate woodlands is typical for its overall phenotypic

similarity, great plasticity and the incidence of interspecific hybridization, which has resulted in continuous disputes concerning species circumscription and delimitation. The group consists of one diploid *D. expansa* and two allotetraploids *D. carthusiana* and *D. dilatata*. Our flow cytometric measurements of 858 plants from 85 localities revealed unique genome sizes in all species and hybrids, allowing their easy and reliable identification for subsequent morphometric multivariate analyses. Even the two tetraploids markedly differ in their genome size (21% difference). This study (**Paper 8**) showed that genome size data may help to resolve taxonomic complexities in this important component of the temperate fern flora and that multivariate analyses may resolve species boundaries. This study together with other thorough examinations of the genus *Dryopteris* (Ekrt et al. 2007, 2009, 2010, 2013) led to a chorological compilation of distribution of all *Dryopteris* species in the Czech Republic (**Paper 9**). Based on a thorough revision of 40 public herbarium collection including the largest ones (e.g., BRNM, BRNU, CB, HR, MP, OL, OLM, PR, PRC, ROZ) the distribution pattern of individual species in the Czech Republic was processed. All revised data from herbarium vouchers used for mapping were georeferenced, and entered into the Pladias database (Wild et al. 2019) and geographically sorted according to the traditionally used CEBA (Central European Basic Area) grid template of Niklfeld (1997). During the following years, based on herbarium revisions and a review of literature, the distribution maps of all fern and lycophytes of the Czech Republic were published (Ekrt in Kaplan et al. 2016, 2017a, 2017b, 2018a, 2018b, 2019, 2020).

Going back to **Paper 8**, a very interesting finding resulted from the study. We found a very varied frequency of particular hybrid taxa that depended primarily on the evolutionary relationships, reproductive biology, and co-occurrence of progenitors. However, the dataset was not designed to prove this, and evidence was insufficient. Therefore, a follow-up study (**Paper 10**) was conducted to focus on this aspect. This also represents the most thorough investigation of hybridization rates in natural populations of ferns. The *Dryopteris carthusiana* group was also chosen as it forms three different hybrid combination and is frequently available in mixed populations. A total 40 mixed (co-occurrence of at least two species) population were sampled and all mature plants in a continuous area containing about 100 individuals were collected and the genome size of all studied individuals (about 3962 plants) was determined using flow cytometry. We found hybrids in 85% of populations. The triploid *D. ×ambroseae* (*D. expansa × D. dilatata*) occurred in every population that included both parent species and is most abundant when the parent species are equally abundant. By contrast, tetraploid *D. ×deweveri* (*D. carthusiana × D. dilatata*) was rare (15 individuals total) and triploid *D. ×sarvelae* (*D. carthusiana × D. expansa*) was completely absent in this study. The parentage of hybrid taxa is considerably asymmetric. Overall, we can say that hybridization rates differ greatly even among closely related fern taxa. In contrast to angiosperms, our data suggest that hybridization rates are highest in balanced parent populations, supporting the notion that some ferns possess very weak barriers to hybridization.

The other application of flow-cytometry and genome size measurement was focusing on the experimental interspecific cytotype variability of the fern *Pteridium aquilinum* (**Paper 11**). In Europe, *P. aquilinum* is considered an aggressive colonizer and a weed, growing in woods, pastures, abandoned fields and various other disturbed habitats. This success is likely due to a combination of sexual and clonal reproduction (Page 1976). The taxon is considered diploid

with a notable exception of the triploids occasionally found in the British Isles (Sheffield et al. 1993). Unlike in most even-ploidy cytotypes, fern triploids are often apomictic or infertile, due to genetic imbalance. An interesting opportunity to study these phenomena has emerged with the discovery of this triploid bracken, a triploid that is fertile but not apomictic. To study the cytotype variation of the species, we analyzed 1456 randomly sampled fronds from 135 population. The diploid cytotype of *P. aquilinum* is dominant in continental Europe with 121 entirely diploid populations found, but we also found 9 mixed and 5 entirely triploid populations. Triploids were also revealed in Norway, Sweden, Germany, Switzerland, the Czech Republic, and Austria, but are rare. There seems to be no obvious geographical patterns in the distribution of cytotypes, so we additionally tested genetic differences (9 microsatellite regions) between ploidy levels as well. It was found that the triploid cytotype is rare and likely originated multiple times from the diploids and relies on clonal and possibly limited sexual reproduction to maintain itself. However, diploids and triploids are often genetically different within a population, indicating that the triploids may also migrate between populations, because 21.4% of triploid plants found were fertile (**Paper 11**).

The intensive and long-term use of flow cytometry in fern biosystematics has finally led us to the challenge of producing the largest cytogeographic study ever preformed on ferns. We focused on the interspecific cytotype variability of the polyploid *Cystopteris fragilis* complex (**Paper 12**). The collection of 5518 individuals from 449 localities across four continents and subsequent analyses of genome size took us five years (thanks to the great help of many colleagues). We revealed five different ploidy levels (2x, 4x, 5x, 6x, 8x) and three species with intraspecific ploidy-level variation: *C. fragilis*, *C. alpina* and *C. diaphana*. Two predominant *C. fragilis* cytotypes, tetraploids and hexaploids, co-occur over most of Europe in a diffuse, mosaic-like pattern. Within this contact zone, 40 % of populations were mixed-ploidy and most also contained pentaploid hybrids. Despite our application of possible ecological preferences, field-recorded parameters and database-extracted climate data, the environmental conditions were only observed to have a limited effect on the distribution of the predominant cytotypes. Flow-cytometric analyses revealed subtle but highly significant differences in monoploid genome size (Cx value) between *C. fragilis* tetraploids and hexaploids. Specifically, the monoploid genome of hexaploids is on average 2.6 % smaller than that of tetraploids. This might be a consequence of 'genome downsizing', a process of systematic DNA loss, which is known to commonly accompany polyploidy (Leitch & Bennett 2004). Based on this study, we can confirm, that both the ploidy level diversity and the frequency of mixed-ploidy populations observed here suggest that, in this respect, ferns can match the well-documented patterns in angiosperms (Kolář et al. 2017). While ploidy coexistence in *C. fragilis* is not driven by environmental factors, it could be facilitated by the perennial life-form of the species, its reproductive modes and the efficient wind dispersal of spores (**Paper 12**).

A follow-up research on *Cystopteris fragilis* complex was built on prior knowledge of the genome size/ploidy level of the plants under study (**Paper 12**) and was focused particularly on the phylogenetic pattern among the members of the complex worldwide, based on phylogenetic analyses of plastid DNA among main cytotypes and spore character (**Paper 13**). We selected a representative set of 87 *C. fragilis* samples reflecting the ploidy level (4x, 5x, 6x) and spore type (spiny/rugose) variability of the group found in Northern Hemisphere populations for

sequencing of two plastid loci (rbcL, trnG-R). The spore character was evaluated in this study as the production of rugose instead of regular spiny spores, is sometimes associated with a potential species called *C. dickieana*. Our plastid DNA analyses revealed two haplotype lineages, which we label the hemifragilis and reevesiana clades, based on their potential relationship to the two presumed diploid parents of *C. fragilis*. The tetraploid, pentaploid and hexaploid accessions did not form monophyletic groups and they were each distributed in both major clades of the *C. fragilis* complex. Rugose spores were rarer overall (26% of samples), but five times more prevalent in the hemifragilis clade and therefore should not be associated with a single name. It is evident, that this study is limited by using only plastid DNA data (**Paper 13**) but further reveals a great genotypic and cytotypic complexity present in this taxonomic complex. The proper delimitation and understanding of *C. fragilis* still remains a great challenge.

3 Future perspectives

In many aspects, ferns as vascular spore-bearing plants have been neglected in comparison to angiosperms. Therefore, there are numerous remaining challenges that show the future directions we can take. Some of the studies presented raise questions for further research of ferns and, possibly, lycophytes. Future research will have to include both the gametophytic and the sporophytic stages. Many other stories may still be unfolding that we will no doubt hear about in the future.

Spore abortion in ferns was until now studied only in temperate leptosporangiate ferns (Quintanilla & Escudero 2006, Paper 1). Eusporangiate ferns as well as lycopods were still not tested. Within ferns, there is an extensive opportunities concerning other regions (arid, tropical) where examining spore abortion will be challenging. Furthermore, the comparison between plants of different habitats corresponding with optimal vs. sub-optimal conditions is still understudied.

Focusing on the gametophyte, its unique system of sex determination and ensuing population demographic control also deserves more interest in the future. More than seventy years after the discovery of antheridiogens by Walter Döpp (Döpp 1950) the vast majority of fern species (98%) remains unexplored. It may be expected that large, species-rich families such as Athyriaceae, Thelypteridaceae, Polypodiaceae and Cyatheaceae, with few, if any, species tested, should be the subject of future inquiries, as should eusporangiate fern lineages or even lycopods. We are now beginning to understand how antheridiogens operate on the molecular level but many questions about their distribution and evolution remain unanswered. Hopefully, the presented comprehensive dataset (Paper 5) can provide a starting point for fern researchers to learn whether their species of interest use this intriguing system of pheromonal control over sexual determination.

Further questions arise about possible gametophyte competition during their development. Of great importance possible are the understudied allelopathic influences that were discovered during the study of *Asplenium ruta-muraria* (Paper 6). The exudates of female gametophytes did not preclude the formation of archegonia among younger gametophytes or stimulate germination in darkness. However, they may have suppressive or facilitative effects on younger gametophytes of various fern species. The mechanics and properties of these effects have been

poorly explored until now, may have evolutionary significance and deserve further study in ferns.

During our study on the formation of fern polyhaploids (Paper 2), we suggested that the apo-sex hybrids might be of certain evolutionary potential, particularly if their polyhaploid offspring are capable of producing viable spores and crossing with sexual species. Many other interesting research topics were raised based on those data, such as the incidence of polyhaploid formation in other fern groups or ploidies, genetic variation of polyhaploid offspring, and especially the occurrence and fertility of polyhaploid descendant from tetraploid hybrids.

Despite continued study focusing on the dynamics of genome evolution and genome size diversity, we still measured the genome size of only about 5.1% of the fern species (Fujiwara et al. 2023). More FCM measurements are needed for a better overview of genome size variability particularly in the still poorly understood fern and lycophyte groups. Our research group is very active in this field. We continuously excerpt published data on genome size of ferns and lycophytes and also obtain genome sizes of still not measured taxa. We believe we will create the largest database of genome size of ferns and lycophytes used for further analyses.

Members of our research group also participate in the Fern and lycophyte genome databank and silica archive (FerDA) project coordinated by L. Ekrt. We are creating the Europe's largest genebank for ferns and lycophytes via a gradual collection (and storing it as silica-dried) of fern and lycophyte material during various field excursions to many European countries. We were inspired by a similar project by our colleagues from the Duke University – Fern labs Database (https://fernlab.biology.duke.edu/). The silica-dried samples stored within FerDA are precisely georeferenced, documented by herbarium vouchers and usually of known genome size or ploidy level. This project is still in progress, but several thousands of samples are ready to be used for possible phylogenetic studies in the future.

4 References

Albertini E., Barcaccia G., Carman J. G. & Pupilli F. (2019): Did apomixis evolve from sex or was it the other way around? – Journal of Experimental Botany 70: 2951–2964.

Barker M. S. (2009): Evolutionary genomic analyses of ferns reveal that high chromosome numbers are a product of high retention and fewer rounds of polyploidy relative to angiosperms. – American Fern Journal 99: 136–141.

Barker M. S. & Wolf P. G. (2010): Unfurling fern biology in the genomics age. – BioScience 60: 177–185.

Barrington D. S. (2020): The biogeography of polyploid ferns across space and time. – American Fern Journal 110: 233–254.

Barrington D. S., Haufler C. H. & Werth C. R. (1989): Hybridization, reticulation, and species concepts in the ferns. – American Fern Journal 79: 55–64.

Braithwaite A. F. (1964): A new type of apogamy in ferns. – New Phytologist 63: 293–305.

Bureš P., Tichý L., Wang Y.-F. & Bartoš J. (2003): Occurrence of Polypodium ×mantoniae and new localities for P. interjectum in the Czech Republic confirmed using flow cytometry. – Preslia 75: 293–310.

Čertner M., Lučanová M., Sliwinska E., Kolář F. & Loureiro J. (2022): Plant material selection, collection, preservation, and storage for nuclear DNA content estimation. – Cytometry Part A 101: 737–748.

Chao Y.-S., Liu H.-Y., Huang Y.-M., & Chiou W.-L. (2010): Reproductive traits of Pteris cadieri and P. grevilleana in Taiwan: Implications for their hybrid origin. – Botanical Studies 51: 209–216.

Cheng F. & Cheng Z. (2015): Research progress on the use of plant allelopathy in agriculture and the physiological and ecological mechanisms of allelopathy. – Frontiers in Plant Science 6: 1020.

Clark J., Hidalgo O., Pellicer J., Liu H., Marquardt J., Robert Y., Christenhusz M., Zhang S., Gibby M., Leitch I. J. & Schneider H. (2016): Genome evolution of ferns: evidence for relative stasis of genome size across the fern phylogeny. – New Phytologist 210: 1072–1082.

Comai L. (2005): The advantages and disadvantages of being polyploid. – Nature Reviews Genetics 6: 836–846.

Conant D. S. & Cooper-Driver G. (1980): Autogamous allohomoploidy in Alsophila and Nephelea (Cyatheaceae): A new hypothesis for speciation in homoploid homosporous ferns. – American Journal of Botany 67: 1269–1288.

Dauphin B., Grant J. & Mráz P. (2016): Ploidy level and genome size variation in the homosporous ferns Botrychium s. l. (Ophioglossaceae). – Plant Systematics and Evolution 302: 575–584.

DeSoto L., Quintanilla L. G., & Méndez M. (2008): Environmental sex determination in ferns: effects of nutrient availability and individual density in Woodwardia radicans. – Journal of Ecology 96: 1319–1327.

Döpp W. (1932): Die Apogamie bei Aspidium remotum Al. Br. – Planta 17:86–152.

Döpp W. (1950): Eine die Antheridienbildung bei Farnen fördernde Substanz in den Prothallien von Pteridium aquilinum (L.) Kuhn. – Berichte der Deutschen Botanischen Gesellschaft 63: 139–147.

Dyer A. F. (1979): The culture of fern gametophytes for experimental investigation. – In Dyer A. F. (ed.), The experimental biology of ferns, pp. 253–305. – Academic Press, London.

Dyer R. J., Pellicer J., Savolainen V., Leitch I. J., & Schneider H. (2013): Genome size expansion and the relationship between nuclear DNA content and spore size in the Asplenium monanthes fern complex (Aspleniaceae). – BMC Plant Biology 13: 219.

Dyer R. J., Savolainen V., & Schneider H. (2012): Apomixis and reticulate evolution in the Asplenium monanthes fern complex. – Annals of Botany 110: 1515–1529.

Ebihara A., Ishikawa H., Matsumoto S., Lin S.-J., Iwatsuki K., Takamiya M., Watano Y. & Ito M. (2005): Nuclear DNA, chloroplast DNA, and ploidy analysis clarified biological complexity of the Vandenboschia radicans complex (Hymenophyllaceae) in Japan and adjacent areas. – American Journal of Botany 92: 1535–1547.

Ekrt L., Lepší M., Boublík K. & Lepší P. (2007): Dryopteris remota rediscovered for the flora of the Czech Republic. – Preslia 79: 69–82.

Ekrt L., Linhartová R., & Štech M. (2013): Rozlišování a revize rozšíření kapradě podobné (Dryopteris expansa) a jejího křížence D. ×ambroseae v České republice. – Zprávy České Botanické Společnosti 48: 263–291.

Ekrt L., Štech M., Lepší M. & Boublík K. (2010): Rozšíření a taxonomická problematika skupiny Dryopteris affinis v České republice. – Zprávy České Botanické Společnosti 45: 25–52.

Ekrt L., Trávníček P., Jarolímová V., Vít P. & Urfus T. (2009): Genome size and morphology of the Dryopteris affinis group in Central Europe. – Preslia 81: 261–280.

Fang Y., Qin X., Liao Q., Du R., Luo X., Zhou Q., Li Z., Chen H., Jin W., Yuan Y., Sun P., Zhang R., Zhang J., Wang L., Cheng S., Yang X., Yan Y., Zhang X., Zhang Z., Bai S., Van de Peer Y., Lucas W. J., Huang S. & Yan J. (2022): The genome of homosporous maidenhair fern sheds light on the euphyllophyte evolution and defences. – Nature Plants 8: 1024–1037.

Fraser-Jenkins C. R. (2007): The species and subspecies in the Dryopteris affinis group. – Fern Gazette 18: 1–26.

Fujiwara T., Liu H., Meza-Torres E. I., Morero R. E., Vega A. J., Liang Z., Ebihara A., Leitch I. J. & Schneider H. (2023): Evolution of genome space occupation in ferns: linking genome diversity and species richness. – Annals of Botany 131: 59–70.

Fujiwara T. & Watano Y. (2020): Independent allopatric polyploidizations shaped the geographical structure and initial stage of reproductive isolation in an allotetraploid fern Lepisorus nigripes (Polypodiaceae). – PLoS ONE 15: e0233095.

Grusz A. L. (2016): A current perspective on apomixis in ferns. – Journal of Systematics and Evolution 54: 656–665.

Guo Z.-Y. & Liu H.-M. (2013): Gametophyte morphology and development of three species of Cyrtogonellum Ching (Dryopteridaceae). – American Fern Journal 103: 153–165.

Haufler C. H. (2002): Homospory 2002: An odyssey of progress in pteridophyte genetics and evolutionary biology. – BioScience 52: 1081–1093.

Haufler C. H. (2014): Ever since Klekowski: Testing a set of radical hypotheses revives the genetics of ferns and lycophytes. – American Journal of Botany 101: 2036–2042.

Haufler C. H., Pryer K. M., Schuettpelz E., Sessa E. B., Farrar D. R., Moran R., Schneller J. J., Watkins J. E. & Windham M. D. (2016): Sex and the single gametophyte: revising the homosporous vascular plant life cycle in light of contemporary research. – BioScience 66: 928–937.

Haufler C. H. & Welling C. B. (1994): Antheridiogen, dark spore germination, and outcrossing mechanisms in Bommeria (Adiantaceae). – American Journal of Botany 81: 616–621.

Henry T. A., Bainard J. D. & Newmaster S. G. (2014): Genome size evolution in Ontario ferns (Polypodiidae): evolutionary correlations with cell size, spore size, and habitat type and an absence of genome downsizing (T. Schwarzacher, Ed.). – Genome 57: 555–566.

Huang Y.-M., Chiou H.-M. & Chiou W.-L. (2004): Density affects gametophyte growth and sexual expression of Osmunda cinnamomea (Osmundaceae: Pteridophyta). – Annals of Botany 94: 229–232.

Huang Y.-M., Chou H.-M., Hsieh T.-H., Wang J.-C. & Chiou W.-L. (2006): Cryptic characteristics distinguish diploid and triploid varieties of Pteris fauriei (Pteridaceae). – Canadian Journal of Botany 84: 261–268.

Jighly A., Lin Z., Forster J. W., Spangenberg G. C., Hayes B. J. & Daetwyler H. D. (2018): Insights into population genetics and evolution of polyploids and their ancestors. – Molecular Ecology Resources 18: 1157–1172.

Kaplan Z., Danihelka J., Chrtek J., Prančl J., Ducháček M., Ekrt L., Kirschner J., Brabec J., Zázvorka J., Trávníček B., Dřevojan P., Šumberová K., Kocián P., Wild J. & Petřík P. (2018a): Distributions of vascular plants in the Czech Republic. Part 7. – Preslia 90: 425–531.

Kaplan Z., Danihelka J., Chrtek J., Zázvorka J., Koutecký P., Ekrt L., Řepka R., Štěpánková J., Jelínek B., Grulich V., Prančl J. & Wild J. (2019): Distributions of vascular plants in the Czech Republic. Part 8. – Preslia 91: 257–368.

Kaplan Z., Danihelka J., Ekrt L., Štech M., Řepka R., Chrtek J., Grulich V., Rotreklová O., Dřevojan P., Šumberová K. & Wild J. (2020): Distributions of vascular plants in the Czech Republic. Part 9. – Preslia 92: 255–340.

Kaplan Z., Danihelka J., Koutecký P., Šumberová K., Ekrt L., Grulich V., Řepka R., Hroudová Z., Štěpánková J., Dvořák V., Dančák M., Dřevojan P. & Wild J. (2017a): Distributions of vascular plants in the Czech Republic. Part 4. – Preslia 89: 115–201.

Kaplan Z., Danihelka J., Lepší M., Lepší P., Ekrt L., Chrtek J., Kocián J., Prančl J., Kobrlová L., Hroneš M. & Šulc V. (2016): Distributions of vascular plants in the Czech Republic. Part 3. – Preslia 88: 459–544.

Kaplan Z., Danihelka J., Šumberová K., Chrtek J., Rotreklová O., Ekrt L., Štěpánková J., Taraška V., Trávníček B., Prančl J., Ducháček M., Hroneš M., Kobrlová L., Horák D. & Wild J. (2017b): Distributions of vascular plants in the Czech Republic. Part 5. – Preslia 89: 333–439.

Kaplan Z., Koutecký P., Danihelka J., Šumberová K., Ducháček M., Štěpánková J., Ekrt L., Grulich V., Řepka R., Kubát K., Mráz P., Wild J. & Brůna J. (2018b): Distributions of vascular plants in the Czech Republic. Part 6. – Preslia 90: 235–346.

Kato M. (1993): Biogeography of ferns: dispersal and vicariance. – Journal of Biogeography 20: 265–274.

Kenrick P. & Crane P. R. (1997): The origin and early evolution of plants on land. – Nature 389: 33–39.

Klekowski E. J. & Lloyd R. M. (1968): Reproductive biology of the Pteridophyta: 1. General considerations and a study of Onoclea sensibilis L. – Journal of the Linnean Society of London, Botany 60: 315–324.

Kolář F., Čertner M., Suda J., Schönswetter P. & Husband B. C. (2017): Mixed-ploidy species: Progress and opportunities in polyploid research. – Trends in Plant Science 22: 1041–1055.

Korpelainen H. (1994): Growth, sex determination and reproduction of Dryopteris filix-mas (L.) Schott gametophytes under varying nutritional conditions. – Botanical Journal of the Linnean Society 114: 357–366.

Laird S. & Sheffield E. (1986): Antheridia and archegonia of the apogamous fern Pteris cretica. – Annals of Botany 57: 139–143.

Landis J. B., Soltis D. E., Li Z., Marx H. E., Barker M. S., Tank D. C. & Soltis P. S. (2018): Impact of whole-genome duplication events on diversification rates in angiosperms. – American Journal of Botany 105: 348–363.

Lavania U. C. (2020): Plant speciation and polyploidy: in habitat divergence and environmental perspective. – Nucleus 63: 1–5.

Leitch I. J. & Bennett M. D. (2004): Genome downsizing in polyploid plants. – Biological Journal of the Linnean Society 82: 651–663.

Leitch A. R. & Leitch I. J. (2012): Ecological and genetic factors linked to contrasting genome dynamics in seed plants. – New Phytologist 194: 629–646.

Leitch I. J. & Leitch A. R. (2013): Genome size diversity and evolution in land plants. – In: Leitch I. J., Greilhuber J., Doležel J., Wendel J. (eds), Plant genome diversity. Berlin: Springer-Press. 2: 307–322.

Liu H.-M., Dyer R. J., Guo Z.-Y., Meng Z., Li J.-H. & Schneider H. (2012): The evolutionary dynamics of apomixis in ferns: A case study from Polystichoid ferns. – Journal of Botany 2012: 1–11.

Lloyd R. M. & Klekowski E. J. (1970): Spore germination and viability in Pteridophyta: Evolutionary significance of chlorophyllous spores. – Biotropica 2: 129–137.

Lopez-Smith R. & Renzaglia K. (2008): Sperm cell architecture, insemination, and fertilization in the model fern Ceratopteris richardii. – Sexual Plant Reproduction 21: 153–167.

Loureiro J., Kron P., Temsch E. M., Koutecký P., Lopes S., Castro M. & Castro S. (2021): Isolation of plant nuclei for estimation of nuclear DNA content: Overview and best practices. – Cytometry 99: 318–327.

Lovis J. D. (1964): Autopolyploidy in Asplenium. – Nature 203: 324–325.

Manton I. (1950): Problems of cytology and evolution in the Pteridophyta. – Cambridge University Press, London.

Manton I. & Walker S. (1954): Induced apogamy in Dryopteris dilatata (Hoffm.) A. Gray and D. filix-mas (L.) Schott emend. and its significance for the interpretation of the two species. – Annals of Botany 18: 377–383.

Martínez de Alba A. E., Elvira-Matelot E. & Vaucheret H. (2013): Gene silencing in plants: A diversity of pathways. – Biochimica et Biophysica Acta 1829: 1300–1308.

Mayrose I., Zhan S. H., Rothfels C. J., Magnuson-Ford K., Barker M. S., Rieseberg L. H. & Otto S. P. (2011): Recently formed polyploid plants diversify at lower rates. – Science 333: 1257–1257.

Moran R. C. (2004): A natural history of ferns. – Timber Press, Portland.

Morzenti V. M. (1962): A first report on pseudomeiotic sporogenesis, a type of spore reproduction by which "sterile" ferns produce gametophytes. – American Fern Journal 52: 69–78.

Mullenniex A., Hardig T. M., & Mesler M. R. (1999): Molecular confirmation of hybrid swarms in the fern genus Polystichum (Dryopteridaceae). – Systematic Botany 23: 421–426.

Näf U. (1956): The demonstration of a factor concerned with the initiation of antheridia in polypodiaceous ferns. – Growth 20: 91–105.

Näf U., Nakanishi K. & Endo M. (1975): On the physiology and chemistry of fern antheridiogens. – Botanical Review 41: 315–359.

Niklfeld H. (1997): Mapping the flora of Austria and the Eastern Alps. – Revue valdôtaine d'histoire naturelle 51: 53–62.

Page C. N. (1976): The taxonomy and phytogeography of bracken-a review. – Botanical Journal of the Linnean Society 73: 1–34.

Pellicer J., Hidalgo O., Dodsworth S. & Leitch I. (2018): Genome size diversity and its impact on the evolution of land plants. – Genes 9: 88.

Petit C., Bretagnolle F. & Felber F. (1999): Evolutionary consequences of diploid–polyploid hybrid zones in wild species. – Trends in Ecology & Evolution 14: 306–311.

PPG I. (2016): A community-derived classification for extant lycophytes and ferns: PPG I. – Journal of Systematics and Evolution 54: 563–603.

Pryer K. M., Schneider H., Smith A. R., Cranfill R., Wolf P. G., Hunt J. S. & Sipes S. D. (2001): Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. – Nature 409: 618–622.

Pryer K. M., Schuettpelz E., Wolf P. G., Schneider H., Smith A. R. & Cranfill R. (2004): Phylogeny and evolution of ferns (Monilophytes) with a focus on the early leptosporangiate divergences. – American Journal of Botany 91: 1582–1598.

Quintanilla L. G. & Escudero A. (2006): Spore fitness components do not differ between diploid and allotetraploid species of Dryopteris (Dryopteridaceae). – Annals of Botany 98: 609–618.

Quintanilla L. G., DeSoto L., Jimenez A. & Mendez M. (2007): Do antheridiogens act via gametophyte size? A study of Woodwardia radicans (Blechnaceae). – American Journal of Botany 94: 986–990.

Raghavan V. (1989): Developmental biology of fern gametophytes. – Cambridge University Press, Cambridge, New York.

Ranker T. A. & Haufler C. H. (2008): Biology and Evolution of Ferns and Lycophytes. – Cambridge University Press, Cambridge, New York.

Regalado Gabancho L., Prada C., & Gabriel y Galán J. M. (2010): Sexuality and apogamy in the Cuban Asplenium auritum–monodon complex (Aspleniaceae). – Plant Systematics and Evolution 289: 137–146.

Reichstein T. (1981): Hybrids in European Aspleniaceae (Pteridphyta). – Botanica Helvetica 91: 89–139.

Rieseberg L. H. & Carney S. E. (1998): Plant hybridization. – New Phytologist 140: 599–624.

Rünk K., Moora M. & Zobel M. (2004): Do different competitive abilities of three fern species explain their different regional abundances? – Journal of Vegetation Science 15: 351–356.

Schedlbauer M. D. & Klekowski E. J. (1972): Antheridogen activity in the fern Ceratopteris thalictroides (L.) Brongn. – Botanical Journal of the Linnean Society 65: 399–413.

Schneider H., Schuettpelz E., Pryer K. M., Cranfill R., Magallón S., & Lupia R. (2004): Ferns diversified in the shadow of angiosperms. – Nature 428: 553–557.

Schneller J. J. (1988): Spore bank, dark germination and gender determination in Athyrium and Dryopteris: results and implications for population biology of Pteridophyta. – Botanica Helvetica 98: 77–86.

Schneller J. (1995): Antheridiogen system in the fern Asplenium ruta-muraria (Aspleniaceae: Pteridophyta). – Fern Gazette 15: 64–70.

Schneller J. J. (2008): Antheridiogens. – In Ranker T. A. & Haufler C. H. (eds.), Biology and evolution of ferns and lycophytes., pp. 134–158. – Cambridge University Press, Cambridge.

Schneller J. J., Haufler C. H. & Ranker T. A. (1990): Antheridiogen and natural gametophyte populations. – American Fern Journal 80: 143–152.

Schuettpelz E. & Pryer K. M. (2007): Fern phylogeny inferred from 400 leptosporangiate species and three plastid genes. – Taxon 56: 1037–1050.

Sessa E. B., Testo W. L. & Watkins J. E. (2016): On the widespread capacity for, and functional significance of, extreme inbreeding in ferns. – New Phytologist 211: 1108–1119.

Sheffield E., Wolf P. G., Rumsey F. J., Robson D. J., Ranker T. A. & Challinor S. M. (1993): Spatial distribution and reproductive behaviour of a triploid bracken (Pteridium aquilinum) clone in Britain. – Annals of Botany 72: 231–237.

Sigel E. M. (2016): Genetic and genomic aspects of hybridization in ferns. – Journal of Systematics and Evolution 54: 638–655.

Sigel E. M., Windham M. D., Huiet L., Yatskievych G. & Pryer K. M. (2011): Species relationships and farina evolution in the cheilanthoid fern genus Argyrochosma (Pteridaceae). – Systematic Botany 36: 554–564.

Soltis D. E., Albert V. A., Leebens-Mack J., Bell C. D., Paterson A. H., Zheng C., Sankoff D., dePamphilils C. W., Wall P. K. & Soltis P. (2009): Polyploidy and angiosperm diversification. – American Journal of Botany 96: 336–348.

Soltis P. S., Marchant D. B., Van de Peer Y. & Soltis D. E. (2015): Polyploidy and genome evolution in plants. – Current Opinion in Genetics & Development 35: 119–125.

Soltis D. E. & Soltis P. S. (1992): The distribution of selfing rates in homosporous ferns. – American Journal of Botany 79: 97–100.

Soltis D. E., Soltis P. S., Tate J. A. (2003): Advances in the study of polyploidy since Plant speciation. New Phytologist 161: 173–191.

Soltis D. E., Visger C. J. & Soltis P. S. (2014): The polyploidy revolution then...and now: Stebbins revisited. – American Journal of Botany 101: 1057–1078.

Suda J., Meyerson L. A., Leitch I. J. & Pyšek P. (2015): The hidden side of plant invasions: the role of genome size. – New Phytologist 205: 994–1007.

Sundue M., Vasco A., Moran R. C. (2011): Cryptochlorophyllous spores in ferns: Nongreen spores that contain chlorophyll. – International Journal of Plant Sciences 172: 1110–1119.

Taylor T. N., Taylor E. L. & Krings M. (2009): Paleobotany: the biology and evolution of fossil plants. – Academic Press, London.

Testo W. & Sundue M. (2016): A 4000-species dataset provides new insight into the evolution of ferns. – Molecular Phylogenetics and Evolution 105: 200–211.

Testo W. L. & Watkins J. E. (2013): Understanding mechanisms of rarity in pteridophytes: competition and climate change threaten the rare fern Asplenium scolopendrium var. americanum (Aspleniaceae). – American Journal of Botany 100: 2261–2270.

Testo W. L., Watkins J. E. & Barrington D. S. (2015): Dynamics of asymmetrical hybridization in North American wood ferns: reconciling patterns of inheritance with gametophyte reproductive biology. – New Phytologist 206: 785–795.

Trewick S. A., Morgan-Richards M., Russell S. J., Henderson S., Rumsey F. J., Pintér I., Barrett J. A., Gibby M. & Vogel J. C. (2002): Polyploidy, phylogeography and Pleistocene refugia of the rockfern Asplenium ceterach: evidence from chloroplast DNA. – Molecular Ecology 11: 2003–2012.

Tryon A. F. & Lugardon B. (1991): Spores of the Pteridophyta: Surface, wall structure, and diversity based on electron microscope studies. – Springer New York, New York.

Tryon R. M. & Vitale G. (1977): Evidence for antheridogen production and its mediation of a mating system in natural populations of fern gametophytes. – Botanical Journal of the Linnean Society 74: 243–249.

Turnbull L. A., Levine J. M., Loreau M. & Hector A. (2013): Coexistence, niches and biodiversity effects on ecosystem functioning. – Ecology Letters 16: 116–127.

Van de Peer Y., Ashman T.-L., Soltis P. S. & Soltis D. E. (2021): Polyploidy: an evolutionary and ecological force in stressful times. – The Plant Cell 33: 11–26.

Vanneste K., Sterck L., Myburg A. A., Van de Peer Y. & Mizrachi E. (2015): Horsetails are ancient polyploids: evidence from Equisetum giganteum. – Plant Cell 27: 1567–1578.

Wagner W. H. & Chen K. L. (1965): Abortion of spores and sporangia as a tool in the detection of Dryopteris hybrids. – American Fern Journal 55: 9–29.

Walker T. G. (1979): The cytogenetic of ferns. – In: Dyer A. F., The Experimental Biology of Ferns, pp. 87–132., Academic Press, London.

Walker T. G. (1962): Cytology and evolution in the fern genus Pteris L. – Evolution 16: 27–43.

Weinberg E. S. & Voeller B. R. (1969): External factors inducing germination of fern spores. – American Fern Journal 59: 153–167.

Werth C. R. & Windham M. D. (1991): A model for divergent, allopatric speciation of polyploid Pteridophytes resulting from silencing of duplicate-gene expression. – The American Naturalist 137: 515–526.

Whittier D. P. (1968): Rate of gametophyte maturation in sexual and apogamous forms of Pellaea glabella. – American Fern Journal 58: 12–19.

Whitton J., Sears C. J., Baack E. J. & Otto S. P. (2008): The dynamic nature of apomixis in the angiosperms. – International Journal of Plant Sciences 169: 169–182.

Wild J., Kaplan Z., Danihelka J., Petřík P., Chytrý M., Novotný P., Rohn M., Šulc V., Brůna J., Chobot K., Ekrt L., Holubová D., Knollová I., Kocián P., Štech M., Štěpánek J., & Zouhar V. (2019): Plant distribution data for the Czech Republic integrated in the Pladias database. – Preslia 91: 1–24.

Willson M. F. (1981): Sex expression in fern gametophytes: Some evolutionary possibilities. – Journal of Theoretical Biology 93: 403–409.

Windham M. D. (1983): The ferns of Elden Mountain, Arizona. – American Fern Journal 73: 85–93.

Windham M. D. & Yatskievych G. (2003): Chromosome studies of cheilanthoid ferns (Pteridaceae: Cheilanthoideae) from the western United States and Mexico. – American Journal of Botany 90: 1788–1800.

Wood T. E., Takebayashi N., Barker M. S., Mayrose I., Greenspoon P. B. & Rieseberg L. H. (2009): The frequency of polyploid speciation in vascular plants. – Proceedings of the National Academy of Sciences 106: 13875–13879.

Wright A. J., Wardle D. A., Callaway R. & Gaxiola A. (2017): The overlooked role of facilitation in biodiversity experiments. – Trends in Ecology & Evolution 32: 383–390.

Appendix 1:
Papers included to the habilitation

Paper 1

Hornych O. & Ekrt L. (2017): Spore abortion index (SAI) as a perspective tool of evaluation of spore fitness in ferns: An insight into sexual and apomictic species. – Plant Systematics and Evolution 303(4): 497–507.

CrossMark

ORIGINAL ARTICLE

Spore abortion index (SAI) as a promising tool of evaluation of spore fitness in ferns: an insight into sexual and apomictic species

Ondřej Hornych[1] · Libor Ekrt[1]

Abstract Ferns reproduce through small and usually haploid spores. The general paradigm states that whereas species produce good shaped spores, hybrids are sterile and form aborted spores. Apomictic fern species represent an unusual case, and it is believed that they produce an unbalanced spore spectrum. Until now, no comprehensive comparison of sexual and apomictic taxa using extensive spore fitness data has been published. Based on a representative data set of 109 plants from 23 fern taxa, we accomplished the first robust analysis of spore fitness using spore abortion index (SAI), the ratio of aborted to all examined spores. One thousand spores were analyzed for each plant. Focusing mainly on two major European fern taxa (*Asplenium*, *Dryopteris*), we compared this trait for different fern reproductive types (sexual/apomicts/hybrids) and ploidy levels (diploid versus polyploid). Our results confirmed the general assumption that shows higher SAI for apomictic taxa (18%) when compared to sexual taxa (3%). Furthermore, hybrids are characterized by having almost all spores aborted (99.8%) with the notable exception of pentaploid *Dryopteris × critica* (93.1%), the hybrid between sexual and apomictic taxa. We found no significant difference in SAI between sexual taxa of various ploidy levels or between sexual taxa of genera *Dryopteris*

and *Asplenium*. Additionally, we carried out an optimization of the SAI method, outlying important guidelines for the use of this method in the future.

Introduction

Generative reproduction represents the basic and recurrent evolutionary force of land plants. Evaluation of reproductive success of plants is universally measured by amounts of offspring or figuratively by amounts of formed seeds (Johnson et al. 2010).

In experimental studies, pollen viability tests, assessing the percentage of viable pollen grains, are widely used to address several topics related to seed plant reproduction (Dafni and Firmage 2000). Similarly, for spore producing vascular plants, spore abortion rate is used as a ratio of aborted spores to all spores in each sample. Unfortunately, until now, this spore abortion index (in this paper abbreviated as SAI) is yet to be standardized and optimized or comparatively and overly evaluated among different ferns types (sexual, apomicts, hybrids).

Ferns are capable of forming spores sexually or via several apomictic ways to form "normal" spores, bad shaped aborted spores or somatic diplospores (Manton 1950; Braithwaite 1964). It was generally believed that sexual species usually form good shaped spores, hybrids are usually sterile (with predominantly aborted spores), and apomictic species are known for an unbalanced spore spectrum (Manton 1950; Lovis 1977). Until now, studies analyzing this topic used only small sample sizes or taxon representation or were otherwise limited (Park and Kato

Handling editor: Martin Lysak.

Electronic supplementary material The online version of this article (doi:10.1007/s00606-016-1386-3) contains supplementary material, which is available to authorized users.

✉ Ondřej Hornych
hornych.ondrej@gmail.com

[1] Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, 370 05 České Budějovice, Czech Republic

2003; Quintanilla and Escudero 2006; Gomes et al. 2006). No generally encompassing study was done. This made it more difficult to research hybrids of sexual and apomictic species (Bär and Eschelmüller 2010; Dyer et al. 2012; Ekrt and Koutecký 2016). In light of recent studies in ferns, it seems that spore abortion index can represent an important and informative tool in fern population biology and biosystematics (Quintanilla and Escudero 2006; Arosa et al. 2009; Nakato et al. 2012; Hernández et al. 2015; Ekrt and Koutecký 2016).

To this day, no unified method exists for assessing SAI. Various amounts of spores are being used for this goal ranging from 100 (Quintanilla and Escudero 2006; Gomes et al. 2006; Hanušová et al. 2014) through 400 (Arosa et al. 2009) up to 1000 (Nakato et al. 2012; Ekrt and Koutecký 2016). Sometimes abortion rates are estimated without presenting a unified number of spores counted (Hernández et al. 2015).

Apart from SAI, studies have employed an alternative method of assessing viability of spores by estimating spore germination rates. Germination rates and inverted SAI seem to correlate well in *Dryopteris* (Quintanilla and Escudero 2006). *Dicksonia sellowiana* (Pr.) Hook. (Dicksoniaceae) produced <10% of spores that appeared viable but did not germinate (Gomes et al. 2006). For *Cornopteris christenseniana* Tagawa (Woodsiaceae), germination rate of viable spores is roughly proportional to their frequency among all spores (Park and Kato 2003). As such, for the purpose of this study we consider germination rates and inverted SAI comparable. However, germination rates may be affected by storage conditions (Kott and Britton 1982; Aragon and Pangua 2004), type of substrate used for germination tests (Kott and Peterson 1973) and age of the collected specimen (Windham and Ranker 1986). The physical appearance of spores does not change significantly over time or by improper storage conditions. Because of this limitation, misguided data could be obtained. Therefore, SAI may be a better method to estimate the formalized ability to produce viable spores for aged or improperly stored specimens.

Our study focused on the largest European fern genera *Asplenium* and *Dryopteris*. Additionally, the genera *Athyrium*, *Gymnocarpium* and *Phegopteris* were represented by one taxon each. We selected 23 taxa that include 14 sexual species, 5 apomictic species and 4 hybrids. The selection includes 8 diploids, 6 triploids, 8 tetraploids and 1 pentaploid. The main goal of our study was (1) a formalized comparison of taxa with different modes of reproduction (sexual, apomictic, hybrids), ploidy levels and generic affiliation in spore abortion and (2) an optimization of the method of assessing spore abortion index for further research.

Materials and methods

Plants used in the study

A total of 109 specimens from 23 fern taxa with monolete non-chlorophyllous spores were used for the study. The majority of the taxa belong to genera *Asplenium* and *Dryopteris*. Three additional genera *Athyrium*, *Gymnocarpium* and *Phegopteris* were added with one taxon each as they represent sexual diploids, sexual tetraploids and apomictic triploids, respectively. The studied taxa encompass varying modes of reproduction (sexual or apomictic) and ploidy levels. Four hybrid taxa were also included. For the purpose of this study, "hybrid" refers to F1 generation hybrids. For each taxon, 3–5 plants have been analyzed (Table 1).

The majority of plants, including every hybrid, have been determined and used from previous systematic studies (Ekrt and Štech 2008; Ekrt et al. 2009, 2010; Ekrt and Koutecký 2016), and some plants have recently been collected for the purpose of this study in the field. We did not collect plants growing in suboptimal conditions (extreme shade, light exposure, etc.). Fronds were collected in their phenological optimum; fronds with immature spores were avoided. A small minority of rare specimens were obtained from public herbaria. Ploidy levels of studied taxa were determined using flow cytometry or micromorphological characters correlated with ploidy levels (e.g., spore size, stoma measurements). Voucher specimens are deposited in the herbarium of the Faculty of Science, University of South Bohemia in České Budějovice (CBFS), or in other public herbaria (Online Resource 1); herbaria acronyms follow Thiers (2016). The nomenclature of taxa under study follows Danihelka et al. (2012) or Blockeel (2006) for taxa not included in the previous.

Evaluation of spore abortion rates

To prepare the spore sample, dried fronds were used. Parts of the frond, which have shed the majority of their spores, were avoided. Using a thick needle, spore material was gently brushed to move spores onto a microscope slide for examination under dry conditions. Before creating a new set of spores for examination, the microscope slide was thoroughly cleaned to avoid contamination. Light microscope (LABO COMFORT 1502, Arsenal) was used to determine the viability of spores under 400× magnification. The microscope slide was examined while making sure that no spore is calculated twice. Spores were considered aborted when exhibiting abortive traits such as collapsed exospore, overly blackish color or anomalous

Table 1 A list of taxa under study with described characteristics (ploidy level, mode of reproduction/hybrid status)

Species	No. of samples	Ploidy level	Mode of reproduction
Asplenium adiantum-nigrum	5	4×	Sexual
Asplenium cuneifolium	5	2×	Sexual
Asplenium onopteris	5	2×	Sexual
Asplenium ruta-muraria	5	4×	Sexual
Asplenium trichomanes nothosubsp. *lusaticum*	4	3×	Hybrid
Asplenium trichomanes subsp. *quadrivalens*	5	4×	Sexual
Asplenium trichomanes subsp. *trichomanes*	5	2×	Sexual
Asplenium viride	5	2×	Sexual
Athyrium filix-femina	5	2×	Sexual
Dryopteris affinis	3	2×	Apomictic
Dryopteris borreri	5	3×	Apomictic
Dryopteris cambrensis	5	3×	Apomictic
Dryopteris carthusiana	5	4×	Sexual
Dryopteris dilatata	5	4×	Sexual
Dryopteris expansa	5	2×	Sexual
Dryopteris filix-mas	5	4×	Sexual
Dryopteris fragrans	5	2×	Sexual
Dryopteris remota	3	3×	Apomictic
Dryopteris × ambroseae	5	3×	Hybrid
Dryopteris × critica	4	5×	Hybrid
Dryopteris × deweveri	5	4×	Hybrid
Gymnocarpium dryopteris	5	4×	Sexual
Phegopteris connectilis	5	3×	Apomictic

shape. Spores of uncommon shapes with a stable exospore and natural color were considered developed.

A total of 1000 spores per each sample were checked to calculate spore abortion index (SAI) as a ratio of aborted spores to all spores in each sample expressed as a percentage. For optimization and the most suitable employment of SAI in the future, SAI was calculated for ten sets of 100 spores independently. Additionally, after 500 spores were analyzed a new set of spores was prepared from a different part of the frond to amount for any discrepancies within the frond. Thus, SAI is available for ten sets of 100 spores, two sets of 500 spores and the total SAI from 1000 spores.

Data analyses

Several Nested ANOVA tests were performed in this study. All of these tests had species affiliation as a random factor nested within the main tested factor. The first analysis was employed to show potential differences between SAI of sexual and apomictic taxa. For the purpose of this analysis, only the genus *Dryopteris* could be used as the other well-represented genus *Asplenium* had no sampled apomictic

species. Sexually reproducing diploids and tetraploids of all applicable genera were also compared in this manner. Additionally, an analysis was performed to ascertain discrepancies of total SAI between *D. ×critica* and other *Dryopteris* hybrids. Finally, a set of Nested ANOVAs was also employed to determine the effects of taxon-related factors. The analysis was used to show possible differences between the apomictic *Phegopteris connectilis* and other apomictic species, all from the genus *Dryopteris*. Furthermore, potential differences in SAI between the genera *Asplenium* and *Dryopteris* were analyzed. Only sexual non-hybrid taxa were used. Other genera are represented by a single taxon each and thus could not be put to the same test. For all samples, the values of SAI were arcsine-transformed for every performed Nested ANOVA and no samples were excluded from their respective analyses. All above-mentioned analyses were performed using Statistica 13 (Dell Inc. 2015).

The values of standard error of estimate for SAI calculation were calculated. The following equation was used:

$$w = \sqrt{\frac{pq}{N}}$$

where N stands for the amount of spores used, p and q stand for the ratio of developed and aborted spores, respectively, and w stands for the standard error of estimate. The values of w were calculated for 100, 500 and 1000 analyzed spores as well as for three p values: 5, 50% and the mean SAI of apomictic samples. To exploratively contrast theoretical results with real data, the difference between partial SAI and total SAI was calculated. Calculations of partial SAI were performed for 100, 200, etc., spores up to a 1000, resulting in ten values total. Each sample of the taxon was used.

To show the potential differences between different parts of the frond, a series of permutation tests was performed. Each sample had the difference in SAI between the two sets of 500 spores calculated. Additionally, two random selections from binomial distribution, with frequency being the total SAI of the sample and N being 500 (to represent 1000 spores calculated), were picked and difference between them was calculated. This pair of selections was performed 9999 times for a total of 10,000 numbers. The sample difference between two parts of the frond was then compared with the modeled distribution, and p value was calculated. To compensate for Type 1 error, p values were adjusted by false discovery rate correction. The permutation analysis and p value adjustments were performed in R 3.1.2 (R Core Team 2014).

Results

Determining aborted spores

A total of 109,000 spores were determined as either aborted or developed. A variety of shapes and sizes was found. Most developed spores looked as represented in Fig. 1a, transparent enough to tell the exospore and colored in light brown. We have found two types of aborted spores. Non-hybrid plants (Fig. 1b) have aborted spores with collapsed exospore and darker colors than the surrounding developed spores. This type of spores is often smaller than developed spores and has an irregular shape but sometimes retains a degree of transparency. In hybrid taxa (Fig. 1c), aborted spores are completely black and vary greatly in size sometimes being much larger than developed spores. These spores lack transparency completely. Additionally, a large amount of tiny black debris is scattered around aborted spores of hybrids. Similar findings have been reported by Wagner and Chen (1965) in the genus *Dryopteris*.
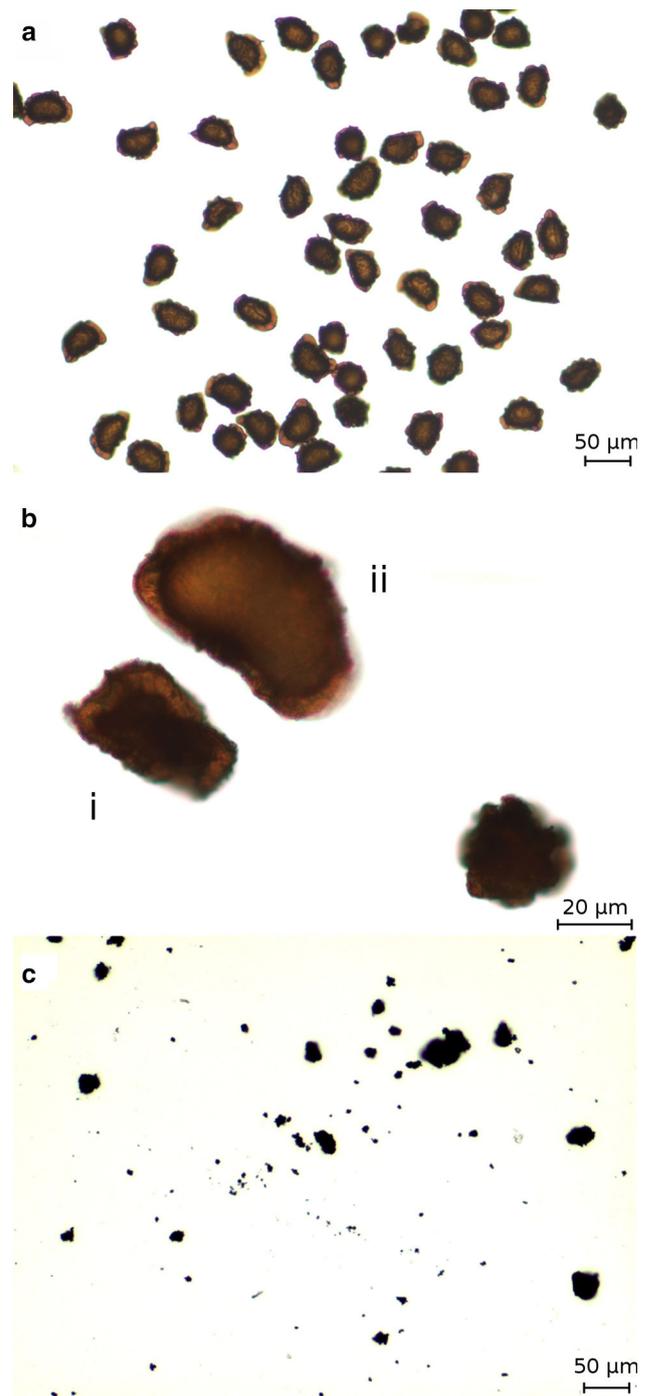
Fig. 1 Different types of spores and its variability in plants under study. **a** *Dryopteris filix-mas*: developed *light brown* spores observed in most non-hybrid sexual taxa. **b** *Dryopteris borreri: i* aborted spore present in *darker colors* in apomictic taxa *ii* well-developed spore, typical for non-hybrid taxa. **c** *Dryopteris × ambroseae: black irregularly shaped* and sized aborted spores typical for hybrids with debris scattered around

Spore abortion index

A variety of SAI values were obtained from the 109 samples tested, ranging from <1 to 100% (Table 2).

Of the total 23 taxa sampled, the diploid sexual *Athyrium filix-femina* has the lowest mean SAI, with all samples having <1% of aborted spores (mean SAI = 0.76%). The sexual tetraploid *Gymnocarpium dryopteris* has mean abortion rate of 1.04%, while having a sample with only three aborted spores out of a thousand (sample 5), the lowest of all sampled plants. Predictably, hybrids occupy the other side of the spectrum with a single sample of both *Dryopteris × ambroseae* and *D. × deweveri* having no developed spores. The majority of hybrid plants samples, except those of the distinct *D. × critica*, have <1% of developed spores.

Overall, sexual taxa have SAI ranging from above-mentioned 0.3% (*G. dryopteris*, sample 5) up to 19% (*D. fragrans*, sample 4). Mean SAI for samples of all sexually reproducing taxa is 3.05%. Meanwhile, apomictic taxa occupy a large gradient of SAI ranging from 1.7 (*D. affinis*, sample 3) to 60.9% (*D. borreri*, sample 5). The SAI of the apomictic *P. connectilis* is similar to SAI of studied *Dryopteris* apomictic taxa (species: $p = 0.443854$, genus: $p = 0.634251$; mean SAI 14.36 and 19.25%, respectively). Mean SAI for all apomictic samples is 18.09%. Regarding ploidy, diploid apomicts have mean SAI of 13.4%, while triploid apomicts abort mean 18.87% of spores. However, the number of samples is unbalanced. A comparison between SAI of various modes of reproduction is shown in Fig. 2.

Comparing SAI of different groups

Highly significant differences in SAI exist between sexual and apomictic taxa of the genus *Dryopteris* (species: $p = 0.3418$, reproduction mode: $p = 0.0022$). For this genus, median SAI values for apomictic and sexual taxa are 19.6 and 3.3%, respectively (Fig. 3). Apomictic taxa form aborted spores with higher frequency.

Potential effects of other taxon-related factors on SAI were tested. Our analyses showed no effect of ploidy level

Table 2 A summary of total SAI (%) of all samples. Each taxon is represented by three to five samples (see Table 1)

Taxon	SAI 1	SAI 2	SAI 3	SAI 4	SAI 5	Mean	S.D.
Asplenium adiantum-nigrum	2.5	1.6	4.4	6.2	2.3	3.40	1.88
Asplenium cuneifolium	2.0	1.8	0.8	1.4	11.6	3.52	4.54
Asplenium onopteris	2.4	2.4	2.5	4.1	1.8	2.64	0.86
Asplenium ruta-muraria	3.3	0.8	1.0	1.2	5.3	2.32	1.95
Asplenium trichomanes nothosubsp. *lusaticum*	99.6	99.9	99.9	99.9	×	99.83	0.15
Asplenium trichomanes subsp. *quadrivalens*	3.3	0.6	1.5	0.7	1.3	1.48	1.09
Asplenium trichomanes subsp. *trichomanes*	0.9	1.0	0.8	0.7	1.8	1.04	0.44
Asplenium viride	4.2	3.0	1.1	2.3	1.4	2.40	1.25
Athyrium filix-femina	0.9	0.6	0.5	0.9	0.9	0.76	0.19
Dryopteris affinis	5.0	33.5	1.7	×	×	13.40	17.49
Dryopteris borreri	4.3	10.2	33.4	21.1	60.9	25.98	22.46
Dryopteris cambrensis	28.2	10.1	7.2	13.5	8.2	13.44	8.59
Dryopteris carthusiana	0.6	1.2	1.7	2.5	0.2	1.24	0.91
Dryopteris dilatata	3.3	3.3	6.9	7.9	3.5	4.98	2.24
Dryopteris expansa	2.6	7.6	1.9	5.3	7.5	4.98	2.67
Dryopteris filix-mas	11.5	4.4	1.8	4.1	15.2	7.40	5.68
Dryopteris fragrans	1.2	3.0	1.1	19.0	3.4	5.54	7.60
Dryopteris remota	30.2	20.9	19.6	×	×	23.57	5.78
Dryopteris × ambroseae	99.0	99.8	100	99.5	99.8	99.62	0.39
Dryopteris × critica	92.6	97.3	89.5	93.5	×	93.23	3.21
Dryopteris × deweveri	95.6	100.0	99.8	99.8	96.6	98.36	2.09
Gymnocarpium dryopteris	1.8	1.5	1.3	0.4	0.3	1.06	0.67
Phegopteris connectilis	14.5	7.4	32.2	5.4	12.4	14.38	10.62

SAI1–5 denotes individual plants given a number 1–5 for each taxon. The cross indicates that less than five samples have been used for the respective taxon
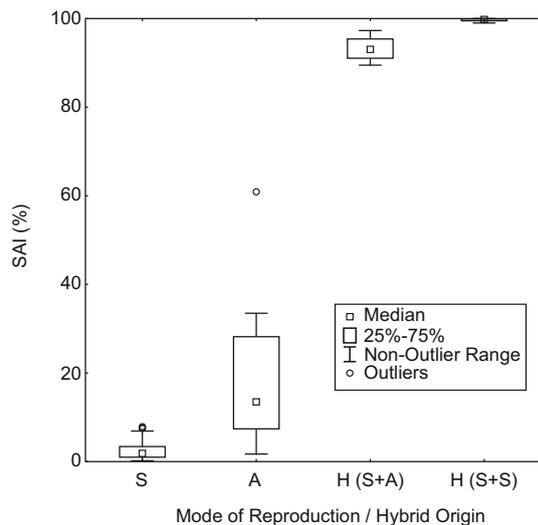
Fig. 2 Spore abortion index (SAI) for all reproduction modes using all samples. *S* sexual taxa, *A* apomictic taxa, *H* (*S* + *A*) hybrids of both apomictic and sexually reproducing parents (represented by *Dryopteris* × *critica* only), *H* (*S* + *S*) hybrids of two sexually reproducing parents
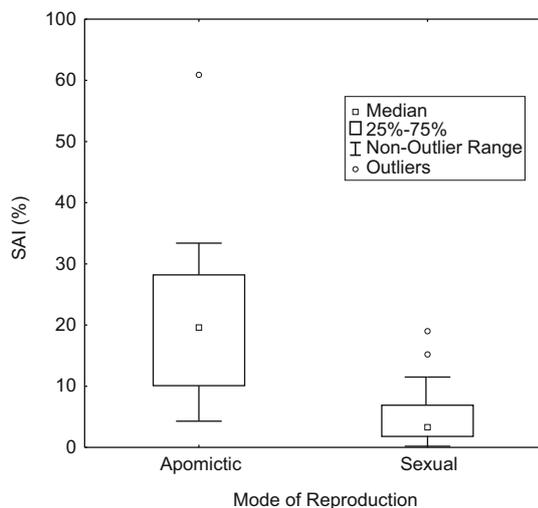
Fig. 3 Spore abortion index (SAI) between sexual and apomictic taxa of the genus *Dryopteris*

on SAI when comparing sexual taxa (species: $p = 0.0008$, ploidy level: $p = 0.8976$), genera *Athyrium* and *Gymnocarpium* included. There seems to be no difference in SAI regarding ploidy levels for sexual species. However, there are significant differences between species.

SAI values not standard for hybrid taxa were found in the *Dryopteris* × *critica* (hybrid of apomictic and sexual taxa). This hybrid differs significantly in SAI from others studied *Dryopteris* hybrids (species: $p = 0.3357$, hybrid origin: $p = 0.0012$). Median SAI value for *Dryopteris* × *critica* is 93.05%, while other hybrids (with sexually reproducing parents) have median SAI 99.8%.
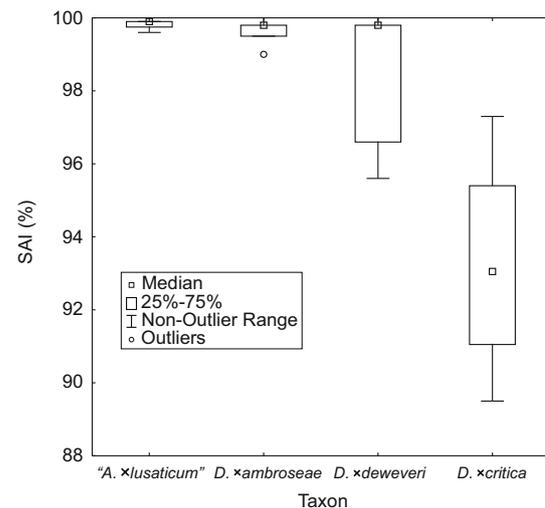
Fig. 4 Spore abortion index (SAI) between hybrid taxa under study, the name "*A.* × *lusaticum*" refers to the hybrid *Asplenium trichomanes* nothosubsp. *lusaticum*

Most of these other hybrids have SAI close to 100% with a notable exception of samples 1 and 5 of *D.* × *deweveri* having SAI of 95.6 and 96.6%, respectively (Fig. 4).

Marginally, significant differences in SAI were found between sexual species of the genera *Asplenium* and *Dryopteris* (species: $p = 0.0925$, genus $p = 0.05353$). The genus *Dryopteris* has a higher median of 3.3% compared to 1.8% of the genus *Asplenium*. Nevertheless, this difference is comparable to the difference between the species within their respective genus.

Optimization of SAI assessment method

Standard error of estimate was calculated (Table 3). This error increases with the proximity of real SAI value to 50% and decreases with the amount of spores used to estimate SAI. At the least optimal scenario (100 spores calculated, real SAI 50%), the standard error of mean is equal to 5%, suggesting that the calculated value will on average be 5 aborted spores off the real value in either direction.

The change in the difference between cumulatively calculated partial SAI and total SAI demonstrates the variance in data (Fig. 5). The following taxa represent the different levels of mean deviation from total SAI in increasing order. The hybrid *D.* × *ambroseae* (Fig. 5a) is very uniform, and SAI never differs more than 1% from total SAI. The example of *A. ruta-muraria* (Fig. 5b) shows little change in estimate after ca 400 spores are calculated. The number of spores needed to provide a close estimate increases to approximately 600 and 900 for *D. dilatata* (Fig. 5c) and *D. cambrensis* (Fig. 5d), respectively.

After p value adjustment, 12 of 109 (11%) samples significantly differed ($p < 0.05$) between the two sets of

Table 3 Calculated values for standard error of estimate of SAI at varying numbers of calculated spores and real SAI values

|	100 spores	500 spores (%)	1000 spores (%)
5% SAI	2.18	0.97	0.69
18% SAI	3.84	1.72	1.21
50% SAI	5.00	2.24	1.58

The value of 18% reflects the mean SAI of sampled apomicts

500 spores, and each estimated from a distinct part of the frond; hence, the SAI value of these plants varies within the frond. Furthermore, ten plants are marginally significantly different ($0.05 < p > 0.1$). Of the significantly differing plants, eight were apomicts, three were sexually reproducing, and one sample was of hybrid origin. One sample (*D. remota* 2) has a surprising 18.2% difference

between two parts of the frond, while total SAI for the sample is 20.9%. See Online Resource 2 for the results of individual tests alongside other measures of variation within sample.

Discussion

Effects of reproduction mode on spore abortion

Our results suggest a high degree of variability in SAI for apomictic taxa. Studied apomictic species had SAI ranging from 1.7 to 60.9% with the mean abortion rate being around 18%. Apomictic taxa of both *Dryopteris* and *Phegopteris* showed similar SAI and wide pattern. Therefore, the rate of aborted spores in apomictic species might
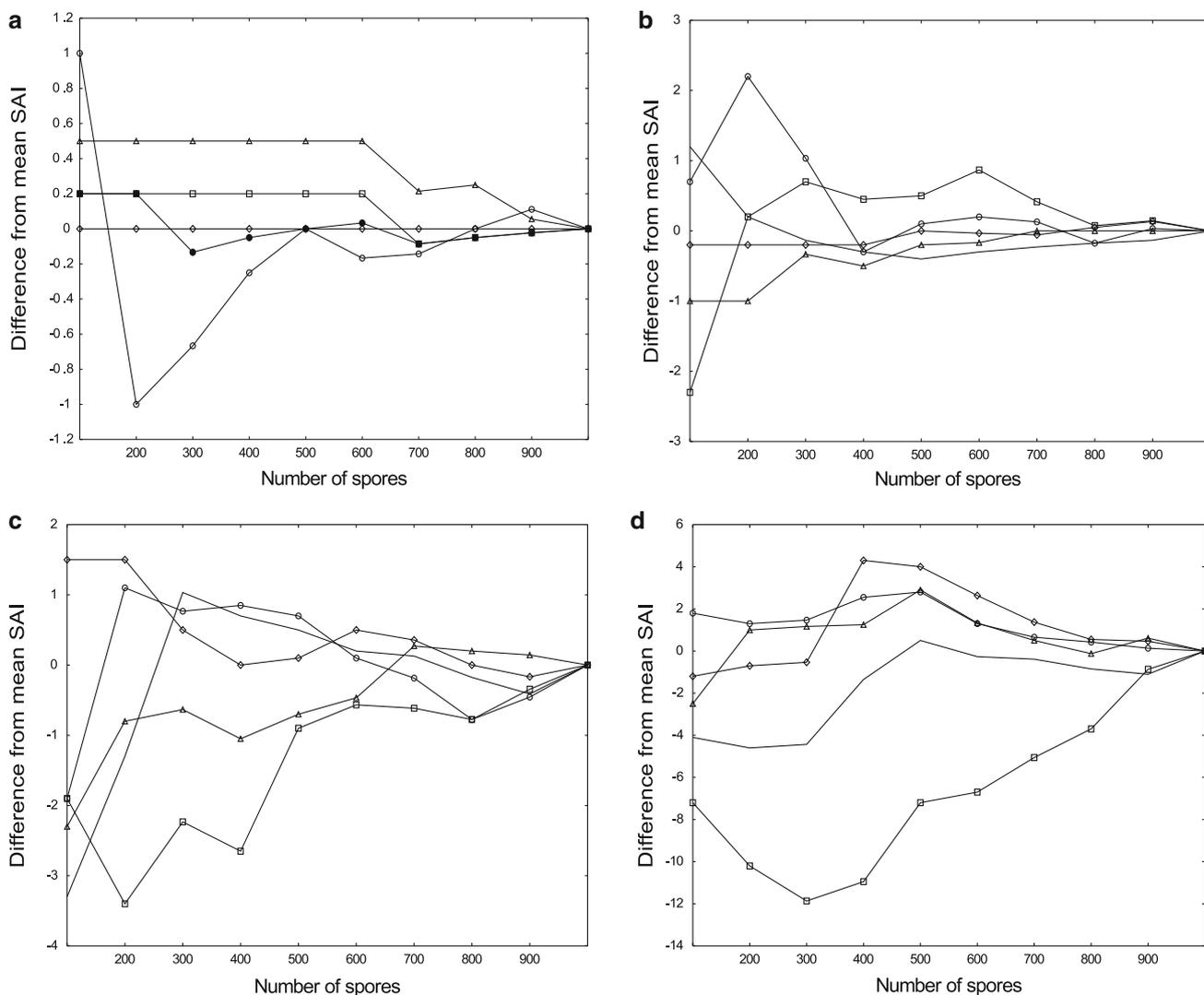
Fig. 5 Change in the difference between partial and total SAI with the increasing amount of spores used to calculate partial SAI. The change is presented for each sample in *Dryopteris × ambroseae* (**a**), *Asplenium ruta-muraria* (**b**), *D. dilatata* (**c**) and *D. cambrensis* (**d**)

permeate throughout family boundaries and is probably not just limited to either genus.

Fern apomicts are formed via apogamy (formation of sporophytes from somatic cells of the prothallium) followed by agamospory (production of unreduced spores). There are several ways of how spores are formed in fern apomicts. Aborted spores are formed via unbalanced meiosis, and (diplo)spores are formed via regular meiosis. Both processes are present simultaneously, so it is generally expected that apomicts usually have a higher incidence of aborted spores when compared to sexual species (Manton 1950; Gastony and Windham 1989). There are several studies that confirm higher spore abortion and greater SAI variability for apomicts. Study of apomictic *Cornopteris christenseniana* revealed 8–99% of aborted spores (Park and Kato 2003). A more detailed examination of apomicts was carried out by Walker (1962). He compared natural apomicts and synthetic apomictic hybrids of *Pteris* resulting in 15–43% and 45–81% SAI, respectively. An extensive series of studies were carried out by Eschelmüller analyzing germination rates of apomicts. These studies suggest very erratic and highly variable germination rates for *Dryopteris affinis* complex (Eschelmüller 1998) and *Dryopteris remota* (Eschelmüller 1993). Our results with a robust and highly comparable dataset confirmed the generally expected notion that apomictic species are mostly capable of forming a high proportion of viable spores but are prone to high levels of abortion. However, published literature is equivocal. There are apomicts with evidence of little or no spore abortion, e.g., triploid species of *Cyrtogonellum* Ching (Dryopteridaceae) (Guo and Liu 2013), tetraploid *Pteris vittata* L. (Pteridaceae) with stated 100% germination rate, therefore, supposed 0% SAI (Khare and Kaur 1983). High germination rates and <10% SAI were revealed in apomictic diploid *Dryopteris affinis* (Quintanilla and Escudero 2006). Similarly 8–10% of aborted spores are produced by apomictic triploid *Argyrochosma nivea* var. *tenera* (Gillies ex Hook.) Ponce (Adiantaceae) (Hernández et al. 2015).

In our study, sexual taxa produced a lesser amount of aborted spores (mean SAI 4.83%) when compared to apomicts (mean SAI 19.25%), in the genus *Dryopteris*. This trend applies more broadly to all studied taxa (mean SAI 3.05 and 18.09%, respectively). The apomictic diploid *Dryopteris affinis* was found to have comparable SAI (mostly around or below 5%) and germination rates to sexual *Dryopteris* species (Quintanilla and Escudero 2006). To our knowledge, no other comparisons of sexual and apomictic taxa in either SAI or germination rates have been published. While results of various germination tests may vary wildly, as demonstrated below, published data suggest low SAI for sexual taxa. Arosa et al. (2009) reported mean SAI lower than 8% for *Culcita macrocarpa* C. Presl

(Dicksoniaceae) and *Woodwardia radicans* (L.) Sm. (Blechnaceae). A set of 55 samples of *Dicksonia sellowiana* produced mean 3.8% of aborted spores (Gomes et al. 2006). It is apparent that sexual taxa commonly produce a vast majority of well-developed spores. Nevertheless, our results show a potential of abortion rates as high as 19% (*Dryopteris fragrans*, sample 4). Although not yet backed by proper experiments, environmental stress is sometimes evoked to explain these abnormalities (Arosa et al. 2009) as various environmental or seasonal factors are known to affect spore production (Odland 1998; Greer and McCarthy 2000; Mesipuu et al. 2009). Braithwaite (1964) studied the apomictic *Aspleniun aethiopicum* Bech. (Aspleniaceae), which produced a high amount of aborted spores after producing an overabundance of viable spores the previous season. It is certainly possible that similar mechanisms can affect SAI in sexual taxa as well. Further studies on the effect of various external and internal conditions on SAI are needed to properly explain abnormal spore abortion of some plants.

In ferns, aborted spores are usually used as an important character for the detection of hybrids (Wagner and Chen 1965; Ekrt et al. 2010). In concordance with general expectations, our study confirmed very high spore abortion rates in both triploids and tetraploid hybrids of sexual species (SAI more than 98%). However, *Dryopteris × critica* represents a special case as a pentaploid hybrid of sexual *D. filix-mas* and apomictic *D. borreri*. This taxon is capable of forming a proportion of developed spores thus produce new entities (Bär and Eschelmüller 2010; Ekrt and Koutecký 2016). Spore abortion rate of *Dryopteris × critica* reached mean 93.2% in this study, and published data indicate 80–95% SAI (Eschelmüller 1998; Fraser-Jenkins 2007; Ekrt and Koutecký 2016). Furthermore, the existence of a minor portion of developed spores in fern hybrids was revealed in several other studies in *Polystichum* Roth. (Dryopteridaceae) (Pinter 1995), *Osmunda* L. (Osmundaceae) (Yatabe et al. 2011) and *Cystopteris* Bernh. (Cystopteridaceae) (Kawakami et al. 2010; Hanušová and Ekrt unpublished data). Further detailed reproductive studies are needed to fully understand this problem, and a standardization of the SAI estimate method may help in future endeavors.

Effects of ploidy levels on spore abortion

No difference in SAI was observed in our study between sexual diploids and tetraploids. Significant differences were observed for the random nested factor of species. Regarding ploidy levels, similar results were reached in several other studies. This factor had no effect on germination rates of herbaria specimens of *Pellaea* Link. (Adiantaceae) (Windham and Ranker 1986). In *Psilotum nudum* (L.) P.Beauv.

(Psilotaceae), plants producing either haploid or diploid spore did not differ in both SAI and germination rates (Whittier and Braggins 1994). Notably Quintanilla and Escudero (2006) observed no difference between diploid and tetraploid *Dryopteris* in both germination rates and SAI. However, in the same study, the authors found a higher SAI in two samples of *D. corleyi* Fraser-Jenk. presuming that the increase in SAI is a result of a relatively recent origin of the not yet stabilized allotetraploid. Some of our samples of sexual species also had higher SAI, including the diploid *Dryopteris fragrans* (sample 4) at 19% abortion. Therefore, it is possible that other factors (environmental, seasonal) may be at play, as mentioned in the chapter above.

Several studies dealing with spore germination rates show a difference between diploids and polyploids. Comparably lower germination rates were found in diploids for the *Polystichum aculeatum* group (Pangua et al. 2003), *Polypodium virginianum* L. (Polypodiaceae) (Kott and Peterson 1973) and *Isoëtes* L. (Isoetaceae), where germination rates increased with ploidy level among diploids, tetraploids and decaploids (Kott and Britton 1982). Polyploids tend to have alternate or wider distribution, ecological niches and are more efficient colonizers, when compared to diploids (Vogel et al. 1999; Haufler et al. 2016). As Kott and Peterson (1973) suggest, the difference in germination rates between diploids and polyploids may be a result of various factors, including substrate preferences of viable spores.

Different rates of spore abortion among genera

Our results show a marginally significant difference in SAI between sexual taxa of the species richest genera *Asplenium* and *Dryopteris*. However, this difference is comparable to the difference between the species within their respective genus. Our sampling covers a phylogenetical cross section of species in *Asplenium* (Schneider et al. 2004) as well as species from several groups within *Dryopteris* including the most basal *D. fragrans* (Sessa et al. 2012). The marginally significant differences could reflect different habitat preferences or different position of phylogeny tree. Spore retention during the season may also be reflected in SAI estimates.

Spore abortion index (SAI) as an informative and standardized tool

We employed SAI in a wide and representative dataset of 109 specimens from 23 fern taxa. The result denoted a robust comparison among particular taxa or particular groups to verify hypotheses of differing amounts of aborted spores in species with different reproduction mode. According to our results, we consider SAI a very promising tool in the study of reproduction in spore producing plants.

Theoretically calculated values of standard error of estimate demonstrate the considerable potential error made by using an insufficient amount of spores. While the error may seem low when counting 100 spores with real SAI being 5%, it is important to consider the proportion of the mistake to the actual SAI. Additionally, exploring cumulatively calculated partial SAI for sampled taxa, it is clear that some sample's partial SAI started approaching total SAI only after more than 500 spores had been calculated. Calculating SAI using only 100 spores is highly insufficient, and for appropriate accuracy of results 1000 spores should be analyzed.

Furthermore a significant level of variation of SAI within a single frond was found for about 10% of plants with almost as much being marginally significant. One sample had the difference between the two parts almost as high as its total SAI, 18.2 and 20.9%, respectively. This factor may considerably affect SAI estimate accuracy when only one part of the frond is used, which is, to our knowledge, common practice. Therefore, using at least two distinct parts of the frond is suitable, at least for apomictic taxa. We also recommend avoiding fronds or parts of fronds that have already shed a majority of spores as well as damaged plants or plants growing in extremely suboptimal conditions. Following these guidelines will hopefully provide an accurate estimate of total SAI taking into account several factors analyzed in this study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Information on Electronic Supplementary Material

Online Resource 1. We list all of the plants used alongside herbaria specimen data and SAI (spore abortion index).
Online Resource 2. We list all of the samples used presented with several measures of variation. Among them are the range of SAI within individual sets of 100 spores as well as the difference between the two parts of the frond.

References

Aragon CF, Pangua E (2004) Spore viability under different storage conditions in four rupicolous *Asplenium* L. Taxa. Amer Fern J

94:28–38. doi:10.1640/0002-8444(2004)094[0028:SVUDSC]2.0.CO;2

Arosa ML, Quintanilla LG, Ramos JA, Ceia R, Sampaio H (2009) Spore maturation and release of two evergreen Macaronesian ferns, *Culcita macrocarpa* and *Woodwardia radicans*, along an altitudinal gradient. Amer Fern J 99:260–272. doi:10.1640/0002-8444-99.4.260

Bär A, Eschelmüller A (2010) Farnstudien: Einige pentaploide Bastarde von *Dryopteris filix-mas* mit triploiden Vertretern der *Dryopteris*-Gruppe. Ber Bayer Bot Ges 80:119–140

Blockeel TL (2006) The liverworts, mosses and ferns of Europe. Harley Books, Colchester

Braithwaite AF (1964) A new type of apogamy in ferns. New Phytol 63:293–305

Dafni A, Firmage D (2000) Pollen viability and longevity: practical, ecological and evolutionary implications. Pl Syst Evol 222:113–132. doi:10.1007/BF00984098

Danihelka J, Chrtek J Jr, Kaplan Z (2012) Checklist of vascular plants of the Czech Republic. Preslia 84:647–811

Dell Inc. (2015) Dell Statistica (data analysis software system), version 13. Available at: software.dell.com

Dyer RJ, Savolainen V, Schneider H (2012) Apomixis and reticulate evolution in the *Asplenium monanthes* fern complex. Ann Bot (Oxford) 110:1515–1529. doi:10.1093/aob/mcs202

Ekrt L, Koutecký P (2016) Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (*Dryopteridaceae*). Ann Bot (Oxford) 117:97–106. doi:10.1093/aob/mcv152

Ekrt L, Štech M (2008) A morphometric study and revision of the *Asplenium trichomanes* group in the Czech Republic. Preslia 80:325–347

Ekrt L, Trávníček P, Jarolímová V, Vít P, Urfus T (2009) Genome size and morphology of the *Dryopteris affinis* group in Central Europe. Preslia 81:261–280

Ekrt L, Holubová R, Trávníček P, Suda J (2010) Species boundaries and frequency of hybridization in the *Dryopteris carthusiana* (*Dryopteridaceae*) complex: a taxonomic puzzle resolved using genome size data. Amer J Bot 97:1208–1219. doi:10.3732/ajb.0900206

Eschelmüller A (1993) *Dryopteris remota* vom "Wachterl" keimt am besten. Mitteilungen des Naturwissenschaftlichen Arbeitskreises Kempten 30:5–22

Eschelmüller A (1998) Keimversuche mit Sporen der triploiden Sippen von *Dryopteris affinis* und ihren Bastarden mit *Dryopteris filix-mas*. Mitt Naturwiss Arbeitskreises Kempten 36:47–78

Fraser-Jenkins CR (2007) The species and subspecies in the *Dryopteris affinis* group. Fern Gaz 18:1–26

Gastony GJ, Windham MD (1989) Species concepts in pteridophytes: the treatment and definition of agamosporous species. Amer Fern J 79:65–77

Gomes SG, Randi AM, Puchalskil A, Santos DDS, dos Reis MS (2006) Variability in the germination of spores among and within natural populations of the endangered tree fern *Dicksonia sellowiana* Hook. (Xaxim). Brazil Arch Biol Technol 49:1–10. doi:10.1590/S1516-89132006000100001

Greer GK, McCarthy BC (2000) Patterns of growth and reproduction in a natural population of the fern *Polystichum acrostichoides*. Amer Fern J 90:60–76. doi:10.2307/1547415

Guo ZY, Liu HM (2013) Gametophyte morphology and development of three species of *Cyrtogonellum* Ching (*Dryopteridaceae*). Amer Fern J 103:153–165. doi:10.1640/0002-8444-103.3.153

Hanušová K, Ekrt L, Vít P, Kolář F, Urfus T (2014) Continuous morphological variation correlated with genome size indicates frequent introgressive hybridization among *Diphasiastrum* species (Lycopodiaceae) in Central Europe. PLoS ONE 9:e99552. doi:10.1371/journal.pone.0099552

Haufler CH, Pryer KM, Schuettlepz E, Sessa EB, Farrar DR, Moran R, Schneller JJ, Watkins JE, Windham M (2016) Sex and the single gametophyte: revising the homosporous vascular plant life cycle in light of contemporary research. BioScience (in press). doi:10.1093/biosci/biw108

Hernández MA, Andrada AR, Páez VDLA, Martínez OG (2015) Ploidy level and obligate apogamy in two populations of *Argyrochosma nivea* var. *tenera* (Pteridaceae). Hoehnea 42:233–237. doi:10.1590/2236-8906-36/2014

Johnson MTJ, Smith SD, Rauscher MD (2010) Effects of plant sex on range distributions and allocation to reproduction. New Phytol 186:769–779. doi:10.1111/j.1469-8137.2010.03201.x

Kawakami SM, Kawakami S, Kato J, Kondo K, Smirnov SV, Damdinsuren O (2010) Cytological study of a fern *Cystopteris fragilis* in Mongolian Altai. Chromosome Bot 5:1–3

Khare PB, Kaur S (1983) Gametophyte differentiation of pentaploid *Pteris vittata* L. Proc Natl Acad Sci India 49:740–742

Kott LS, Britton DM (1982) A comparative study of spore germination of some *Isoëtes* species of northeastern North America. Canad J Bot 60:1679–1687

Kott LS, Peterson RL (1973) A comparative study of gametophyte development of the diploid and tetraploid races of *Polypodium virginianum*. Canad J Bot 52:91–96

Lovis JD (1977) Evolutionary patterns and processes in ferns. Advances Bot Res 4:229–415

Manton I (1950) Problems of cytology and evolution in the Pteridophyta. Cambridge University Press, Cambridge

Mesipuu M, Shefferson RP, Kull T (2009) Weather and herbivores influence fertility in the endangered fern *Botrychium multifidum* (S.G. Gmel.). Rupr. Pl Ecol 203:23–31. doi:10.1007/s11258-008-9501-3

Nakato N, Ootsuki R, Murakami N, Masuyama S (2012) Two types of partial fertility in a diploid population of the fern *Thelypteris decursive-pinnata* (Thelypteridaceae). J Pl Res 125:465–474. doi:10.1007/s10265-011-0461-7

Odland A (1998) Size and reproduction of *Thelypteris limbosperma* and *Athyrium distentifolium* along environmental gradients in Western Norway. Nordic J Bot 18:311–321. doi:10.1111/j.1756-1051.1998.tb01882.x

Pangua E, Quintanilla LG, Sancho A, Pajarón S (2003) A comparative study of the gametophytic generation in the *Polystichum aculeatum* group (Pteridophyta). Int J Pl Sci 164:295–303. doi:10.1086/346165

Park C, Kato M (2003) Apomixis in the interspecific triploid hybrid fern *Cornopteris christenseniana* (*Woodsiaceae*). J Pl Res 116:93–103. doi:10.1007/s10265-003-0081-y

Pinter I (1995) Progeny studies of the fern hybrid *Polystichum × bicknellii*. Fern Gaz 15:25–40

Quintanilla LG, Escudero A (2006) Spore fitness components do not differ between diploid and allotetraploid species of *Dryopteris* (*Dryopteridaceae*). Ann Bot (Oxford) 98:609–618. doi:10.1093/aob/mcl137

R Core Team (2014) R: A language and environment for statistical-computing. R Foundation for Statistical Computing. Vienna, Austria. http://www.R-project.org/

Schneider H, Russell SJ, Cox CJ, Bakker FT, Henderson S, Rumsey F, Barrett J, Gibby M, Vogel JC (2004) Chloroplast phylogeny of asplenioid ferns based on rbcL and trnL-F spacer sequences (*Polypodiidae*, *Aspleniaceae*) and its implications for biogeography. Syst Bot 29:260–274. doi:10.1600/036364404774195476

Sessa EB, Zimmer EA, Givnish TJ (2012) Phylogeny, divergence times, and historical biogeography of New World *Dryopteris* (*Dryopteridaceae*). Amer Fern J 99:730–750. doi:10.3732/ajb.1100294

Thiers B (2016) Index Herbariorum: a global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. Available at: http://sciweb.nybg.org/science2/IndexHerbariorum.asp. Accessed Aug 2016

Vogel JC, Rumsey FJ, Schneller JJ, Barrett JA, Gibby M (1999) Where are the glacial refugia in Europe? Evidence from pteridophytes. Biol J Linn Soc 66:23–37. doi:10.1111/j.1095-8312.1999.tb01915.x

Wagner WH Jr, Chen LC (1965) Abortion of spores and sporangia as a tool in the detection of Dryopteris hybrids. Amer Fern J 55:9–29

Walker TG (1962) Cytology and evolution in the fem genus Pteris L. Evolution 16:27–43

Whittier DP, Braggins JE (1994) Spore germination in the Psilotaceae. Canad J Bot 72:688–692. doi:10.1139/b94-089

Windham MD, Ranker TA (1986) Factors affecting prolonged spore viability in herbarium collections of three species of Pellaea. Amer Fern J 76:141–148. doi:10.2307/1547722

Yatabe Y, Yamamota K, Tsutsumi C, Shinohara W, Murakami N, Kato M (2011) Fertility and precocity of Osmunda × intermedia offspring in culture. J Pl Res 124:265–268. doi:10.1007/s10265-010-0374-x

Paper 2

Ekrt L. & Koutecký P. (2016): Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (Dryopteridaceae). – Annals of Botany 117: 97–106.

Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (Dryopteridaceae)

Libor Ekrt* and Petr Koutecký

Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760,
CZ-370 05 České Budějovice, Czech Republic
* For correspondence. E-mail libor.ekrt@gmail.com

● **Background and Aims** In ferns, apomixis is an important mode of asexual reproduction. Although the mechanisms of fern reproduction have been studied thoroughly, most previous work has focused on cases in which ferns reproduce either exclusively sexually or exclusively asexually. Reproduction of ferns with potentially mixed systems and inheritance of apomixis remains largely unknown. This study addresses reproduction of the pentaploid *Dryopteris × critica*, a hybrid of triploid apomictic *D. borreri* and tetraploid sexual *D. filix-mas*.
● **Methods** Spore size, abortion percentage and number of spores per sporangium were examined in pentaploid plants of *D. × critica* grown in an experimental garden. The sporangial content of leaf segments was cultivated on an agar medium, and DNA ploidy levels were estimated by DAPI flow cytometry in 259 gametophytes or sporophytes arising from the F_2 generation of the pentaploid hybrid.
● **Key Results** The hybrid is partly fertile (89–94 % of aborted spores) and shows unstable sporogenesis with sexual and apomictic reproduction combined. The number of spores per sporangium varied from approx. 31 to 64. Within a single sporangium it was possible to detect formation of either only aborted spores or various mixtures of aborted and well-developed reduced spores and unreduced diplospores. The spores germinated in viable gametophytes with two ploidy levels: pentaploid ($5x$, from unreduced spores) and half of that (approx. $2·5x$, from reduced spores). Moreover, 2–15 % of gametophytes (both $2·5x$ and $5x$) formed a viable sporophyte of the same ploidy level due to apogamy.
● **Conclusions** This study documents the mixed reproductive mode of a hybrid between apomictic and sexual ferns. Both sexual reduced and apomictic unreduced spores can be produced by a single individual, and even within a single sporangium. Both types of spores give rise to viable F_2 generation gametophytes and sporophytes.

Key words: Apogamy, apomixis, diplospores, *Dryopteris affinis* agg., ferns, flow cytometry, frequency of hybridization, hybrid fertility, plant mating system, spore abortion percentage, sporogenesis.

INTRODUCTION

While sexual reproduction is a process that creates a new genetic entity by combining the genetic material of two parental individuals, asexual reproduction is confined to one genetic entity and maintains its integrity even in the case of imperfect reproductive isolation from other entities. Apomixis produces progeny asexually by different means both in flowering plants (Ozias-Akins, 2006; Krahulcová *et al.*, 2013) and in ferns (Döpp, 1939; Manton, 1950). Among ferns, apomixis evolved several times independently and its frequency is at least 3 %, a value much higher than in other major plant groups (Liu *et al.*, 2012). However, most apomictic fern species are concentrated in just four families (Liu *et al.*, 2012).

Apomixis in ferns includes apogamy – the formation of sporophytes from somatic cells of the prothallium – and agamospory – the production of unreduced (diplo)spores (Manton, 1950; Lovis, 1977; Walker, 1979; Gastony and Windham, 1989). The archesporial cell of sexual fern species usually undergoes four mitoses to produce 16 spore mother cells that

undergo regular meiosis, resulting in 64 reduced spores in 16 tetrads. Under the prevailing type of agamospory (Döpp–Manton scheme) the last (premeiotic) mitosis fails, resulting in eight spore mother cells that undergo regular meiosis, producing 32 diplospores in eight tetrads (Döpp, 1939; Manton, 1950; Walker, 1979). Rarely (Braithwaite scheme), the first meiotic division fails, which results in 32 diplospores in 16 diads (Braithwaite, 1964). Unlike in angiosperms, regular meiosis is present under the Döpp–Manton type of agamospory. Homologous pairing and crossing-over are thus present and were recently recognized as the possible mechanisms of formation of genetically different spores (Lin *et al.*, 1992). Genetic variation among apomictic offspring has been documented (Ishikawa *et al.*, 2003; Schneller and Krattinger, 2010; Ootsuki *et al.*, 2012). In contrast to flowering plants, the fern apomicts are obligate (Lovis, 1977). The only reported case of facultative apomixis among ferns, *Asplenium hallbergii*, remains under study (Dyer *et al.*, 2012). Autopolyploidy, hybridization or fusion of reduced and unreduced gametes may play a role in

formation in the apomictic polyploid ferns (Barrington *et al.*, 1989; Park and Kato, 2003; Grusz *et al.*, 2009; Hunt *et al.*, 2011; Chao *et al.*, 2012; Liu *et al.*, 2012).

In sexual fern hybrids, an abnormal meiosis yields variable percentages of aborted (non-viable) or atypical spores of different size and shape from regular spores (Wagner and Chen, 1965; Gabriel y Galán and Prada, 2011; Zhang *et al.*, 2013) and these hybrids are sterile or nearly so (Wagner and Chen, 1965; Reichstein, 1981).

Hybridization of fern apomicts with related sexual taxa can give rise to new fertile apomictic taxa of higher ploidy levels via diplospores of the apomictic parent (Gastony and Windham, 1989; Fraser-Jenkins, 2007; Grusz *et al.*, 2009; Regalado Gabancho *et al.*, 2010; Dyer *et al.*, 2012). The prothallia of apomictic ferns normally lack functional archegonia but may possess functional antheridia, releasing unreduced spermatozoids that are capable of fertilizing the archegonia of sexual species. It is believed that the resulting hybrids inherit the apomictic mode of reproduction from their male parents (Döpp, 1955; Walker, 1979; Gastony and Windham, 1989; Windham and Yatskievych, 2003; Regalado Gabancho *et al.*, 2010; Liu *et al.*, 2012). However, some studies have reported mixed meiosis in these hybrids with joint existence of eight-celled (apomictic) and 16-celled (sexual-like) meiosis in one plant (Schneller, 1975; Dyer *et al.*, 2012). Thus, spore formation and offspring constitution and viability remain unresolved in the sexual × apomictic fern hybrids.

In Europe, the most thoroughly studied group was the apomictic complex *Dryopteris affinis* agg. (Manton, 1950; Döpp, 1955; Schneller, 1975; Fraser-Jenkins, 2007; Bär and Eschelmüller, 2010; Schneller and Krattinger, 2010). In Central Europe it consists of diploid *D. affinis* and triploid *D. borreri* and *D. cambrensis* (see Ekrt *et al.*, 2009, for ploidy levels and genome sizes). The widespread sexual *D. filix-mas* is capable of hybridization with apomictic taxa, resulting in tetraploid and pentaploid hybrids. Hybrids form both aborted and well-developed spores (Schneller, 1975; Eschelmüller, 1998; Fraser-Jenkins, 2007; Ekrt *et al.*, 2009; Bär and Eschelmüller, 2010) that are able to germinate. Manton (1950), in her famous study of fern cytology and reproduction, described experiments with 'pentaploid *D. borreri*' (i.e. *D.* × *critica*) in which she observed apparently functional spores beside aborted ones and germination of gametophytes from these spores, including probably also those from sexual-like sporangia with 16 spore mother cells. However, no further information on these offspring is available (and could hardly be so using the methods of that time). Schneller (1975) also reported the occurrence of karyologically variable and mostly aneuploid offspring (gametophytes) of the pentaploid *D.* × *critica*.

Our case study follows the above studies. We focused on fertility and offspring viability of the pentaploid hybrid *Dryopteris* × *critica* ($2n = 205$), which is the hybrid between triploid apomictic *D. borreri* ($2n = 123$) and tetraploid sexual *D. filix-mas* ($2n = 164$). Here, we attempt to answer the following questions: (1) What is the portion of aborted and viable spores? (2) Does the pentaploid F_1 hybrid produce viable F_2 offspring, and if so, are any offspring gametophytes able to form sporophytes? (3) What is the pattern of genome size/ploidy levels among maternal plants and gametophytes/sporophytes arisen from spores of

the F_1 pentaploid hybrid? (4) What is the frequency of hybridization at sites of common occurrence of parental taxa *D. borreri* and *D. filix-mas* in the wild?

METHODS

Spore size and abortion

During a previous study of *Dryopteris affinis* agg. in Central Europe (Ekrt *et al.*, 2009), only a few pentaploid plants (*Dryopteris* × *critica*) were detected in the wild. Two plants from different locations (STO, KUR, see Table 1) were transplanted into the experimental garden. To avoid contamination with fern spores from the surrounding area, one leaf of each plant was enveloped with several layers of UHELON 130T Extra textile, 25-μm mesh size (Silk & Progress, s.r.o., Brněnec, Czech Republic). The mature fertile fronds were collected at the start of spontaneous snapping, wrapped in paper sheets and dried at room temperature to release sporangial contents. Spore size and abortion percentage of the experimental plants were studied to estimate spore fitness. Spores were investigated under a light microscope (Olympus CH30) at 400× magnification. The spore abortion percentage was estimated in a random sample of 1000 spores per plant. Spores were considered to be aborted when they lacked the protoplast or were collapsed (Quintanilla and Escudero, 2006). Exospore length was examined in a random sample of 200 well-shaped spores at 1000× magnification. The central part of a frond bearing ripe and still undehisced sporangia was fixed in 50 % ethanol. Under the light microscope, a sporangium was opened by a thin needle in a drop of water and sporangial content was examined. The number of spores (including aborted spores) per sporangium and exospore length of well-developed spores were recorded in 15 separate sporangia.

Because we observed a clearly bimodal distribution of exospore length, we analysed it as a mixture of two types of spores using R 3.1.2 software (R Development Core Team, 2014). As dependence of variance on a mean and on positively skewed lognormal distributions can be expected and was apparent from preliminary analysis, we log-transformed the data. We then modelled the log-transformed data as a mixture of two Gaussian distributions using the normalmixEM function from the mixtools package (Benaglia *et al.*, 2009).

Spore germination

Sporangial content of two leaf segments per experimental plant was poured out and cultivated in four replicates on Petri dishes (6 cm in diameter) with mineral agar BG11 (Stanier *et al.*, 1971) at 19 °C, light intensity of approx. 50 μE and 16/8 h light–dark. The dishes were sealed with Parafilm to reduce contamination and prevent excessive water loss. Young gametophytes were transplanted into Petri dishes with a sterilized peat/sand mixture (3 : 1) and were placed approx. 0·5 cm from one another. During 5 months of cultivation, well-developed gametophytes and young sporophytes (if present) were examined by flow cytometry (FCM).

Table 1. *Localization of plants used in the study; herbarium vouchers are deposited in the herbarium CBFS*

Locality code	Location	Altitude (m)	Coordinates (WGS 84)	No. of plants examined
STO	Czech Republic, Šumava Mts, Stožec: beech forest in the Stožec Mt approx. 750 m E of the summit of Mt Stožec	915	48°52′55.6″N, 13°49′52.8″E	31
KUR	Slovakia, Malá Fatra Mts, Krasňany: bottom part of Kúr valley approx. 3.5 km SE of the church in the village of Krasňany	605	49°11′41.6″N, 18°55′45.5″E	143
KNE	Czech Republic, Moravskoslezské Beskydy Mts, Čeladná: massif of Kněhyně Mt, valley of Korábský stream in foothills of Malá Stolová Mt approx. 4.4 km SSW of the church in the village of Čeladná	640	49°30′39.1″N, 18°19′44.6″E	90
PEC	Czech Republic, Šumava Mts, Nová Pec: deforested line in the N slope of Smrčina Mt approx. 1.6 NNE of the summit	930	48°45′34.2″N, 13°55′50.4″E	81

Screening of wild populations

Localities of both experimental plants (STO, KUR) and two other localities (KNE, PEC) were screened for genome size variation using FCM (Table 1). At each locality, the study plot of approx. 100×150 m was established and leaves of all plants with *D. affinis* agg. morphology and juvenile (undeterminable) individuals were collected. We also collected a smaller number of individuals of *D. filix-mas*, which is dominant in the localities and their surroundings. The leaves were stored moist in plastic bags up to 4 d for FCM analyses. Voucher specimens are stored in the herbarium CBFS.

Flow cytometry

Relative DNA content and DNA ploidy levels were determined using a Partec PA II flow cytometer (Partec GmbH., Münster, Germany) equipped with a mercury arc lamp. Fresh material was analysed (field-collected leaves or cultivated gametophytes/sporophytes). Samples were prepared following the simplified two-step protocol of Doležel *et al.* (2007). For adult leaves, approx. $2 \, cm^2$ of intact leaf tissue was chopped with a sharp razor blade together with approx. $0.25 \, cm^2$ of an internal standard leaf (*Vicia faba* 'Inovec', $2C = 26.90$ pg; Doležel *et al.*, 1992) in a plastic Petri dish containing 0.5 mL of ice-cold Otto I buffer (0.1 M citric acid, 0.5 % Tween-20). The suspension was filtered through a 42-μm nylon mesh and incubated for at least 5 min at room temperature. After incubation, 1 mL of the staining solution was added. The staining solution consisted of 1 mL of Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$), 2-mercaptoethanol ($2 \, \mu L \, mL^{-1}$) and the fluorochrome DAPI ($4 \, \mu L \, mL^{-1}$). Samples were run on the flow cytometer after approx. 1 min of staining and the fluorescence intensity of 3000–5000 particles was recorded. For screening of ploidy levels, pooled samples of up to five individuals could be used as we utilized high-resolution histograms and owing to the absence of endopolyploidy. Nevertheless, each plant was separately re-analysed if the occurrence of more DNA ploidy levels in the pooled sample was suspected. For gametophytes and young sporophytes, the same method was used but the amount of available plant material was much smaller. Only large well-developed gametophytes were measurable (yielded enough nuclei). We used the whole gametophyte/one young leaf of a sporophyte and small amount ($<2 \times 2$ mm) of the internal standard. Although we ran the whole volume of the sample, we

were usually not able to record the usual 3000 particles per sample even with the largest gametophytes; however, the scored peaks were clear and included at least several hundred nuclei. We did not use pooled samples for gametophytes/young sporophytes.

For calibration, cultivated individuals of triploid *Dryopteris borreri* and tetraploid *D. filix-mas* with known chromosome counts (Ekrt *et al.*, 2009) were analysed. The fluorescence histograms were evaluated using FloMax 2.6 software provided by Partec.

Differences between mean DNA contents of two groups within one DNA ploidy level were compared using *t*-tests with separate variance estimates and Welch approximation of the degrees of freedom.

RESULTS

Spore size and abortion

The majority of spores were aborted on both experimental plants of *D.* × *critica* (hereafter E_STO and E_KUR): 93·6 and 88·6 %, respectively, based on 1000 spores each (Fig. 1). The exospore length of well-developed spores showed a clear bimodal distribution (Fig. 2) in both experimental plants. Despite the relatively low number of observations ($N = 200$ for both plants) the statistical model shows similar values for both experimental plants: the mode of exospore length is estimated to be approx. 33 μm for the smaller spores and 47–51 μm for the larger spores (Fig. 2).

Number of spores per sporangium

The content of single sporangia of the experimental plant E_STO, which produces viable reduced and unreduced spores (see below), was examined in detail. We studied 15 randomly selected sporangia. The number of spores per sporangium varied markedly from 31 to 64. The counts did not fit the textbook apomictic/sexual number of 32/64 spores per sporangium. The vast majority of spores were aborted (72·6 %). The number of well-developed spores per sporangium was variable, ranging from zero to 29. The two size classes of well-developed spores were apparent. Several different types of sporangia were detected: sporangia with all spores aborted, sporangia with a mixture of aborted and small spores or a mixture of aborted and
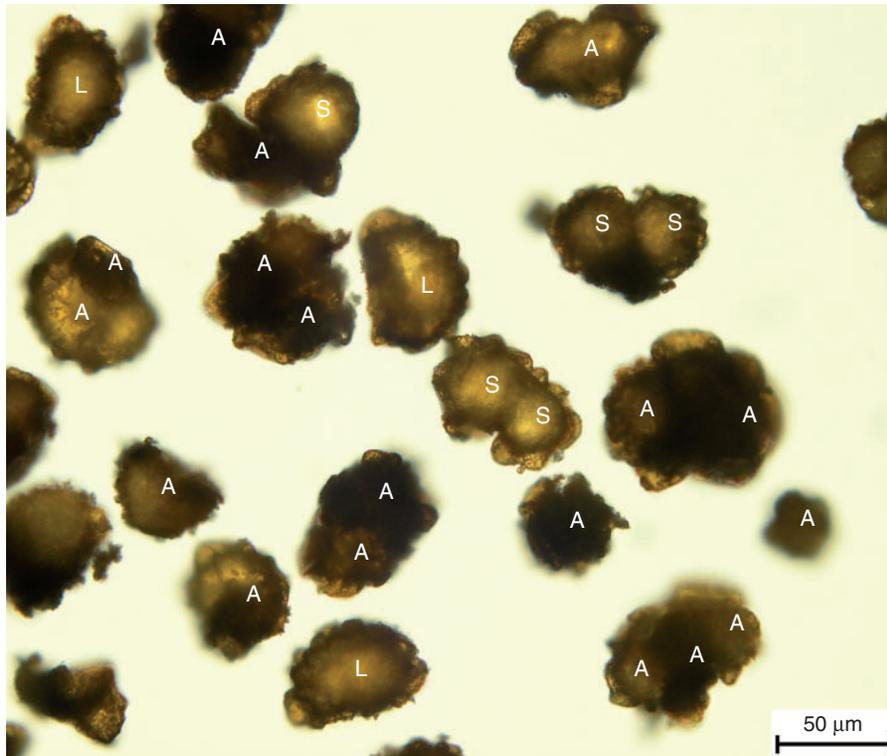
Fig. 1. Three types of spores detected in the experimental plant E_KUR. L = large well-developed spores; S = small well-developed spores; A = aborted spores (lacking the protoplast, collapsed or of irregular shape).

large spores, and sporangia with a mixture of aborted, small and large spores together (Table 2, Fig. 3).

Spore germination and sporophyte formation

All gametophytes germinated from spores of pentaploid *D.* × *critica* were filamentous at the beginning and followed the normal trend of development to the predominantly cordate phase. The vast majority of the gametophytes remained in the gametophyte stage. Only a small percentage formed antheridia and yielded sporophytes: 14·7 % (89 out of 606) in E_STO and 1·7 % (15 out of 877) in E_KUR. Gametophytes that did not form sporophytes survived for approx. 8–32 months and then died. Sporophytes were formed from the central or lower region of a gametophyte either as single 'normal' viable plants or rarely through a callus-like sporophytic growth (several plants originating from E_KUR). In some cases, sporophytes were deformed, having enormous pinna segmentation or split terminal leaf segments. An origin from apogamy appeared to be obligate in all the sporophytes studied.

Genome size of F$_2$ offspring

FCM screening of the offspring (gametophytes) of pentaploid *Dryopteris* × *critica* surprisingly revealed two cytotypes (Fig. 4, Table 3). One of them corresponded to the maternal plants and other pentaploids found in natural populations (Table 3). The other has a genome size approximately half of the pentaploids and is tentatively marked as $2·5x$ in this paper. The two experimental plants differed strongly in the frequency of cytotypes: in E_KUR only $2·5x$ offspring were found ($N = 110$), while in E_STO 55 % of offspring were $2·5x$ and 45 % were pentaploids ($N = 149$).

There was considerable variation in the relative DNA content (genome size) among F_2 gametophytes (Table 3). In the $2·5x$ cytotype, genome size variation between gametophytes originating from one maternal plant reached 18·5 and 23·4 % in E_KUR and E_STO, respectively. The differences between individual gametophytes were corroborated also by simultaneous FCM analysis (Fig. 4D). The mean values of these two groups were also significantly different ($t = 4·28$, d.f. $= 144·90$, $P = 3 \times 10^{-5}$). Among pentaploid gametophytes (E_STO maternal plant), variation reached 5·9 %. Although this variation is higher than among field-collected pentaploid sporophytes (3·6 %), we did not observe any bifurcated peaks in simultaneous analyses of additional gametophytes and, taking the relatively low number of nuclei (lower precision of the analyses) into account, this variation might be attributed to random measurement error. Interestingly, there was a small but significant difference between mean relative DNA contents of the experimental pentaploid gametophytes and field-collected pentaploid sporophytes ($t = 4·54$, d.f. $= 35·08$, $P = 6 \times 10^{-5}$).

Relative genome sizes of gametophytes and sporophytes emerging from them were compared to confirm apomictic (apogamous) formation of the F_2 sporophytes. We analysed 24 gametophyte–sporophyte pairs from the E_STO experimental plant (12 of $5x$ ploidy level and 12 of $2·5x$ ploidy level) and
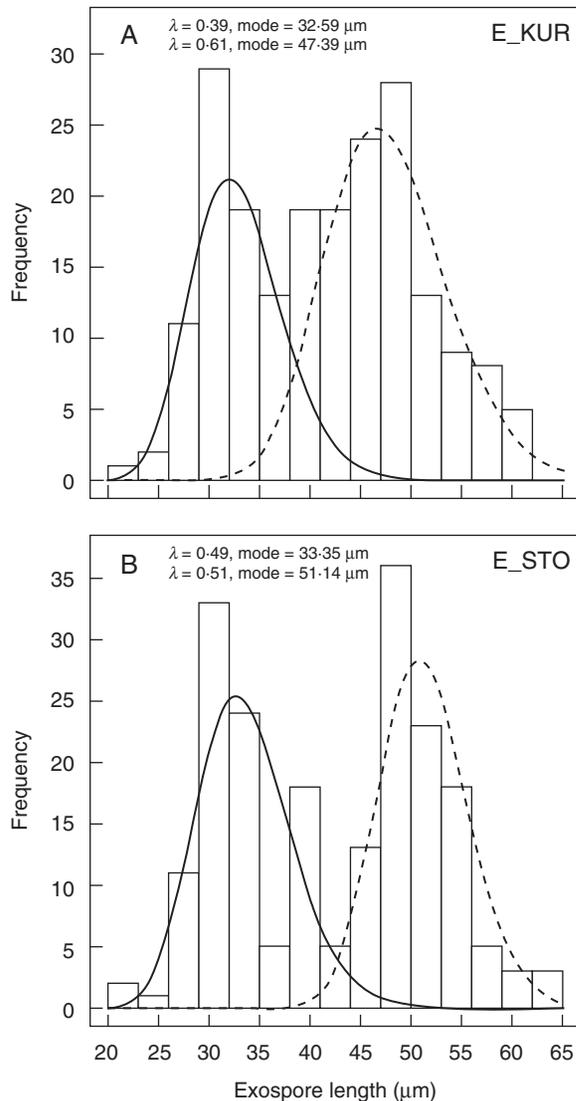
FIG. 2. Histograms of the exospore length of well-developed spores. Two size classes are apparent in each plant, and are visualized as two lognormal distributions (*λ*, proportion of spores that belong to the particular distribution). The parameters of the lognormal distributions were obtained from modelling a mixture of two Gaussian distributions based on log-transformed data.

five pairs from the E_KUR experimental plant (all of $2.5x$ ploidy level). In all cases the relative genome sizes were identical within the gametophyte–sporophyte pair.

Screening of the wild populations

Three ploidy levels were revealed in all four sites. Plants of *D. affinis sensu lato* (*s.l.*) morphology comprised triploids (*D. borreri*) and pentaploids (*D. × critica*). The hybrids were not always recognizable from *D. borreri* based on frond morphology. The proportion of hybrids among *D. affinis s.l.* plants was similar within three sites (KUR, KNE, PEC; $10.5–16.3\%$ of hybrids), but there were many more hybrids in the fourth site STO (71.4% of hybrids). Plants of *D. filix-mas* morphology

TABLE 2. *Summary of spore counts in single sporangia (sg) in the pentaploid plant* Dryopteris × critica *(E_STO); well-developed spores were classified into two size classes (see Fig. 2): small spores (exospore length 20–42 μm) and large spores (42–65 μm)*

Sporangium no.	Spore count			
	Total	Aborted	Small	Large
sg 1	55	55	–	–
sg 2	64	64	–	–
sg 3	60	60	–	–
sg 4	42	35	7	–
sg 5	63	40	23	–
sg 6	43	27	8	8
sg 7	47	10	8	29
sg 8	31	15	8	8
sg 9	36	23	5	8
sg 10	39	15	12	12
sg 11	51	44	–	7
sg 12	51	30	–	21
sg 13	35	26	–	9
sg 14	61	41	–	20
sg 15	53	44	–	9

were all tetraploid; no tetraploid of *D. affinis s.l.* morphology was found. In the whole sample set of 345 plants, no $2.5x$ individuals were detected. Genome size variation within taxa did not exceed 3.5% and is well within the usual random measurement error (Table 3).

DISCUSSION

Spore viability in fern hybrids

We experimentally confirmed the fertility of the pentaploid hybrid *D. × critica*. Both aborted and well-developed spores were detected. The spore abortion rate was approx. 89–94 %. Similar proportions of 80–95 % of aborted spores were recorded also in previous studies of the *D. affinis* group (Eschelmüller, 1998; Fraser-Jenkins, 2007). Eschelmüller (1998) also studied spore viability: in the pentaploid *D. × critica*, 80·5 % (mean from nine plants) of spores were non-viable. Similar rates of approx. 66–80 % of non-viable spores (depending on time of spore evaluation) were observed in pentaploid *D. × critica* by Bär and Eschelmüller (2010). A much wider scale of spore abortion rate in hybrids of sexual and apomictic taxa was detected in the genus *Pteris*, where the proportion of aborted spores produced by the synthetic apomictic hybrids varied from 45 to 89 % (Walker, 1962). Compared with apomictic species, hybrids between sexual fern species are either completely sterile (Reichstein, 1981; Ekrt *et al.*, 2010) or produce only a minor proportion of viable spores (Vida and Reichstein, 1975; Pinter, 1995; Yatabe *et al.*, 2011).

In the present study, we did not focus on spore germination/viability but rather on sporophyte formation. We also had the opportunity to estimate ploidy level using FCM, which was not available to earlier researchers. The germinated gametophytes remained mostly in the gametophyte stage but 1·7 % E_KUR (all $2.5x$) and 14·7 % E_STO (both $2.5x$ and $5x$) developed sporophytes through apogamy. The existence of viable sporophytes of the F_2 generation arisen from the pentaploid F_1 hybrid was observed for the first time. The low rate of sporophyte
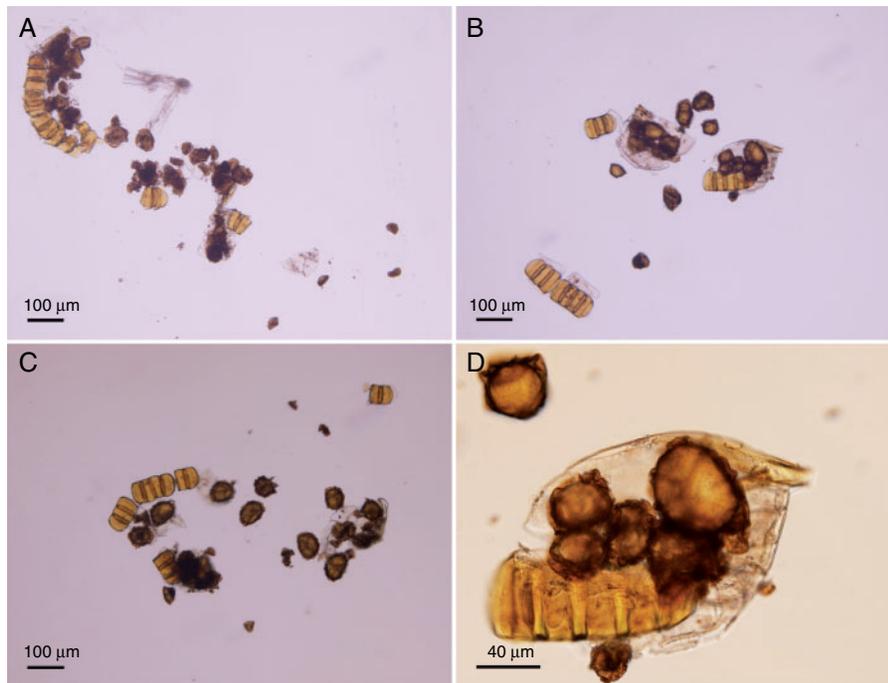
Fig. 3. Single sporangia content: (A) approx. 55 aborted spores (the sporangium sg 1); (B) a mixture of 15 aborted, eight small and eight large spores (sg 8); (C) a mixture of 26 aborted and nine large spores (sg 13); and (D) detail from B (sg 8) showing five small, one large and three aborted spores present in one sporangium.

production may be caused both by an unbalanced number of chromosomes in gametophytes originating from reduced (approx. 2·5*x*) spores or suboptimal environmental conditions for germination and growth.

Spore and genome size variation

We found two size classes of spores and two ploidy levels among gametophytes. The logical explanation would be that smaller spores are reduced while larger spores are unreduced. However, this simple theory is somewhat hampered by the fact that in the experimental plant E_KUR, only reduced 2·5*x* gametophytes were detected, although a bimodal distribution of spore sizes was present. This result would mean that the larger (unreduced) spores were unviable for some unknown reason. The correlation between genome size and spore size has recently been challenged in the *Asplenium monanthes* complex (Dyer *et al*., 2013). This analysis was based on between-species comparisons. When phylogenetic contrasts are applied, the relationship is likely to be valid within a species or between closely related species, and was also evident from the raw data in the *Asplenium monanthes* complex (Dyer *et al*., 2013).

In our data, the genome size variation among 2·5*x* gametophytes was enormous. However, such a result might be expected because (1) the maternal plant is of odd-ploidy level and regular chromosome pairing in meiosis is not possible and (2) the fourth mitosis-forming restitution nuclei in Döpp–Manton type agamospory may be irregular (see below). As a result of both the problems mentioned above, many spores are aborted and even those that are well developed vary somewhat in chromosome number/genome size. Nevertheless, a

small proportion of nearly balanced spores are able to germinate and some of the resultant gametophytes are even able to produce viable sporophytes. Chromosome number variation among the progeny of *D*. × *critica* was also observed by Schneller (1975).

We observed slightly higher but statistically significantly different genome size of the pentaploid gametophytes compared with more or less invariable pentaploid plants from natural populations (including the experimental maternal plant E_STO; the gametophytes are different even from this plant in a one-sample *t*-test). We are not aware of any mechanism that could explain such a difference; indeed, we attribute this result to technical issues. Besides the smaller numbers of nuclei in the gametophytes (i.e. lowering precision of the analysis) such a small shift might be caused by different levels of cytosolic compounds between the gametophytes and mature (sporophyte) leaves, which can influence fluorescence staining (Doležel *et al*., 2007).

Sporogenesis

Sporogenesis of apomictic *Dryopteris affinis* agg., including pentaploid *D*. × *critica*, was comprehensively studied by Manton (1950) (note that all cytotypes are marked as *D. borreri* in that study) and Schneller (1975) (under the names *D. pseudomas* and *D*. × *tavelii*). Whereas in apomictic diploids and triploids the eight-celled type of sporangium prevails, in tetraploid and pentaploid hybrids of an apomictic and sexual species, the 16-celled type predominates. The sporangia of hybrids are exceptional and combine the normal apomictic development with aborted and well-developed spores. Manton (1950) proposed
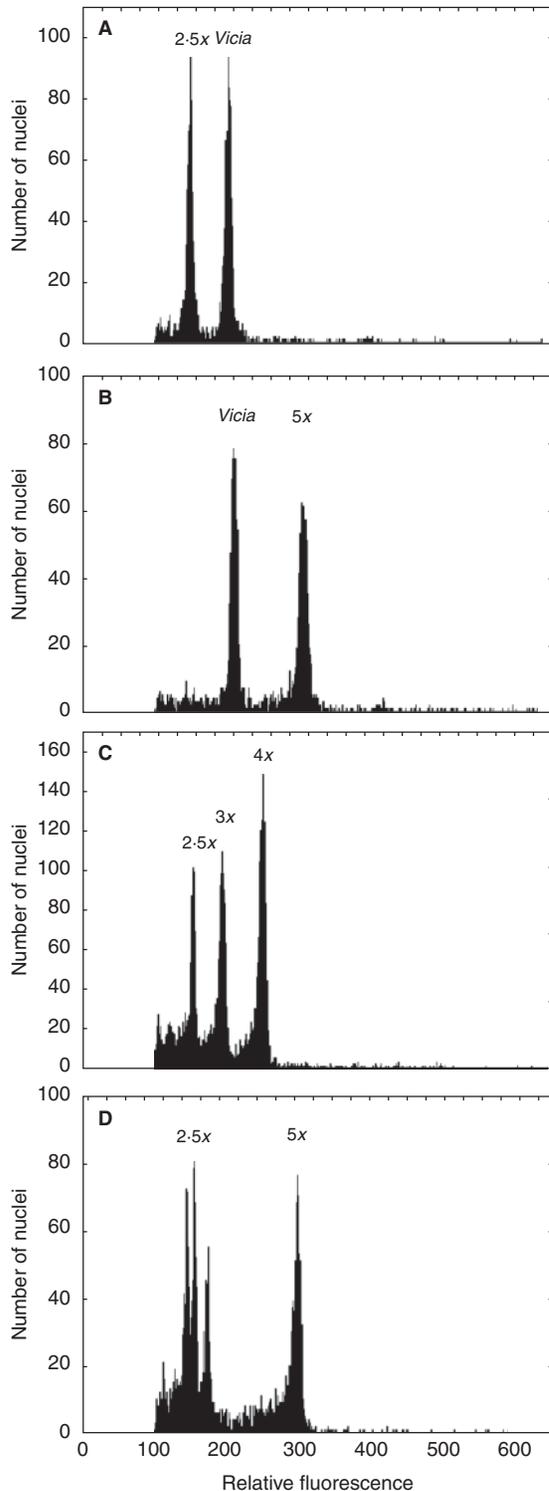
Fig. 4. Flow cytometric profiles (DAPI staining) of F_2 gametophytes of penta-ploid *Dryopteris × critica*: (A) 2.5x gametophyte and apogamous sporophyte analysed with the internal standard *Vicia faba*; (B) 5x gametophyte and apoga-mous sporophyte analysed with the internal standard *V. faba*; (C) simultaneous analysis of 2.5x F_2 gametophyte of *D. × critica*, triploid *D. borreri* and tetra-ploid *D. filix mas* (both with known chromosome count; Ekrt *et al.*, 2009); (D) simultaneous analysis of three 2.5x gametophytes and one 5x gametophyte arisen from one maternal plant (E_STO) – three separate peaks of 2.5x gameto-phytes are clearly visible, corroborating variation in the genome size between the gametophytes.

TABLE 3. *Relative DNA content of* F_2 *gametophytes (type = G) and field-collected sporophytes (type = S) assessed using FCM with DAPI staining; the value is expressed as the ratio to the in-ternal standard* Vicia faba *'Inovec', which is given a unit value*

Group	Type	N	Relative DNA content			CV (%)	Within-group variation (%)
			Mean	SE	Range		
2.5x (E_KUR)	G	98	0·702	0·003	0·639–0·757	1·54–3·97	18·5
2.5x (E_STO)	G	66	0·722	0·003	0·655–0·808	1·76–3·94	23·4
3x (field)	S	266	0·885	0·001	0·869–0·900	1·08–1·90	3·6
4x (field)	S	24	1·127	0·001	1·121–1·139	1·24–1·62	1·6
5x (E_STO)	G	37	1·465	0·003	1·422–1·506	1·52–3·94	5·9
5x (field)	S	55	1·442	0·001	1·412–1·460	1·09–1·90	3·4

N, number of observations – note that some lower-quality analyses (low number of nuclei or high peak CVs) could be classified to the ploidy level but were excluded from summary statistics of the relative genome sizes; s.e., stan-dard error of the mean; CV, coefficient of variation of the sample peak. Within-group variation describes genome size differences among samples from the respective group; it is expressed as the difference between the group maximum and minimum, which is set to 100 %.

that in these plants, there are a few large good spores that ger-minate and produce gametophytes and consequently sporo-phytes. These plants (F_2 generation) were not successfully analysed by Manton (1950) and died in culture. We repeated Manton's experiment and surprisingly revealed two size classes of spores and the 2.5x (reduced) plants and 5x (unreduced) plants arisen from the F_1 pentaploid hybrid. The existence of viable plants originating from reduced spores of the odd-ploidy-level parent has never before been observed in ferns.

Manton (1950) and Schneller (1975) also examined sporo-genesis of the same (or similar) hybrids in the *Dryopteris affinis* agg. They described the predominant formation of 16-celled (sexuality-like forming 64 spores) together with eight-celled (apomictic type forming 32 spores) sporangia on one plant. Manton (1950) also discovered an 'intermediate' type of sporangium and described its sporogenesis in detail in *Dryopteris borreri* s.l., *D. atrata*, *D. remota* and *Pteris cre-tica*. She observed that one or several restitution nuclei in an apomictic-type sporangium may exhibit irregularities leading to division into two unequal parts. Meiosis is then regular even in small nuclei, but due to unbalanced numbers of chro-mosomes the spores abort (Manton, 1950, p. 166). Because not all restitution nuclei are involved in this process, the resul-tant number of spores higher than 32 (no irregular division) and lower than 64 (division of all nuclei) and a mixture of un-balanced aborted spores together with 'normal' diplospores within a sporangium may be expected. These counts were usually studied in immature sporangia. Further deviation from the standard apomictic/sexual pathway was documented by Schneller (1975) who observed not only sporangia with either eight or 16 spore mother cells but also sporangia with inter-mediate counts and unequal cell size. This may indicate that some spore mother cells in a sporangium underwent the last mitosis (are reduced) while others did not (unreduced). Moreover, Schneller (1975) also reported the extremely rare occurrence of sporangia with only four spore mother cells that had probably twice as many chromosomes as the maternal plant due to failure of two mitotic divisions.
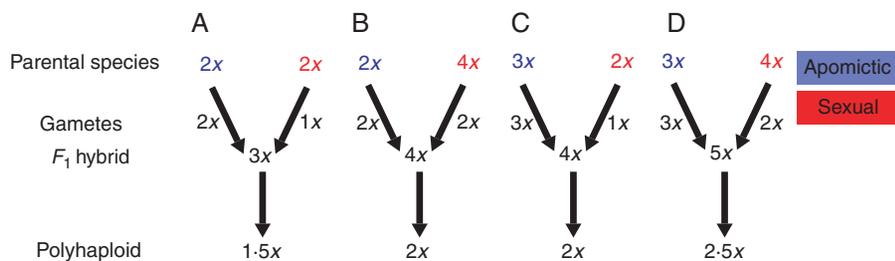
Fig. 5. Theoretical scheme of the most likely crosses between sexual and apomictic fern species. Formation of hypothetical polyhaploids is considered.

Hitherto, studies focused on the number of spores per sporangium reported either 32 spores per sporangium for apomictic species or 64 for sexual species without exceptions (e.g. Gastony and Haufler, 1976; Regalado Gabancho *et al*., 2010; Huang *et al*., 2011; Dyer *et al*., 2012). The first indication of an unbalanced spore number in a single sporangium was recently presented in the peculiar case of diploid sexual *Phegopteris decursive-pinnata*; the variation was caused probably by rare mutations disturbing meiosis (Nakato *et al*., 2012). Our study for the first time suggests the presence of different (apomictic vs. sexual) modes of spore mother cell development in a single sporangium. Formation of only aborted spores or a mixture of aborted and either type or both types of well-developed spores (the two size classes, probably corresponding to reduced and unreduced spores) in a single sporangium was recorded (Table 2; Fig. 3). The number of spores was between 32 (expected for full apomixis) and 64 (full sexuality). Together, these facts show that all the different processes are included not only in one sorus but even in one sporangium. It seems that spore mother cells are more or less independent and each can develop into different types of spores, and moreover that irregularities described first by Manton (1950) (see above) may co-occur. The numbers of well-developed spores per sporangium were multiples of four or close to it (Table 2, this study). This suggests that when reduced and/or unreduced well-developed spores occur, they are produced in whole tetrads. Deviations from exact multiples of four might be caused by occasional abortion of some spores and/or counting errors (especially in case of one 'excessive' spore, as the distinction between aborted and well-developed spores is not always clear-cut and an error of ± 1 could occur).

The formation of unreduced diplospores in apomictic fern hybrids that give rise to new sporophytes has been reported by several studies (e.g. Walker, 1984; Rabe and Haufler, 1992; Chao *et al*., 2012). In contrast, evidence for a mixture of reduced and unreduced spores on one plant is very sparse. The first evidence was provided by Hickok and Klekowski (1973) in *Ceratopteris* hybrids. Their study indicated the presence of meiotic adaptations within hybrid sporophytes that allow for the production of viable unreduced spores and gametophytes as well as reduced spores. Dyer *et al*. (2012) reported the occurrence of presumably reduced (64 spores per sporangium) and unreduced (32 per sporangium) spores on one individual of apomictic *Asplenium hallbergii*. Joint production of aborted, reduced and unreduced spores in different plants of the same population of *Phegopteris decursive-pinnata* was recently studied by Nakato *et al*. (2012).

Evolutionary implications

Our study revealed an unusual pattern of ploidy levels among F_2 offspring of pentaploid *Dryopteris* $\times$ *critica*. The finding that pentaploid hybrids can produce new viable plants of reduced $2\cdot 5x$ ploidy level, or even reduced and unreduced offspring on one plant, is particularly important for understanding the possibility of ploidy level reduction in ferns. Production of viable reduced spores, instead of aborted or unreduced diplospores, has important consequences only for hybrid formation. This contrasts with the general expectation that apomixis is likely to be established in triploid and pentaploid hybrids to avoid their sterility (Liu *et al*., 2012). In particular, the formation of reduced spores from odd-ploidy hybrids arising from sexual and apomictic species can be an important mechanism for the formation of new entities (see also similar cases by Rabe and Haufler, 1992; Nakato *et al*., 2012) and possible diploidization in polyploid ferns. The existence of meiosis and consecutive ploidy reduction in a hybrid polyploid entity may play an important and yet undetected role in fern speciation.

In Fig. 5, we present the most likely hybridization schemes of the sexual and apomictic fern species, considering the formation of polyhaploids. Lovis (1977) speculated that most apomictic ferns are triploids (50–70 %) or diploids (20–35 %). In two cases (B and C, hybridization of an apomictic diploid and a sexual tetraploid and of an apomictic triploid and a sexual diploid), the resulting hybrids are tetraploid, which allows formation of diploid reduced offspring of the new genetic composition. It can be expected that these reduced diploids are genetically stable due to even numbers of chromosome sets, especially in case B, in which the tetraploid hybrid has two chromosome sets from each parent. The other two cases (A and D) lead to odd-ploidy hybrids resulting in possibly unstable aneuploid polyhaploids (D being the case of *D*. $\times$ *critica* in the present study).

Although we have not found polyhaploids ($2\cdot 5x$ plants) in natural populations, we believe they might have certain evolutionary potential. There are several reasons that make detection of polyhaploids in wild populations difficult. In our case, the pentaploid F_1 hybrids are rare in most of the studied populations (approx. 10 % of *D. affinis s.l.* plants) and only a small part (approx. 10 %) of their spores are not aborted (compared with most of the viable spores in parental species). Moreover, only a small proportion of hybrid F_2 gametophytes produced sporophytes (approx. 10 %). Combined together, these three frequencies determine that the overall frequency of polyhaploids is much below 1 %, even if we assume the same fitness

of all types of gametophytes and sporophytes (which might not be the case). However, in small populations, some of these frequencies (especially the frequency of hybrid plants) might be enhanced, resulting in a more significant frequency of polyhaploids. On the other hand, such populations are difficult to find in the field and sampling the representative number of individuals (i.e. finding and analysing many such populations) is nearly impossible. We should also consider that *D.* × *critica* has an odd ploidy level, which leads to chromosomally unstable polyhaploids. In the case of tetraploid hybrids and especially the case shown in Fig. 5B, more regular formation of polyhaploids (fewer aborted spores, higher rate of sporophyte formation) can be expected, leading to higher polyhaploid frequencies.

CONCLUSIONS AND FUTURE RESEARCH

Our study has demonstrated the occurrence of the mixed reproductive mode in an apomictic × sexual fern hybrid. Two types of functionally viable spores are produced: unreduced (apomictic) $5x$ diplospores and reduced (sexual) $2·5x$ spores. The existence of reduced viable spores and the occurrence of both types on one plant and even in one sporangium together is unexpected and novel. Moreover, both spore types are capable of successful sporophyte production, which has not previously been observed. The pentaploid hybrid is capable of autonomous reproduction. In general, the apomictic × sexual hybrids might be of certain evolutionary potential, particularly if their polyhaploid offspring are capable of producing viable spores and crossing with sexual species. To investigate this, we will continue cultivation of polyhaploids until they reach maturity. Many other interesting research topics are raised based on our data, such as the incidence of polyhaploid formation in other fern groups or ploidies, genetic variation of polyhaploid offspring, and especially the occurrence and fertility of polyhaploids descendant from tetraploid hybrids (the scheme in Fig. 5B).

ACKNOWLEDGEMENTS

LITERATURE CITED

Barrington DS, Haufler HC, Werth CR. 1989. Hybridization, reticulation, and species concepts in the ferns. *American Fern Journal* **79**: 55–64.

Bär A, Eschelmüller A. 2010. Farnstudien: Einige pentaploide Bastarde von *Dryopteris filix-mas* mit triploiden Vertretern der *Dryopteris*-Gruppe. *Berichte der Bayerischen Botanischen Gesellschaft* **80**: 119–140.

Benaglia T, Chauveau D, Hunter DR, Young D. 2009. Mixtools: an R package for analyzing finite mixture models. *Journal of Statistical Software* **32**: 1–29.

Braithwaite, AF. 1964. A new type of apogamy in ferns. *New Phytologist* **63**: 293–305.

Chao YS, Liu HY, Ching YC, Chiou WL. 2012. Polyploidy and speciation in *Pteris* (Pteridaceae). *Journal of Botany* **2012**: 1–6. doi:10.1155/2012/817920.

Doležel J, Sgorbati S, Lucretti S. 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Physiologia Plantarum* **85**: 625–631.

Doležel J, Greilhuber J, Suda J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* **2**: 2233–2244.

Döpp W. 1939. Cytologische und genetische Untersuchungen innerhalb der Gattung *Dryopteris*. *Planta* **29**: 481–533.

Döpp W. 1955. Experimentell erzeugte Bastarde zwischen *Dryopteris filix-mas* (L.) Schott und *D paleacea*. (Sw.) C. Chr. *Planta* **46**: 70–91.

Dyer RJ, Savolainen V, Schneider H. 2012. Apomixis and reticulate evolution in the *Asplenium monanthes* fern complex. *Annals of Botany* **110**: 1515–1529.

Dyer RJ, Pellicer J, Savolainen V, Leitch IJ, Schneider H. 2013. Genome size expansion and the relationship between nuclear DNA content and spore size in the *Asplenium monanthes* fern complex (Aspleniaceae). *BMC Plant Biology* **13**: 219. doi:10.1186/1471-2229-13-219.

Eschelmüller A. 1998. Keimversuche mit Sporen der triploiden Sippen von *Dryopteris affinis* und ihren Bastarden mit *Dryopteris filix-mas*. *Mitteilungen des Naturwissenschaftlichen Arbeitskreises Kempten* **36**: 47–78.

Ekrt L, Trávníček P, Jarolímová V, Vít P, Urfus T. 2009. Genome size and morphology of the *Dryopteris affinis* group in Central Europe. *Preslia* **81**: 261–280.

Ekrt L, Holubová R, Trávníček P, Suda J. 2010. Species boundaries and frequency of hybridization in the *Dryopteris carthusiana* (Dryopteridaceae) complex: a taxonomic puzzle resolved using genome size data. *American Journal of Botany* **97**: 1208–1219.

Fraser-Jenkins CR. The species and subspecies in the *Dryopteris affinis* group. *Fern Gazette* **18**: 1–26.

Gabriel y Galán JM, Prada C. 2011. Pteridophyte spores viability. In: H Fernandéz, A Kumar, MA Revilla, eds. *Working with ferns: issues and applications*. London: Springer, 193–205.

Gastony GJ, Haufler CH. 1976. Chromosome numbers and apomixis in the fern genus *Bommeria* (Gymnogrammaceae). *Biotropica* **8**: 1–11.

Gastony GJ, Windham MD. 1989. Species concepts in pteridophytes: the treatment and definition of agamosporous species. *American Fern Journal* **79**: 65–77.

Grusz AL, Windham MD, Pryer KM. 2009. Deciphering the origins of apomictic polyploids in the *Cheilanthes yavapensis* complex (Pteridaceae). *American Journal of Botany* **96**: 1636–1645.

Hickok LG, Klekowski EJ. 1973. Abnormal reductional and nonreductional meiosis in *Ceratopteris*: alternatives to homozygosity and hybrid sterility in homosporous ferns. *American Journal of Botany* **60**: 1010–1022.

Huang YM, Hsu SY, Hsieh TH, Chou HM, Chiou WL. 2011. Three *Pteris* species (Pteridaceae: Pteridophyta) reproduce by apogamy. *Botanical Studies* **52**: 79–87.

Hunt HV, Ansell SW, Russell SJ, Schneider H, Vogel JC. 2011. Dynamics of polyploid formation and establishment in the allotetraploid rock fern *Asplenium majoricum*. *Annals of Botany* **108**: 143–157.

Ishikawa H, Ito M, Watano Y, Kurita S. 2003. Electrophoretic evidence for homologous chromosome pairing in the apogamous fern species *Dryopteris nipponensis* (Dryopteridaceae). *Journal of Plant Research* **116**: 165–167.

Krahulcová A, Rotreklová O, Krahulec F. 2013. The detection, rate and manifestation of residual sexuality in apomictic population of *Pilosella* (Asteraceae, Lactuceae). *Folia Geobotanica* **49**: 239–258.

Lin SJ, Kato M, Iwatsuki K. 1992. Diploid and triploid offspring of triploid agamosporous fern *Dryopteris pacifica*. *The Botanical Magazine* **105**: 443–452.

Liu HM, Dyer RJ, Guo ZY, Meng Z, Li JH, Schneider H. 2012. The evolutionary dynamics of apomixis in ferns: a case study from polystichoid ferns. *Journal of Botany* **2012**: 1–11, doi:10.1155/2012/510478.

Lovis JD. 1977. Evolutionary patterns and processes in ferns. *Advances in Botanical Research* **4**: 229–415.

Manton I. 1950. *Problems of cytology and evolution in the Pteridophyta*. Cambridge: Cambridge University Press.

Nakato N, Ootsuki R, Murakami N, Masuyama S. 2012. Two types of partial fertility in a diploid population of the fern *Thelypteris decursive-pinnata* (Thelypteridaceae). *Journal of Plant Research* **125**: 465–474.

Ootsuki R, Sato H, Nakato N, Murakami N. 2012. Evidence of genetic segregation in the apogamous fern species *Cyrtomium fortunei* (Dryopteridaceae). *Journal of Plant Research* **125**: 605–612.

Ozias-Akins P. 2006. Apomixis: developmental characteristics and genetics. *Critical Reviews in Plant Sciences* **25**: 199–214.

Park C, Kato M. 2003. Apomixis in the interspecific triploid hybrid fern *Cornopteris christenseniana* (Woodsiaceae). – *Journal of Plant Research* **116**: 93–103.

Pinter I. 1995. Progeny studies of the fern hybrid *Polystichum × bicknellii*. *Fern Gazette* **15**: 25–40.

Quintanilla LG, Escudero A. 2006. Spore fitness components do not differ between diploid and allotetraploid species of *Dryopteris* (Dryopteridaceae). *Annals of Botany* **98**: 609–618.

R Development Core Team. 2014. *R: A language and environment for statistical computing.* Vienna: R Foundation for Statistical Computing.

Rabe EW, Haufler CH. 1992. Incipient polyploid speciation in the maidenhair fern (*Adiantum pedatum*; Adiantaceae)? *American Journal of Botany* **79**: 701–707.

Regalado Gabancho L, Prada C, Gabriel y Galán JM. 2010. Sexuality and apogamy in the Cuban *Asplenium auritum–monodon* complex (Aspleniaceae). *Plant Systematics and Evolution* **289**: 137–146.

Reichstein T. 1981. Hybrids in European Aspleniaceae (Pteridophyta). *Botanica Helvetica* **91**: 89–139.

Schneller J. 1975. Untersuchungen an einheimischen Farnen, insbesondere der Dryopteris *filix-mas*-Gruppe. 2. Teil, Cytologische Untersuchungen. *Berichte der Schweizerischen Botanischen Gesellschaft* **85**: 1–17.

Schneller J, Krattinger K. 2010. Genetic composition of Swiss and Austrian members of the apogamous *Dryopteris affinis* complex (Dryopteridaceae, Polypodiopsida) based on ISSR markers. *Plant Systematics and Evolution* **286**: 1–6.

Stanier RY, Kuniswawa R, Mandel R. 1971. Purification and properties of unicellular blue-green algae (Order Chroococcales). *Bacteriological Review* **35**: 171–205.

Vida G, Reichstein T. 1975. Taxonomic problems in the fern genus *Polystichum* caused by hybridization. In: SM Walters, ed. *Floristic and taxonomic studies*. Faringdon, UK: E. W. Classey, 126–135.

Wagner WH, Chen KL. 1965. Abortion of spores and sporangia as a tool in the detection of *Dryopteris* hybrids. *American Fern Journal* **55**: 9–29.

Walker TG. 1962. Cytology and evolution in the fern genus *Pteris* L. *Evolution* **16**: 27–43.

Walker TG. 1979. The cytogenetics of ferns. In: AF Dyer, ed. *The experimental biology of ferns*. London: Academic Press, 87–132.

Walker TG. 1984. Chromosomes and evolution in pteridophytes. In: AK Sharma, A Sharma, eds. *Chromosomes in evolution of eukaryotic groups*, vol. 2. Boca Raton, FL: CRC Press, 103–141.

Windham MD, Yatskievych G. 2003. Chromosome studies of cheilanthoid ferns (Pteridaceae: Cheilanthoideae) from the western United States and Mexico. *American Journal of Botany* **90**: 1788–1800.

Yatabe Y, Yamamota K, Tsutsumi C, Shinohara W, Murakami N, Kato M. 2011. Fertility and precocity of *Osmunda × intermedia* offspring in culture. *Journal of Plant Research* **124**: 265–268.

Zhang R, Liu T, Wu W, *et al.* 2013. Molecular evidence for natural hybridization in the mangrove fern genus *Acrostichum*. *BMC Plant Biology* **13**: 74, doi:10.1186/1471-2229-13-74.

Paper 3

Hornych O., Férová A., Hori K., Košnar J. & Ekrt L. (2022): Apomictic fern fathers: An experimental approach to the reproductive characteristics of sexual, apomict and hybrid fern gametophytes. – American Journal of Botany 109(4): 628–644.

RESEARCH ARTICLE

Botany
American Journal of

Apomictic fern fathers: an experimental approach to the reproductive characteristics of sexual, apomict, and hybrid fern gametophytes

Ondřej Hornych[1] | Alžběta Férová[1] | Kiyotaka Hori[2] | Jiří Košnar[1] | Libor Ekrt[1]

[1]Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, České Budějovice, CZ-37005, Czech Republic

[2]The Kochi prefectural Makino Botanical Garden, Godaisan 4200-6, Kochi 781-8125, Japan

Correspondence
Ondřej Hornych, Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, České Budějovice, CZ-37005, Czech Republic.
Email: hornych.ondrej@gmail.com

Abstract

Premise: Apomixis and hybridization are two essential and complementary factors in the evolution of plants, including ferns. Hybridization combines characteristics from different species, while apomixis conserves features within a lineage. When combined, these two processes result in apo-sex hybrids. The conditions leading to the formation of these hybrids are poorly understood in ferns.

Methods: We cultivated spores from 66 fern samples (43 apomicts, 7 apo-sex hybrids, and 16 sexuals), and measured their development in vitro over 16 weeks. We evaluated germination, lateral meristem formation rates, sexual expression, and production of sporophytes and then compared ontogenetic patterns among the three groups.

Results: The three examined groups formed antheridia (male gametangia) but differed in overall gametophyte development. Sexual species created archegonia (female, 86% of viable samples), but no sporophytes. Apomicts rarely created nonfunctional archegonia (8%) but usually produced apogamous sporophytes (75%). Surprisingly, apomictic and sexual species showed similar development speed. The sexually reproducing parents of viable studied hybrids formed about twice as many meristic gametophytes as the apomictic parents (39% vs. 20%, respectively).

Conclusions: We present the most thorough comparison of gametangial development of sexual and apomictic ferns, to date. Despite expectations, apomictic reproduction might not lead to earlier sporophyte formation. Apomicts produce functional sperm and thus can contribute this type of gamete to their hybrids. The development patterns found in the parents of hybrids indicate a possible increase of hybridization rates by antheridiogens. The apo-sex hybrids always inherit the apomictic reproductive strategy and are thus capable of self-perpetuation.

KEYWORDS

agamospory, antheridia, apogamy, apo-sex hybrid, archegonia, *Dryopteris*, gametes, hybridization, pteridophytes, wood fern

Apomixis (asexual reproduction via seed or spore) and hybridization are considered two essential factors involved in plant evolution (Šarhanová et al., 2017; Patel et al., 2018; Carman et al., 2019). These two processes work in opposite directions. Hybridization allows for the combination of characteristics of two different species, sometimes resulting in speciation or hybrid vigor (Chen, 2010; Sigel, 2016; Goulet et al., 2017) at the cost of highly increased or even total spore abortion in ferns (Wagner and Chen, 1965; Hornych and Ekrt, 2017). By contrast, apomixis allows for the formation of progeny without mixing with other individuals and the restoration of spore viability, while limiting genetic variability (Grusz, 2016; Albertini et al., 2019). When working in tandem, they may balance each other's disadvantages.

Apomixis has long been established by researchers in plants. There are several means of apomictic seed formation in angiosperms (Tucker and Koltunow, 2009), which tend to co-occur alongside sexuality, rather than being obligatory (Tucker et al., 2003; Krahulcová et al., 2014). Some species, referred to as pseudogamous apomicts, even require pollen for successful asexual seed formation (Mogie, 1992).

Various facets of apomixis have inspired considerable research in angiosperms (Lepší et al., 2019; Chrtek et al., 2020; Doležal et al., 2020), including studies focused on the fixation of positive characteristics in crops (Scheben and Hojsgaard, 2020; Fiaz et al., 2021). Apomixis is also known in ferns, where it is usually an obligate trait, and in some clades very prevalent. Specifically, it has been estimated that between 3% and 10% of fern species are apomictic (Walker, 1985; Liu et al., 2012), whereas the estimate for angiosperms is <1% (Whitton et al., 2008). Unlike sexual species, fern apomicts form unreduced (diplo)spores (2n) via a process called agamospory. Ferns show two types of agamospory (reviewed by Grusz, 2016), the more prevalent premeiotic endomitosis (PE, Döpp-Manton sporogenesis; Döpp, 1932; Manton, 1950) and the rare meiotic first division restitution (MFDR, Braithwaite sporogenesis; Braithwaite, 1964). Both types yield spores germinating into gametophytes that can form sporophytes apogamously, without syngamy. By contrast, sexual species produce reduced spores (n) resulting in gametophytes capable of forming antheridia (male gametangia) and archegonia (female gametangia). These gametangia produce gametes that allow mating and, potentially, hybridization.

Hybridization involves a fusion of gametes originating from two different species. The rate of hybridization (Koutecký et al., 2011; Ma et al., 2014; Zanella et al., 2016) and its limiting barriers (Rieseberg and Carney, 1998; Baack et al., 2015) have been studied extensively in angiosperms. Even though ferns are considered to hybridize frequently (e.g., Testo et al., 2015), hybridization rates in fern populations have rarely been assessed quantitatively (Hornych et al., 2019). Hybridization barriers and promoters have likewise received relatively little attention (Xiang et al., 2000; Testo et al., 2015; Hornych et al., 2019). Nevertheless, it is well established (Regalado Gabancho et al., 2010; Dyer et al., 2012; Ekrt and Koutecký, 2016) that apomixis and hybridization can meet in ferns (Manton, 1950)—when an apomictic species hybridizes with a sexually reproducing species, an apo-sex (apomictic) hybrid is formed, which we refer to as an apo-sex hybrid in this study.

Little is known about apo-sex hybrids in ferns. Unlike in angiosperms, fern apo-sex hybrids differ considerably from both sexual hybrids (sexual × sexual) and regular apomictic species. First, fern apo-sex hybrids have spore abortion rates of 80%–95% in some groups (Eschelmüller, 1998; Fraser-Jenkins, 2007; Ekrt and Koutecký, 2016), which compares favorably to the ~99% spore abortion rate in sexual hybrids (Hornych and Ekrt, 2017). Regular apomictic species, which can start as apo-sex hybrids, produce a minority of aborted spores (Quintanilla and Escudero, 2006; Guo and Liu, 2013; Hornych and Ekrt, 2017), probably because selective pressures reduce spore abortion over time. Due to the generally high spore abortion rates of fern hybrids, most of them are considered as F1 generation and backcrossing is only rarely considered (e.g., Kentner and Mesler, 2000). While the situation may be more complicated in apo-sex

fern hybrids, we will consider them as F1 in this study. Second, fern apo-sex hybrids are capable of producing viable reduced and unreduced spores, a characteristic rare among ferns (Windham, 1983; Sigel et al., 2011; Ekrt and Koutecký, 2016). Like their apomictic parents, fern apo-sex hybrids can produce gametangia, which may be dysfunctional, and apogamously formed sporophytes (Walker, 1962; Regalado Gabancho et al., 2010; Ekrt and Koutecký, 2016). Although the information is limited, these findings indicate a possible involvement of apo-sex hybrids in sexual as well as apomictic reproduction.

To fully know the story of fern apo-sex hybrids, reproductive features of apomictic and sexually reproducing species must be understood on the gametophytic level. By default, sexual species need both female and male gametangia—otherwise, they cannot reproduce (Haufler et al., 2016; Sessa et al., 2016). By contrast, the gametangia of apomicts are superfluous, serving only for possible outcrossing with conspecific sexual individuals, or hybridization. Apomictic taxa are mostly capable of forming only viable antheridia, and the reported archegonia of apomicts are generally inviable (Walker, 1985; Chiou et al., 2006; Huang et al., 2011; Grusz et al., 2021). Therefore, apomicts are considered to be the paternal parents of fern apo-sex hybrids exclusively (Walker, 1962; Grusz et al., 2009; Jaruwattanaphan et al., 2013), although there is indirect evidence in the opposite direction (Hori et al., 2014, 2016, 2018; Hori and Murakami, 2019). A broader assessment of gametangia production by apomicts will improve our understanding of apomictic hybridization in ferns. However, it is impossible to understand dynamic interactions between apomictic and sexual species based on just knowing which gametangia apomicts create.

While angiosperm gametophytes are generally attached to sporophyte (maternal) tissue, fern gametophytes live in independent microhabitats. Within these microhabitats, growth speed is strongly affected by abiotic factors (Korpelainen, 1994; Quintanilla et al., 2007; Testo et al., 2014), but apomicts are assumed to grow faster than sexually reproducing species (Haufler et al., 2016). Even small differences in growth speed can rapidly translate into ontogenetic shifts and changes in the timing of gametangia formation, which could, in turn, affect the odds of crossing between apomictic and sexual individuals. Biotic factors may also play a major role, most prominently through interactions involving the antheridiogen system (Döpp, 1950; Atallah and Banks, 2015; Hornych et al., 2021). Antheridiogens are pheromones released by meristic (often archegoniate) gametophytes, that are themselves insensitive to this chemical message (Näf, 1958; Näf et al., 1975; Tanaka et al., 2014). These pheromones are perceived by nearby asexual gametophytes, which respond by forming antheridia exclusively (Döpp, 1950; Schneller, 2008; Atallah and Banks, 2015). This system promotes dioecy and outcrossing, and, consequently, hybridization may be promoted as well. Apomicts are as likely to respond to antheridiogens as sexually reproducing species (Hornych et al., 2021), with

two possible opposing effects on hybridization. On the one hand, antheridiogens may push gametophytes of apomictic species into prematurely developing antheridia, increasing the odds of hybridization. On the other hand, faster-growing apomicts may suppress the formation of archegonia in nearby sexual species, reducing the odds of hybridization. Under these pheromone interactions, minor differences in growth rate may further alter hybridization rates in mixed apomict-sexual populations, and deserve further investigation.

To better understand the establishment and self-perpetuation of apo-sex hybrids, this study attempts to answer the following questions. (1) Do the growth capabilities of gametophytes of various apomictic and sexually reproducing species differ? (2) Do developmental features of apomictic and sexually reproducing species affect the likelihood of hybridization? (3) What characteristics affect the reproduction of apo-sex hybrids and their backcrossing with sexual congeners?

MATERIALS AND METHODS

Plant material and cultivation

Spores from 66 plants of 54 fern taxa (43 apomicts, 7 apomict × sexual hybrids, 16 sexuals) were obtained from the author's and other collectors' personal herbaria, garden cultivation, the Carolina Biological Supply Company (Burlington, North Carolina, USA), and the Spore Exchange of the American Fern Society (Appendix S1). Reproductive mode of vouchered samples was verified via comparing genome sizes of gametophytes and sporophytes using flow cytometry (for details see Ekrt and Koutecký, 2016). Fronds of fresh specimens were allowed to air dry between two sheets of paper to facilitate spore release and then moved to paper envelopes for storage. Other spores were sown directly (approximate sowing density: 10 spores per cm^2) from the packages, either after puncture via a pinhead or directly by using cotton wool. Cotton wool temporarily held the spores, and by light tapping the spores were released onto the medium.

Sown spores were cultivated in a growth chamber (MLR-352 Climatic Test Chamber, PHC Europe B.V., Etten-Leur, Netherlands) under a 12 h dark:12 light (PPFD: 1700 $\mu mol \cdot m^{-2} \cdot s^{-1}$) regimen at 20°C. Spores were sown on Petri dishes, 9 cm in diameter, containing the inorganic Murashige and Skoog medium (Murashige and Skoog, 1962) at original concentrations to avoid growth limitation due to nutrient shortages. For most samples, three dishes were used (Table 1).

Cultivated samples were observed for 16 weeks using a bifocal microscope (Olympus S7X7). Germination percentage (visibly aborted spores excluded) was calculated by evaluating 50 randomly selected spores per dish every week for the first four weeks. Similarly, the percentage of meristic gametophytes (possessing lateral meristem; Figure 1A) was calculated using

50 gametophytes (ungerminated spores excluded) per dish every week for four weeks following the emergence of meristic gametophytes for a given species (week 3 or 4 of cultivation). Every two weeks, from week 6 to week 16, at least 10 gametophytes (rarely minimum 5 if too few were present) from each dish were stained with 1% acetocarmine for ≥30 min (similar to Pangua et al. [2019] but without the heating) and subsequently observed for the presence of gametangia under a light microscope. The gametophytes were scored as either asexual, male, female, or hermaphroditic, based on the presence or absence of antheridia and archegonia (Figure 1B, C). Finally, 20 gametophytes per dish were observed for the presence of sporophytic tissue every two weeks, from week 6 to week 16 (Figure 1D).

Data analysis

To compare cultivation results, we examined germination (at week 4), meristems (at weeks 4 and 6), and the occurrence of antheridia, archegonia, and sporophytes (at weeks 6–16). Only samples that formed at least one of either antheridium, archegonium, or apogamously formed sporophyte were included. This subset was used for partial redundancy analyses (RDA, constrained multivariate analysis) with reproductive type (apomict, hybrid, sexual), spore travel by post (yes, no), and spore age used as explanatory variables. Spore age was calculated as year of collection minus year of cultivation (range: 0–16 yr; Table 1). First, the data were analyzed with spore travel by post as the sole explanatory variable, with reproductive type and spore age included as covariates. Second, four data sets: all samples, all non-hybrid samples, *Dryopteris* samples, and non-hybrid *Dryopteris* samples were tested using reproduction type as the sole explanatory variable, with spore travel by post and spore age as covariates. All RDAs were performed in Canoco 5 (ter Braak and Šmilauer, 2012).

The relationship between spore age and germination rate was tested using generalized linear models (GLMs) with binomial distribution and testing significance by the chi-squared criterion. Spore travel by post was also used as an explanatory variable in this model. Additionally, sporophyte potential was compared between apomicts and sexual species. Sporophyte potential was defined as the percentage of archegoniate gametophytes in sexual species and the percentage of sporophyte-bearing gametophytes in apomicts. This variable allows for a comparison of reproductive success between the reproductive types despite no sexual species forming sporophytes and no apomict forming viable archegonia. Sporophyte potential was compared by generalized linear mixed effects models using reproduction type and observation time (6–16 weeks) as explanatory variables. Additionally, spore source identity (unique for each sample) and spore age were used as random variables. Three data sets were tested: all (non-hybrid) samples, only *Dryopteris* non-hybrids, and

TABLE 1 Overview of the samples used in this study. Spore age is number of full years elapsed between spore sample collection and sowing. Germination rate is based on observations made in week 4 of cultivation.

Taxon	Sample ID	Dishes (*n*)	Spore age (yr)	Germination rate	Reproduction type
Adiantum hispidulum Sw.	1	3	4	14.7%	Apomict
Anemia tomentosa (Sav.) Sw.	2	3	9	79.3%	Apomict
Asplenium adiantum-nigrum L.	3	3	1	58.0%	Sexual
Asplenium aethiopicum (Burm.f.) Bech.	4	3	14	0.0%	Apomict
Asplenium flabellifolium Cav.	5	3	1	66.7%	Apomict
Asplenium monanthes L.	6	3	14	0.0%	Apomict
Asplenium septentrionale (L.) Hoffm.	7	3	0	76.7%	Sexual
Ceratopteris richardii Brongn.	8	3	1	76.0%	Sexual
Cheilanthes distans (R.Br.) Mett.	53	3	2	32.7%	Apomict
Cheilanthes viridis (Forssk.) Sw.	59	3	2	20.0%	Apomict
Cyrtomium atropunctatum Sa.Kurata	9	3	2	22.0%	Apomict
Cyrtomium falcatum (L.f.) C.Presl	10	3	3	10.0%	Apomict
Cyrtomium fortunei J.Sm.	11	3	3	0.0%	Apomict
Cyrtomium macrophyllum (Makino) Tagawa	12	3	3	0.0%	Apomict
Diplazium taiwanense Tagawa	13	3	1	0.0%	Apomict
Doodia caudata (Cav.) R.Br.	14	3	14	0.0%	Sexual
Doodia caudata (Cav.) R.Br.	15	3	1	68.7%	Sexual
Dryopteris × *alpirsbachensis* Freigang, Zenner, Bujnoch, S.Jess. & Magauer (*D. carthusiana* × *D. remota*)	16	3	0	0.0%	Hybrid
Dryopteris × *complexa* Fraser-Jenk. (*D. affinis* × *D. filix-mas*)	18	3	2	42.7%	Hybrid
Dryopteris × *complexa* Fraser-Jenk. (*D. affinis* × *D. filix-mas*)	17	3	1	56.7%	Hybrid
Dryopteris × *critica* (Fraser-Jenk.) Fraser-Jenk. (*D. borreri* × *D. filix-mas*)	21	3	7	0.0%	Hybrid
Dryopteris × *critica* (Fraser-Jenk.) Fraser-Jenk. (*D. borreri* × *D. filix-mas*)	22	3	0	68.0%	Hybrid
Dryopteris × *critica* (Fraser-Jenk.) Fraser-Jenk. (*D. borreri* × *D. filix-mas*)	19	3	1	0.0%	Hybrid
Dryopteris × *critica* (Fraser-Jenk.) Fraser-Jenk. (*D. borreri* × *D. filix-mas*)	20	3	2	0.0%	Hybrid
Dryopteris affinis (Lowe) Fraser-Jenk.	23	3	1	90.0%	Apomict
Dryopteris affinis (Lowe) Fraser-Jenk.	24	3	2	100.0%	Apomict
Dryopteris bissetiana (Baker) C.Chr.	25	3	3	12.7%	Apomict
Dryopteris bissetiana (Baker) C.Chr.	26	3	6	92.7%	Apomict
Dryopteris borreri Newman	27	3	8	0.0%	Apomict
Dryopteris borreri Newman	28	3	0	90.7%	Apomict
Dryopteris borreri Newman	29	3	0	98.7%	Apomict
Dryopteris cambrensis (Fraser-Jenk.) Beitel & W.R.Buck	30	3	0	0.0%	Apomict
Dryopteris cambrensis (Fraser-Jenk.) Beitel & W.R.Buck	31	3	0	98.0%	Apomict
Dryopteris carthusiana (Vill.) H.P.Fuchs	32	3	0	98.0%	Sexual
Dryopteris caucasica (A.Braun) Fraser-Jenk. & Corley	33	3	1	65.3%	Sexual
Dryopteris crassirhizoma Nakai	34	3	16	0.0%	Sexual
Dryopteris dilatata (Hoffm.) A.Gray	35	3	1	81.3%	Sexual

TABLE 1 (Continued)

Taxon	Sample ID	Dishes (*n*)	Spore age (yr)	Germination rate	Reproduction type
Dryopteris expansa (C.Presl) Fraser-Jenk. & Jermy	36	3	0	96.7%	Sexual
Dryopteris filix-mas (L.) Schott	37	1	1	100.0%	Sexual
Dryopteris filix-mas (L.) Schott	38	2	2	92.0%	Sexual
Dryopteris filix-mas (L.) Schott	39	3	0	100.0%	Sexual
Dryopteris formosana (Christ) C.Chr.	40	3	2	0.0%	Apomict
Dryopteris kinokuniensis Sa.Kurata	41	3	4	0.0%	Apomict
Dryopteris lacunosa S.Jess., Zenner, Chr.Stark & Bujnoch	42	3	3	0.0%	Apomict
Dryopteris oreades Fomin	43	3	1	46.7%	Sexual
Dryopteris pacifica (Nakai) Tagawa	44	3	3	0.0%	Apomict
Dryopteris purpurella Tagawa	45	3	5	70.7%	Apomict
Dryopteris remota (Döll) Druce	46	3	1	59.3%	Apomict
Dryopteris sparsa (D.Don) Kuntze	47	3	7	30.0%	Apomict
Dryopteris subarborea (Baker) C.Chr.	48	3	3	8.0%	Apomict
Dryopteris tsugiwoi Sa.Kurata	49	3	5	0.0%	Apomict
Dryopteris tsushimensis K.Hori & N.Murak.	50	5	1	97.3%	Apomict
Dryopteris villarii (Bellardi) Woyn. ex Schinz & Thell.	51	3	0	41.3%	Sexual
Dryopteris wallichiana (Spreng.) Hyl.	52	3	6	7.3%	Apomict
Myriopteris rufa Fée	54	3	4	42.7%	Apomict
Myriopteris tomentosa (Link) Fée	55	3	3	0.0%	Apomict
Myriopteris wootonii (Maxon) Grusz & Windham	56	3	4	51.3%	Apomict
Oreopteris limbosperma (All.) Holub	58	3	1	20.7%	Sexual
Paragymnopteris marantae (L.) K.H.Shing	57	3	6	89.3%	Sexual
Phegopteris connectilis (Michx.) Watt	60	1	6	0.0%	Apomict
Phegopteris connectilis (Michx.) Watt	61	3	1	8.7%	Apomict
Polystichum luctuosum (Kunze) T.Moore	64	3	0	0.0%	Apomict
Polystichum polyblepharum (Roem. ex Kunze) C.Presl	62	3	3	0.0%	Apomict
Polystichum setiferum (Forssk.) Moore ex Woyn.	63	3	1	0.0%	Sexual
Pteris cretica L.	65	3	7	0.0%	Apomict
Pteris multifida Poir.	66	3	1	48.0%	Apomict
Pteris semipinnata L.	67	3	1	0.0%	Apomict

only samples with non-zero sporophyte potential. All GLMs were tested with binomial distribution using the chi-squared criterion in R (R Development Core Team, 2020), and the analyses including random variables were performed using the package lme4 (Bates et al., 2015).

A review of gametangial expression by apomicts and apo-sex hybrids was compiled from the literature (Liu et al., 2012) and combined with our results for further analysis (Table 2). To prevent false positives, the synthetic artificial hybrids reported by Walker (1962) were labeled as a single-taxon "*Pteris* synthetic hybrids," as the results were not presented for each hybrid separately.

RESULTS

Cultivation

Overall, 42 of 66 (64%) cultivated samples germinated as well as developed antheridia during the observation period
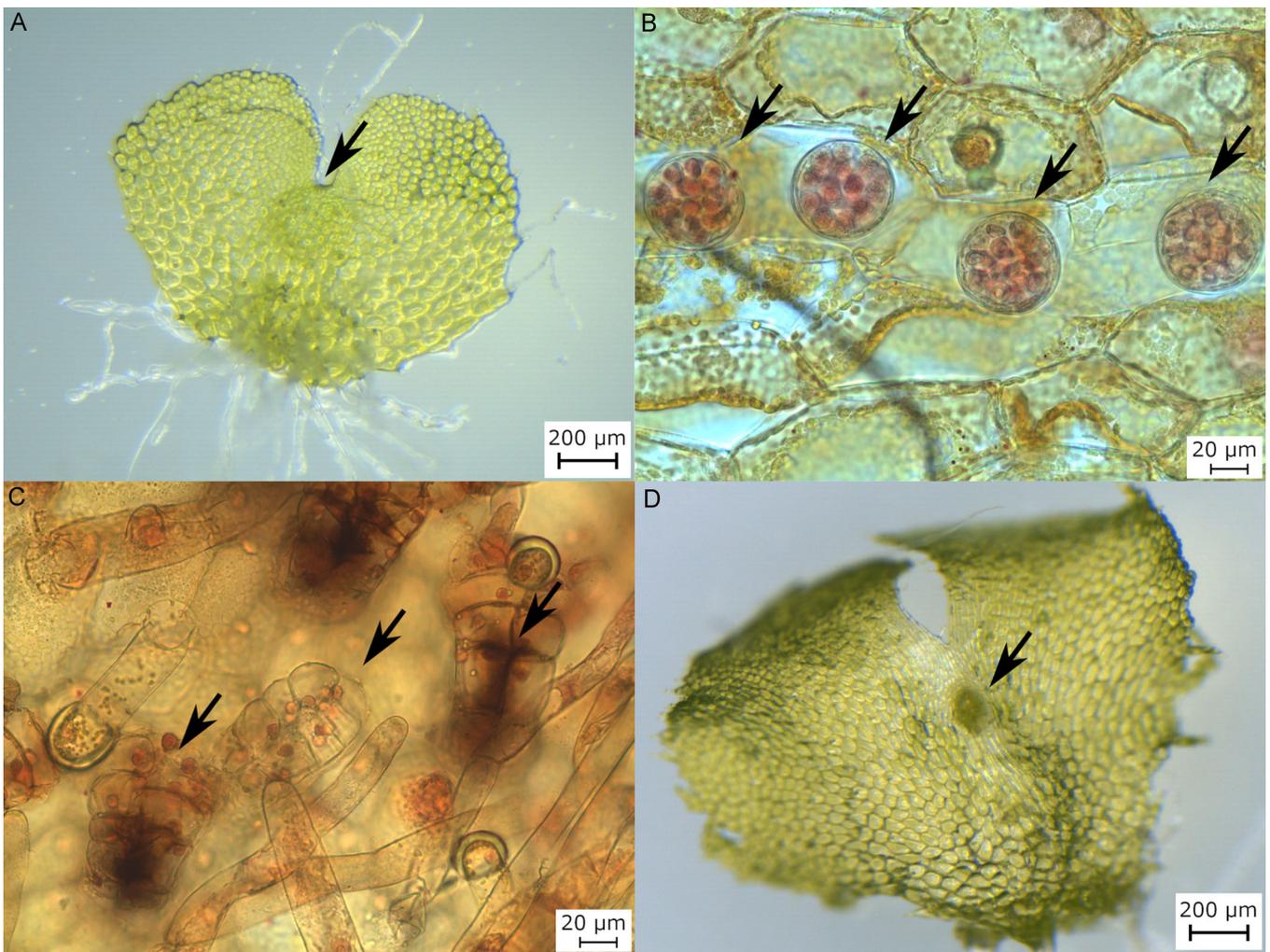
FIGURE 1 Four characters measured in fern gametophytes (A, B, C: *Dryopteris filix-mas*—sexual; D: *D. borreri*—apomict) cultivated in this study: (A) meristic gametophyte, lateral meristem highlighted with an arrow; (B) antheridiate gametophyte, sperm inside antheridia (highlighted) stained red with acetocarmine; (C) archegoniate gametophyte, archegonia highlighted; and (D) gametophyte bearing an apogamous sporophyte, the initial stage of the sporophyte highlighted.

(Table 1). The remaining 24 samples showed no germination and were deemed inviable. Among the viable samples, germination rates at week 4 were similar in apomicts (median 51%) and hybrids (median 57%), lower than in sexual species (median 77%; Figure 2). At week 6, apomictic and sexual species were more similar in lateral meristem formation rate (median 25% and 35%, respectively) compared to hybrids (median 10%; Figure 2). Of the 24 viable apomicts, 18 (75%) apogamously formed sporophytes at some point, and two species (8%) formed inviable archegonia (*Dryopteris purpurella* Tagawa, *D. tsushimensis* K.Hori & N.Murak.) after producing sporophytes. Additionally, 12 of 14 (86%) viable sexual species formed archegonia. No sexual species formed any sporophytes. Overall, neither gametangial type emerged systematically sooner than the other (i.e., both male and female gametes were generally available at the same time). Hermaphroditic gametophytes were extremely rare (15 gametophytes total spanning six taxa).

Viable apo-sex hybrids (*Dryopteris ×complexa* Fraser-Jenk. and *D. ×critica* (Fraser-Jenk.) Fraser-Jenk.) had lower spore germination rates than their parental taxa (*D. affinis* (Lowe) Fraser-Jenk., *D. borreri* Newman—apomict, *D. filix-mas* (L.) Schott—sexual). At week 4, an average 10% of gametophytes arising from hybrid individuals were meristic, fewer than their apomictic parents (20%), and much fewer than the sexual *D. filix-mas* (39%). Apo-sex hybrids, like their apomictic parents, also formed no archegonia but abundant sporophytes, unlike sexual samples examined. These results are congruent with the differences among all viable taxa, as presented above. The complete observation data set can be found in Appendix S2.

Multivariate analyses

Spore travel by post significantly affected overall gametophyte performance (RDA: pseudo-F = 3.4, P = 0.003). Spores

TABLE 2 A list of apomictic taxa and their observed gametangial expression, based on published records and the results of this study.

| Species	Family	Archegonia	Antheridia	Notes	References
Adiantum hispidulum Sw.	Pteridaceae	No	Yes		This study
Adiantum philippense L. = *A. lunatum*	Pteridaceae	No	Yes		Sareen et al., 2014
Aleuritopteris formosana (Hayata) Tagawa = *Cheilanthes farinosa*	Pteridaceae	No	Yes	Verma (1977) found no antheridia	Manton et al., 1966; Verma, 1977
Anemia tomentosa (Sav.) Sw.	Pteridaceae	No	Yes		This study
Argyrochosma tenera (Gillies ex Hook.) M.Kessler & A.R.Sm. = *A. nivea* var. *tenera*	Aspleniaceae	No	No		Hernández et al., 2015
Asplenium ×lellingerianum C.Sánchez & L.Regalado	Aspleniaceae	Yes	Yes	Antheridia rare and sperm dysfunctional	Regalado Gabancho et al., 2010
Asplenium aethiopicum (Burm.fil.) Bech.	Aspleniaceae	Yes	Yes	Gametangia dysfunctional	Braithwaite, 1964
Asplenium auritum Sw. = *A. macilentum*	Aspleniaceae	Yes	Yes	Gametangia dysfunctional	Braithwaite, 1964
Asplenium flabellifolium Cav.	Aspleniaceae	No	Yes		This study
Asplenium monodon Liebm.	Aspleniaceae	Yes	Yes	Antheridia rare and sperm dysfunctional	Regalado Gabancho et al., 2010
Asplenium resiliens Kunze	Aspleniaceae	No	Yes		Whittier, 1970
Astrolepis sinuata (Lag. ex Sw.) D.M.Benham & Windham	Pteridaceae	No	Yes		Döpp, 1959
Bommeria pedata (Sw.) E. Fourn.	Pteridaceae	Yes	Yes		Haufler and Gastony, 1978
Cheilanthes distans (R.Br.) Mett.	Pteridaceae	No	Yes		This study; Döpp, 1959
Cheilanthes eatonii	Pteridaceae	No	Yes		This study
Cheilanthes nudiuscula (R.Br.) Moore = *Cheilanthes hirsuta*	Pteridaceae	Yes	Yes	Referenced in Huang et al., 2011	Huang et al., 2009
Cheilanthes viridis (Forssk.) Sw. = *Pellaea viridis*; *P. adiantoides*	Pteridaceae	No	Yes		This study; Steil, Steil, 1918; Voeller, 1964; Gemmrich, 1986
Cornopteris christenseniana Tagawa	Athyriaceae	No	No		Park and Kato, 2003
Ctenitis falciculata (Raddi) Ching = *Aspidium chrysolobum*	Dryopteridaceae	Yes	Yes		Steil, 1918
Cyrtomium atropunctatum (L.f.) C.Presl	Dryopteridaceae	No	Yes		This study
Cyrtomium falcatum (L.f.) C.Presl	Dryopteridaceae	No	Yes	Irregular and tard antheridial formation	This study; Voeller, 1964; Yatskievych, 1993
Cyrtomium fortunei J.Sm.	Dryopteridaceae	No	Yes	Irregular and tard antheridial formation	Yatskievych, 1993
Cyrtomium macrophyllum (Makino) Tagawa	Dryopteridaceae	No	Yes	Irregular and tard antheridial formation	Yatskievych, 1993

(Continues)

TABLE 2 (Continued)

Species	Family	Archegonia	Antheridia	Notes	References
Diplazium megaphyllum (Baker) Christ	Athyriaceae	Yes	Yes	Archegonia inviable, most gametophytes asexual	Chiou et al., 2006
Dryopteris ×complexa Fraser-Jenk.	Dryopteridaceae	No	Yes		This study
Dryopteris ×critica (Fraser-Jenk.) Fraser-Jenk.	Dryopteridaceae	No	Yes		This study; Ekrt and Koutecký, 2016
Dryopteris affinis (Lowe) Fraser-Jenk. = *D. pseudomas*	Dryopteridaceae	Yes	Yes	Archegonia rarely found by Duncan, 1943	This study; Duncan, 1943; Schneller, 1981, 1988
Dryopteris bissetiana (Baker) C.Chr.	Dryopteridaceae	Yes	Yes	This study found only antheridia	This study; Momose, 1967
Dryopteris borreri Newman	Dryopteridaceae	Yes	Yes	Archegonia rarely found by Duncan (1943); Whittier (1970) found only archegonia	This study; Duncan, 1943; Poelt, 1960; Whittier, 1970
Dryopteris cambrensis (Fraser-Jenk.) Beitel & W.R.Buck	Dryopteridaceae	No	Yes		This study
Dryopteris munchii A.R.Sm.	Dryopteridaceae	No	Yes		Reyes Jaramillo et al., 2008
Dryopteris pacifica (Nakai) Tagawa	Dryopteridaceae	Yes	Yes		Momose, 1967; Lin et al., 1992
Dryopteris purpurella Tagawa	Dryopteridaceae	Yes	Yes		This study
Dryopteris remota (Döll) Druce	Dryopteridaceae	No	Yes		This study
Dryopteris sparsa (D.Don) Kuntze	Dryopteridaceae	No	Yes		This study
Dryopteris subarborea (Baker) C.Chr.	Dryopteridaceae	No	Yes		This study
Dryopteris tsushimensis K.Hori & N.Murak.	Dryopteridaceae	Yes	Yes		This study
Dryopteris wallichiana (Spreng.) Hyl.	Dryopteridaceae	No	Yes		This study
Gaga marginata (Kunth) Fay W.Li & Windham	Pteridaceae	Yes	Yes		Martínez et al., 2017
Myriopteris alabamensis (Buckley) Grusz & Windham = *Cheilanthes alabamensis*	Pteridaceae	No	Yes		Whittier, 1970
Myriopteris gracilis Fée = *Cheilanthes feei*	Pteridaceae	No	Yes		Whittier, 1970
Myriopteris myriophylla (Desv.) J.Sm.	Pteridaceae	Yes	Yes		Martínez et al., 2017
Myriopteris rufa Fée = *Cheilanthes castanea*	Pteridaceae	Yes	Yes	This study found only antheridia	This study; Whittier, 1970
Myriopteris tomentosa (Link) Fée = *Cheilanthes tomentosa*	Pteridaceae	No	Yes		Whittier, 1970
Myriopteris wootonii (Maxon) Grusz & Windham	Pteridaceae	No	Yes		This study
Pecluma dispersa (A.M.Evans) M.G.Price = *Polypodium dispersum*	Polypodiaceae	No	No		Evans, 1964
Pellaea atropurpurea (L.) Link	Pteridaceae	No	Yes		Whittier, 1970

TABLE 2 (Continued)

Species	Family	Archegonia	Antheridia	Notes	References
Pellaea glabella Mett. ex Kuhn	Pteridaceae	No	Yes		Whittier, 1968, 1970
Phegopteris connectilis (Michx.) Watt	Thelypteridaceae	Yes	Yes	This study found only antheridia	This study; Whittier, 1970
Pityrogramma calomelanos (L.) Link	Pteridaceae	N/A	Yes		Dubey and Roy, 1985
Polystichum fraxinellum (Christ) Diels = *Cyrtogonellum fraxinellum*	Dryopteridaceae	Yes	Yes	Sperm active, archegonia not involved with sporophyte formation	Guo and Liu, 2013
Polystichum luctuosum (Kunze) T.Moore = *Polystichum tsus-simense*	Dryopteridaceae	N/A	Yes		Näf, 1966
Polystichum minimum (Y.T.Hsieh) Li Bing Zhang = *Cyrtogonellum inaequale*	Dryopteridaceae	No	Yes	Sperm active	Guo and Liu, 2013
Polystichum tenuius (Ching) Li Bing Zhang = *Cyrtogonellum caducum*	Dryopteridaceae	No	Yes	Sperm active	Liu et al., 2012; Guo and Liu, 2013
Pteris argyraea Moore = *Pteris confusa*	Pteridaceae	No	Yes		Walker, 1958
Pteris biaurita L. = *Pteris sulcata*	Pteridaceae	No	Yes		Steil, 1918
Pteris cretica L.	Pteridaceae	Yes	Yes	Inviable archegonia, Whittier (1970) and Huang et al. (2011) found no archegonia	Steil, 1918; Whittier, 1970; Laird and Sheffield, 1986; Huang et al., 2011
Pteris khasiana (C.B.Clarke) Hieron. subsp. *fauriei* (Hieron.) Fraser-Jenk. = *Pteris fauriei*	Pteridaceae	No	Yes		Huang et al., 2006
Pteris multifida Poir.	Pteridaceae	No	Yes		This study
Pteris subesquirolii Y.S.Chao = *Pteris pellucidifolia*	Pteridaceae	No	Yes		Huang et al., 2011
Pteris synthetic hybrids	Pteridaceae	No	Yes	Sluggish sperm when compared to regular apomicts	Walker, 1962
Pteris wulaiensis C.M.Kuo	Pteridaceae	Yes	Yes	Archegonia sterile	Huang et al., 2011
Total 58 species, four hybrids	**Total six families**	**Total 21 of 60 (35%)**	**Total 59 of 62 (95%)**		
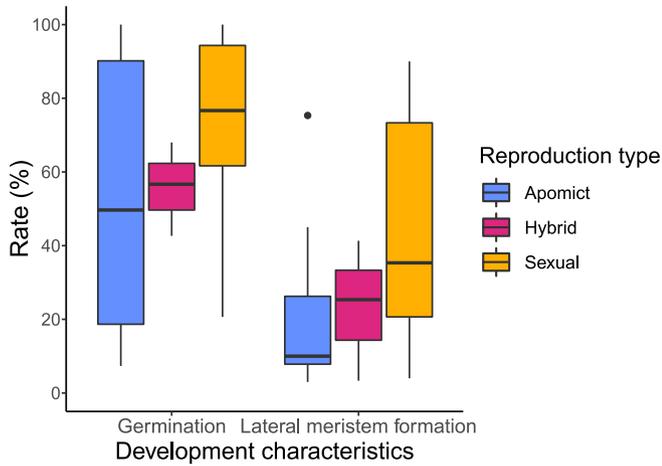
FIGURE 2 Germination (at week 4 of culture) and lateral meristem formation (week 6) in viable (germination >0%) samples of the three reproductive types: apomictic species ($n = 24$), apo-sex hybrids ($n = 3$, hybrids between apomictic and sexual species), and sexual species ($n = 15$).

that traveled yielded generally less viable gametophytes. The spores traveled by post from Japan, USA, and Germany to Czechia. Consequently, spore travel by post was therefore used as a covariate in other analyses.

Further analyses of gametophyte development demonstrated a significant difference between apomicts, sexual species (Figure 3A), and apo-sex hybrids (Figure 3B), regardless of whether all samples or just *Dryopteris* were used (all samples: RDA pseudo-$F = 5.3$, $P < 0.001$; all non-hybrid samples: RDA pseudo-$F = 8.7$, $P < 0.001$; *Dryopteris* samples: RDA pseudo-$F = 3.6$, $P < 0.001$; non-hybrid *Dryopteris* samples: RDA pseudo-$F = 5.8$, $P < 0.001$). Apomict and sexual samples differed markedly in archegonia and sporophyte production, as outlined above. Apomicts and sexual species also produced somewhat more antheridiate (average 4% more by week 16) and meristic gametophytes (average 27% more by week 6), respectively. Apo-sex hybrids differed considerably from both apomicts and sexual species, mostly in having lower germination (average 6% less by week 4), meristem formation (average 5% less by week 6), and late antheridia formation (average 5% less by week 16) rates.

Germination rate and sporophyte potential

Increasing spore age was significantly inversely associated with germination rates ($\chi^2 = 6.68$, df $= 1$, $P = 0.009$) but spore travel by post did not correlate significantly ($\chi^2 = 1.59$, df $= 1$, $P = 0.207$). Germination rates ranged from 0 to 100% with the average rate of 58% for germinated samples.

Sporophyte potential did not significantly differ between apomicts and sexual species (Figure 4), regardless of the samples tested (all samples: $\chi^2 = 0.96$, df $= 1$, $P = 0.326$; *Dryopteris*: $\chi^2 = 1.37$, df $= 1$, $P = 0.242$; non-zero potential: $\chi^2 = 1.60$, df $= 1$, $P = 0.206$). There were insignificantly more archegoniate sexual gametophytes than sporophyte-bearing apomictic gametophytes overall.
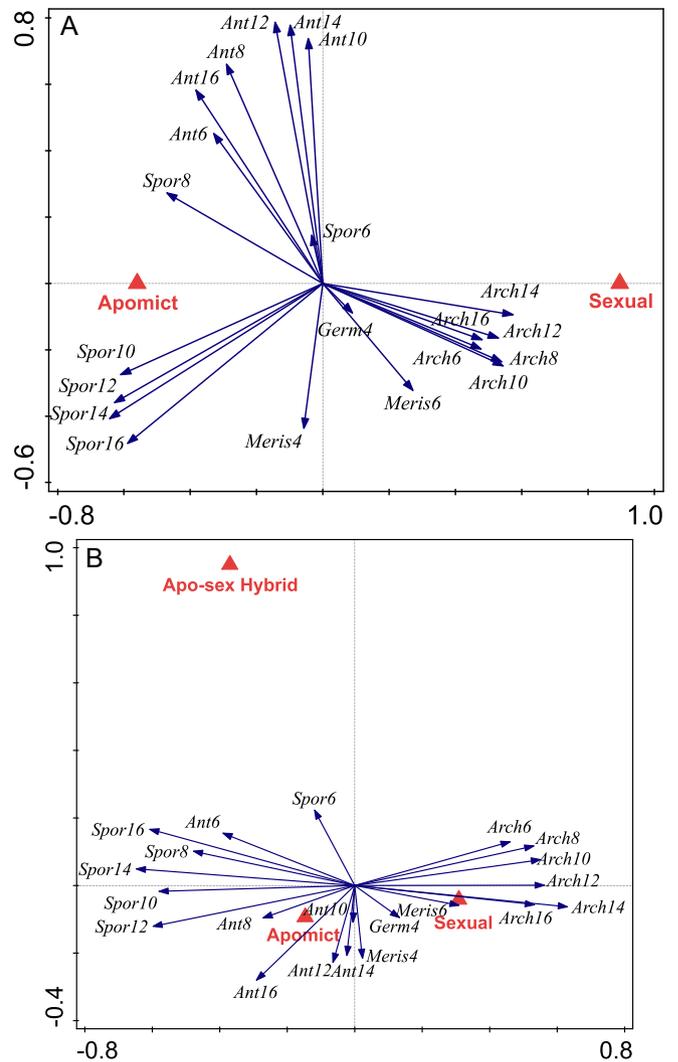
FIGURE 3 Germination rate, meristem formation rate, and reproductive characteristics significantly differ between (A) viable sexual and apomictic ferns newly examined in this study (RDA: pseudo-$F = 8.7$, $P < 0.001$), in which the first axis explains 20% variation; and (B) viable sexual, apomictic, and apo-sex hybrid ferns sampled (RDA: pseudo-$F = 5.3$, $P < 0.001$), in which the first two axes explain 20% and 22% variation, cumulatively. Tested characteristics: Germ = percent germinated spores, Meris = percent meristic gametophytes, Ant = percent gametophytes bearing antheridia, Arch = percent gametophytes bearing archegonia, and Spor = percent gametophytes with apogamous sporophytes. All characteristics are further labeled by the week of observation (4–16) throughout the experiment.

During week 6, three of 24 (13%) apomicts formed sporophyte-bearing gametophytes, and three of 15 (20%) sexual species formed archegoniate gametophytes. At week 8, 11 of 24 (46%) apomictic species sampled generated sporophytes from somatic, gametophyte tissue, and seven of 15 (47%) sexual species formed archegoniate gametophytes. The maximal proportion of archegoniate and sporophyte-bearing samples was also found during week 16 in the sexually reproducing *Dryopteris filix-mas* (65% archegoniate) and the apomictic *D. borreri* (73% sporophyte-bearing). Two sexually reproducing species (*D. filix-mas* and *Ceratopteris*
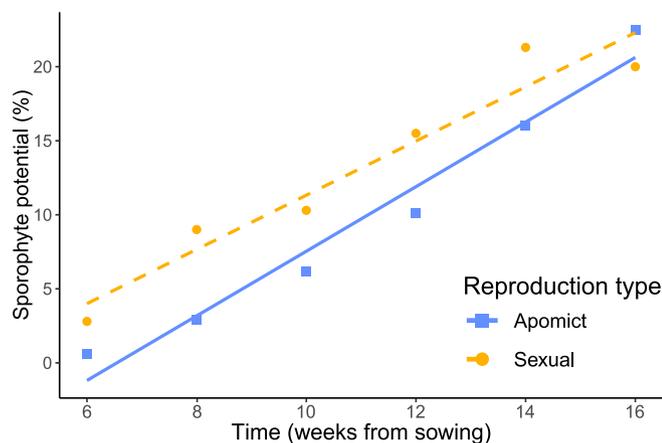
FIGURE 4 Changes in average sporophyte potential over the 16 weeks of the experiment (measurements began at week 6) in apomictic and sexual fern species with non-zero potential (forming either archegonia or sporophytes at some point). Sporophyte potential expresses the percentage of gametophytes bearing apogamous sporophytes and gametophytes bearing archegonia for apomictic and sexual species, respectively. The difference between reproductive types was not significant ($p = 0.206$; see text for details). Apo-sex hybrids were not included in this analysis.

richardii Brongn.), two hybrids (*D.* ×*complexa* and *D.* ×*critica*), and three apomictic species (*D. borreri*, *D. cambrensis* (Fraser-Jenk.) Beitel & W.R.Buck, *Myriopteris rufa* Fée) produced ≥50% of archegoniate or sporophyte-bearing gametophytes at some point, mostly at week 16.

Apomicts and gametangia in literature

Our list of gametangial expression in fern apomicts contains 62 taxa (Table 2), including three apo-sex hybrids and an additional group of "*Pteris* synthetic hybrids," 14 of which were added through our cultivation. Excluding the synthetic hybrids, our list represents 26% of the known apomict diversity (242 taxa; Liu et al., 2012). Considering all listed taxa, 59 (95%) formed antheridia and 21 (35%) formed archegonia. Overall, antheridia were considered viable, with multiple exceptions (e.g., in species forming spores via MFDR and in synthetic apomicts). Archegonia were universally deemed inviable or not taking part in sporophyte formation, where reported. Seven of the 14 reports with multiple references were conflicting, sometimes antheridia (*Aleuritopteris formosana* (Hayata) Tagawa, *Dryopteris borreri*), or archegonia (*D. affinis*, *D. bissetiana* (Baker) C.Chr., *D. borreri*, *Myriopteris rufa*, *Phegopteris connectilis* (Michx.) Watt, *Pteris cretica* L.) were not found by all authors.

DISCUSSION

This study analyzed several developmental features of fern apomictic and sexual species as well as their hybrids. The continuous existence of apo-sex hybrids in natural populations is driven by two key factors: their origin via hybridization and their independent reproduction, including backcrossing. Regarding origin, the co-occurrence of apomictic and sexual taxa not only allows for hybridization, but forces the two reproductive strategies into competition. Elucidating these competitive interactions is necessary to understand the frequency of hybridization. The ultimate form of competition in the gametophytic stage is the timely formation of sporophytes.

Apomictic and sexual species do not differ in sporophyte potential

Apogamous sporophytes are generally considered to form earlier than sporophytes originating from the sexual process (Haufler et al., 2016). For example, apogamously induced sporophytes were reported to form on apomict gametophytes before archegonia on sexual congeners in *Asplenium* (Regalado Gabancho et al., 2010), *Pellaea* (Whittier, 1968), and *Pteris* (Huang et al., 2006). Furthermore, as sexual species might need extra time for fertilization, apogamous sporophytes would gain a considerable competitive advantage over conspecific sexual sporophytes (Whittier, 1968). Contrary to the results of Laird and Sheffield (1986), the two apomicts forming both archegonia and sporophytes formed sporophytes before archegonia in our study, indicating a possible time advantage for the apogamous embryo over the sexual one. However, sexual species did not form sporophytes in our study, despite producing both gamete types, possibly due to the lack of a water film on top of the agar medium precluding sperm movement. Therefore, we cannot directly compare the time of origin for sporophytes between apomictic and sexual species. Nevertheless, there was no marked overall or early difference in sporophytic potential between reproductive types (Figure 4). The sporophytic potential was insignificantly larger in sexual species, but any possible advantage of sexual species would likely just be compensating for the lack of fertilization in some unsuccessful archegoniate gametophytes. Antheridia were generally present in gametophyte populations at the time of archegonial formation, so sexual gametophytes need not wait a significant amount of time before fertilization; thus, sexually formed sporophytes may originate at around the same time as competing apogamous sporophytes. A possible exception may occur in dry environments, where sperm may be ready for fertilization but cannot swim to the surrounding archegonia, due to the lack of water. For that reason, apomixis is sometimes viewed as an adaptation for dry climates (Haufler et al., 2016; Grusz et al., 2021), which our data cannot dispute, as we mostly focused on non-xerophytic fern apomicts.

Differences in gametophyte development of parents affect hybridization potential

Although apomicts may not necessarily have a time advantage in creating sporophytes, time may play a key

role from a different perspective. Apomicts seem to utilize antheridiogens at the same rate as sexual species (Hornych et al., 2021). Antheridiogens are pheromones produced by meristic gametophytes that affect the surrounding gametophytes of antheridiogen-sensitive taxa (Döpp, 1950; Atallah and Banks, 2015). These affected gametophytes forgo meristem formation and focus on creating antheridia instead (Näf et al., 1975; Schneller, 2008). Meristic gametophytes themselves are insensitive to this message (Näf, 1958). In a mixed population, a lower proportion of meristic gametophytes in one reproductive type may increase its susceptibility to antheridiogens and reduce the number of gametophytes capable of sporophyte formation. On the other hand, the amount of available sperm would increase, providing certain advantages.

We propose three scenarios, illustrated in Figure 5, presuming a similar abundance of apomicts and sexuals in a population and similar effects of antheridiogens on gametophytes of the same or different species. First, if apomicts have proportionally more meristic gametophytes than sexual competitors, the amount of male gametophytes and outcrossing rates of sexual species would increase (Schedlbauer and Klekowski, 1972; Haufler and Welling, 1994), thus increasing their genetic variability and, potentially, the growth vigor of young sporophytes. Sexual species would have an improved chance of eventually succeeding in competition on the sporophytic level, and the hybridization rate would be low. Second, if meristic sexuals and apomicts are equally abundant, neither reproductive strategy would gain an advantage and the hybridization rate would be intermediate. Third, if sexual species have proportionally more meristic gametophytes, the abundant sperm of apomicts might overwhelm most of the available sexual archegonia and force the sexual competition to raise

progeny with a substantial proportion of the apomict's genome and, eventually, inherit the apomictic reproductive strategy (as discussed below). Thus, the hybridization rate would be high. In accordance with the second scenario, we observed no considerable differences in meristem formation between apomictic and sexual species overall, especially during week 4 (Figure 3A). Nevertheless, regarding the parents of the two studied and viable apo-sex hybrids, the sexual *Dryopteris filix-mas* formed about twice as many meristic gametophytes (39%) as the apomicts (*D. affinis* and *D. borreri*, 20%), supporting the third scenario for these species combinations. Antheridiogens may therefore increase the amount of sperm formed by the apomicts and, consequently, hybridization rate. Nevertheless, due to our broad approach, we cultivated only a few of the parental species' samples and more cultivation experiments are needed to support this scenario.

Apomicts hybridize via sperm

The point of origin for any fern hybrid, including apomictic, is the merger of two gamete types, sperm and egg. Sexuality requires that both types are functional, and sexual species can theoretically contribute either gamete type. By contrast, apomicts are capable of self-perpetuating (i.e., producing sporophytes) without producing any gametes. Thus, selective pressures conserving the formation of viable gametangia are limited. Previous research indicates that fern apomicts are generally capable of forming antheridia (Table 2), but archegonia are rare and dysfunctional.

In our study, all taxa whose spores germinated formed antheridia at some point within the 16-week observation period. Between 3% and 77% of gametophytes formed antheridia. Apomictic ferns are clearly capable of forming antheridia (Table 2). Nevertheless, the formation of antheridia does not necessarily mean the production of viable sperm. Spermatogenesis in ferns is influenced by the type of agamospory in apomictic species. Species utilizing MFDR seem incapable of forming viable sperm (Walker, 1985; Regalado Gabancho et al., 2010). However, the production of sperm by most apomictic ferns, which form spores via PE, allows them to serve as paternal parents to apo-sex hybrids (Walker, 1962; Grusz et al., 2009; Jaruwattanaphan et al., 2013).

A process homologous to antheridia formation is the formation of viable pollen in angiosperms; both are required to produce male gametes. Pollen itself is a (micro)spore hosting the male gametophyte, so it is the inside of the pollen grain that is comparable to male fern gametophytes. Angiosperm apomicts are generally capable of creating viable pollen, sometimes in lesser quantities (Whitton et al., 2008; Podio et al., 2012; Hajrudinović et al., 2015; Rotreklová and Krahulcová, 2016). In apomicts, the principal difference between fern and angiosperm male gamete production lies in the fact that angiosperm apomicts can produce reduced haploid (n) pollen (Caetano et al.,
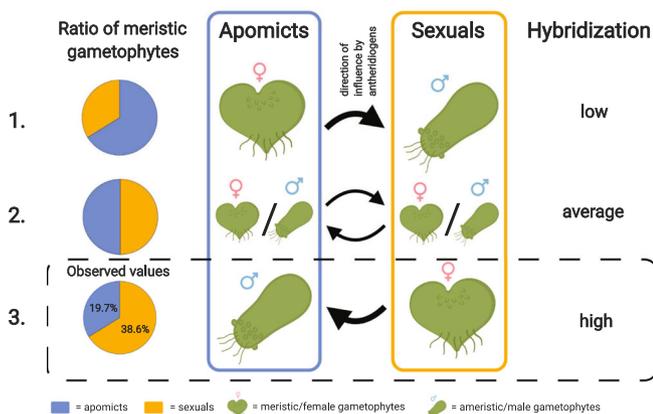
FIGURE 5 Three possible scenarios of how the proportion of meristic gametophytes could be affecting hybridization rates, presuming a similar abundance of apomicts and sexuals in a population: 1. Meristic apomicts dominate, leading to a low rate of hybridization. 2. The abundance of meristic apomictic and sexual gametophytes is equal, resulting in intermediate rates of hybridization. 3. Meristic sexual gametophytes dominate, leading to a high rate of hybridization. The third scenario best fits the results of this study.

2013; Lepší et al., 2019), while fern apomicts only form viable diploid (2n) spores, and the haploid spore pathway leads to abortion in ferns (Manton, 1950).

Despite cultivating 21 viable apomictic spore samples, only two of our tested species produced archegonia (*D. purpurella* and *D. tsushimensis*). Including previously published reports, 34% of fern apomicts are capable of forming archegonia, mostly considered inviable (Table 2). However, some apomictic species capable of forming archegonia under certain conditions may be reported as only antheridiate. For example, our two samples of *D. affinis* only formed antheridia, but rarely occurring archegonia were reported for *D. pseudomas* (synonym of *D. affinis* and *D. borreri*) by Duncan (1943). It is therefore possible that a higher proportion of apomictic taxa would form archegonia under further evaluation, because the data for most species is backed by just one published report. Archegonia of fern apomicts are therefore perhaps not as rare as previously considered (Manton, 1950), but likely have zero reproductive potential. Because of that, apomictic ferns are generally considered incapable of being the maternal parent of apo-sex hybrids. However, research on various apomictic complexes indicates that apomicts may be the maternal parents of some apomictic species (Hori et al., 2014, 2016, 2018; Hori and Murakami, 2019), although the evidence is indirect, and yet undiscovered taxa may have fulfilled that role instead. It is impossible to exclude the possibility of viable archegonia of apomictic ferns serving as a place of origin for hybrids. Nevertheless, such an occurrence would probably be incredibly rare.

In contrast to apomictic ferns, angiosperm apomicts may often serve as maternal parents. Obligate apomixis in angiosperms is rare, and most apomicts engage in sexual reproduction, including the provision of eggs (Tucker et al., 2003; Hörandl, 2010; Krahulcová et al., 2014). In some cases, the contribution of unreduced eggs may be considerably higher than that of unreduced pollen in apo-sex hybrids (Krahulcová et al., 2004).

Apo-sex hybrids self-perpetuate and are capable of backcrossing

Although our sampling and cultivation strategy limits our ability to compare individual taxa, several considerable differences exist between the three viable hybrid samples and their parental taxa. Apo-sex hybrids had lower germination rates than their parental species, indicating a genomic imbalance (chromosomal incompatibilities during meiosis) caused by the hybridization event and leading to post-zygotic hybridization barriers. This effect is multiplied by their considerably higher spore abortion rate (~90%; Ekrt and Koutecký, 2016; Hornych and Ekrt, 2017). Thus, even of the ~10% of spores that appear viable, about half fail to germinate. All germinated apo-sex hybrids produced antheridia and abundant apogamous sporophytes. In concordance with the overall trend in apomicts, archegonia were absent. The same was previously described for one of

the tested hybrids, *Dryopteris ×critica* (Ekrt and Koutecký, 2016). Walker (1962) found that synthetic apo-sex hybrids of *Pteris* also produced antheridia but their sperm was sluggish and unable to merge with eggs. The motility of sperm may thus be low initially, but subject to selective pressure, improving to the levels seen in regular apomictic species (Walker, 1962). By contrast, nonfunctional archegonia and antheridia were found in the apo-sex hybrid *Asplenium × lellingerianum* C.Sánchez & L.Regalado, although these findings were attributed to the rare MFDR agamospory type utilized by the hybrid and its parent *A. monodon* Liebm. (Regalado Gabancho et al., 2010). In summary, apo-sex hybrids tend to behave like their apomictic parents rather than the sexual ones but suffer from an early disadvantage in the form of lower germination rates.

Apo-sex hybrids independently reproduce via apomixis and, in theory, are further capable of mixing with sexual species via sperm. The pentaploid *Dryopteris ×critica* was found to uniquely create viable spores of two ploidy levels, pentaploid and polyhaploid (2.5x; Ekrt and Koutecký, 2016). The tetraploid *D. ×complexa* formed antheridia in our cultivation. If capable of producing viable reduced spores, as the related *D. ×critica* does, *D. ×complexa* could fertilize the common tetraploid *D. filix-mas* with its diploid sperm, leading to a tetraploid hybrid swarm. The individuals in this swarm would be perhaps the first facultative apomicts confirmed in ferns. Further studies on the reproductive capabilities of even-ploidy apo-sex hybrids are needed to test this hypothesis.

Methodological remarks

While fern spores may germinate after tens of years of dormancy (Lloyd and Klekowski, 1970), overall spore viability and germination rates decrease with spore age (Smith and Robinson, 1975; Windham et al., 1986; Beri and Bir, 1993). A similar pattern was seen in our study, since germination rates significantly decreased with spore age. Thus, older samples (>5 yr), such as from spore banks and herbaria, should be used with caution. To estimate spore viability, spore abortion may be a more reliable character in these samples (Hornych and Ekrt, 2017).

Interestingly, in our redundancy analyses, spores traveling by post from several different locations (in Europe, USA, and Japan) developed significantly differently than non-traveling spores. However, the effect of travel on germination was insignificant. We are unsure whether these results have a valid biological explanation or are just an artifact. Nevertheless, future research working with a mixture of spores, some of which traveled by post, should consider taking this factor into account.

Because a lack of nutrients may hinder gametophyte growth, a full-strength Murashige and Skoog medium was used to lessen the impact of resource competition. However, media that are too concentrated may also negatively impact

gametophytes (Korpelainen, 1994). Due to these two effects and the commonly observed variability of sexual expression in fern species (Huang et al., 2004; DeSoto et al., 2008), cultivation results are best interpreted as general principles rather than details on individual species, unless proper replication is performed. The conflicting results of archegonia observation within apomictic species (Table 2) are an example of this principle.

CONCLUSIONS

Based on a robust data set of 24 apomictic, 18 sexual, and three apo-sex hybrid (hybrid of an apomict and a sexual species) viable fern taxa, we examined the differences in gametophyte development. We defined the term *sporophytic potential* to evaluate the joint percentage of archegoniate gametophytes in sexual species and the percentage of sporophyte-bearing gametophytes in apomicts. Unexpectedly, this research demonstrated that sporophyte potential did not differ between sexual and apomictic species. The noticeable difference in the overall developmental patterns of these two reproductive types may lead to major evolutionary consequences, affecting potential interspecific competition and the likelihood of forming an apo-sex hybrid. Furthermore, the formation of archegonia in fern apomicts is not as rare as previously reported, but the archegonia are likely of zero reproductive potential. Instead, apomicts rely on the apogamous formation of sporophytes. Apo-sex hybrids inherit the apomictic reproductive strategy almost exclusively, are capable of forming male gametangia and may established independent apomictic lineages, especially once they overcome challenges limiting their germination rate. Consequently, apomicts may use hybridization to absorb and perpetuate genetic variability found in sexual congeners, thus adapting to ever-changing conditions.

AUTHOR CONTRIBUTIONS
O.H. and L.E. conceived the study and performed statistical analyses. O.H., K.H., and L.E. collected spore material. O.H., A.F., and J.K. performed laboratory analyses. All authors contributed to writing the manuscript and gave final approval for publication.

DATA AVAILABILITY STATEMENT
The appendix files are deposited online at Zenodo at https://zenodo.org/record/5818039#.Yi98v7hOlao.

ORCID
Ondřej Hornych https://orcid.org/0000-0002-5093-3103
Jiří Košnar https://orcid.org/0000-0001-5128-593X
Libor Ekrt https://orcid.org/0000-0003-3947-787X

REFERENCES
Albertini, E., G. Barcaccia, J. G. Carman, and F. Pupilli. 2019. Did apomixis evolve from sex or was it the other way around? *Journal of Experimental Botany* 70: 2951–2964.

Atallah, N. M., and J. A. Banks. 2015. Reproduction and the pheromonal regulation of sex type in fern gametophytes. *Frontiers in Plant Science* 6: 100.

Baack, E., M. C. Melo, L. H. Rieseberg, and D. Ortiz-Barrientos. 2015. The origins of reproductive isolation in plants. *New Phytologist* 207: 968–984.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67: 1–48.

Beri, A., and S. S. Bir. 1993. Germination of stored spores of *Pteris vittata* L. *American Fern Journal* 83: 73–78.

ter Braak, C., and P. Šmilauer. 2012. Canoco reference manual and 'user's guide: software of ordination (version 5.0). Microcomputer Power (Ithaca, NY, USA).

Braithwaite, A. F. 1964. A new type of apogamy in ferns. *New Phytologist* 63: 293–305.

Caetano, A. P. S., S. P. Teixeira, E. R. Forni-Martins, and S. M. Carmello-Guerreiro. 2013. Pollen insights into apomictic and sexual *Miconia* (Miconieae, Melastomataceae). *International Journal of Plant Sciences* 174: 760–768.

Carman, J. G., M. Mateo de Arias, L. Gao, X. Zhao, B. M. Kowallis, D. A. Sherwood, M. K. Srivastava, et al. 2019. Apospory and diplospory in diploid *Boechera* (Brassicaceae) may facilitate speciation by recombination-driven apomixis-to-sex reversals. *Frontiers in Plant Science* 10: 724.

Chen, Z. J. 2010. Molecular mechanisms of polyploidy and hybrid vigor. *Trends in Plant Science* 15: 57–71.

Chiou, W.-L., Y.-M. Huang, T.-H. Hsieh, and S.-Y. Hsu. 2006. *Diplazium megaphyllum* (Bak.) Christ, a rare fern in Taiwan, reproduces by apogamy. *Taiwan Journal of Forest Science* 21: 39–47.

Chrtek, J., P. Mráz, A. Belyayev, L. Paštová, V. Mrázová, P. Caklová, J. Josefiová, et al. 2020. Evolutionary history and genetic diversity of apomictic allopolyploids in *Hieracium* s.str.: morphological versus genomic features. *American Journal of Botany* 107: 66–90.

DeSoto, L., L. G. Quintanilla, and M. Méndez. 2008. Environmental sex determination in ferns: effects of nutrient availability and individual density in *Woodwardia radicans*. *Journal of Ecology* 96: 1319–1327.

Doležal, J., A. Krahulcová, T. Urfus, and F. Krahulec. 2020. Residual sexuality of the apomict *Pilosella rubra* under natural conditions in the Krkonoše Mts. *Preslia* 92: 403–428.

Döpp, W. 1932. Die Apogamie bei *Aspidium remotum* Al. Br. 17: 86–152.

Döpp, W. 1950. Eine die Antheridienbildung bei Farnen fördernde Substanz in den Prothallien von *Pteridium aquilinum* (L.) Kuhn. *Berichte der Deutschen Botanischen Gesellschaft* 63: 139–147.

Döpp, W. 1959. Über eine hemmende und eine fördernde Substanz bei der Antheridienbildung in den Prothallien von *Pteridium aquilinum*. *Berichte der Deutschen Botanischen Gesellschaft* 72: 11–24.

Dubey, J. P., and S. K. Roy. 1985. A new antheridiogen from the fern *Pityrogramma calomelanos* (L.) Link. *Proceedings of the Indian National Science Academy* 95: 173–179.

Duncan, R. E. 1943. Origin and development of embryos in certain apogamous forms of *Dryopteris*. *Botanical Gazette* 105: 202–211.

Dyer, R. J., V. Savolainen, and H. Schneider. 2012. Apomixis and reticulate evolution in the *Asplenium monanthes* fern complex. *Annals of Botany* 110: 1515–1529.

Ekrt, L., and P. Koutecký. 2016. Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (Dryopteridaceae). *Annals of Botany* 117: 97–106.

Eschelmüller, A. 1998. Keimversuche mit Sporen der triploiden Sippen von *Dryopteris affinis* und ihren Bastarden mit *Dryopteris filix-mas*. *Mitteilungen des Naturwisssenschaftlichen Arbeitskreises Kempten* 36: 47–78.

Evans, A. M. 1964. Ameiotic alternation of generations: A new life cycle in the ferns. *Science* 143: 261–263.

Fiaz, S., X. Wang, A. Younas, B. Alharthi, A. Riaz, and H. Ali. 2021. Apomixis and strategies to induce apomixis to preserve hybrid vigor for multiple generations. *GM Crops & Food* 12: 57–70.

Fraser-Jenkins, C. R. 2007. The species and subspecies in the *Dryopteris affinis* group. *Fern Gazette* 18: 1–26.

Gemmrich, A. R. 1986. Antheridiogenesis in the fern *Pteris vittata* II) hormonal control of antheridium formation. *Journal of Plant Physiology* 125: 157–166.

Goulet, B. E., F. Roda, and R. Hopkins. 2017. Hybridization in plants: Old ideas, new techniques. *Plant Physiology* 173: 65–78.

Grusz, A. L. 2016. A current perspective on apomixis in ferns. *Journal of Systematics and Evolution* 54: 656–665.

Grusz, A. L., M. D. Windham, and K. M. Pryer. 2009. Deciphering the origins of apomictic polyploids in the *Cheilanthes yavapensis* complex (Pteridaceae). *American Journal of Botany* 96: 1636–1645.

Grusz, A. L., M. D. Windham, K. T. Picard, K. M. Pryer, E. Schuettpelz, and C. H. Haufler. 2021. A drought-driven model for the evolution of obligate apomixis in ferns: evidence from pellaeids (Pteridaceae). *American Journal of Botany* 108: 263–283.

Guo, Z.-Y., and H.-M. Liu. 2013. Gametophyte morphology and development of three species of *Cyrtogonellum* Ching (Dryopteridaceae). *American Fern Journal* 103: 153–165.

Hajrudinović, A., S. Siljak-Yakovlev, S. C. Brown, F. Pustahija, M. Bourge, D. Ballian, and F. Bogunić. 2015. When sexual meets apomict: genome size, ploidy level and reproductive mode variation of *Sorbus aria s.l.* and *S. austriaca* (Rosaceae) in Bosnia and Herzegovina. *Annals of Botany* 116: 301–312.

Haufler, C. H., and G. J. Gastony. 1978. Antheridiogen and the breeding system in the fern genus *Bommeria*. *Canadian Journal of Botany* 56: 1594–1601.

Haufler, C. H., and C. B. Welling. 1994. Antheridiogen, dark spore germination, and outcrossing mechanisms in *Bommeria* (Adiantaceae). *American Journal of Botany* 81: 616–621.

Haufler, C. H., K. M. Pryer, E. Schuettpelz, E. B. Sessa, D. R. Farrar, R. Moran, J. J. Schneller, et al. 2016. Sex and the single gametophyte: revising the homosporous vascular plant life cycle in light of contemporary research. *BioScience* 66: 928–937.

Hernández, M. A., A. R. Andrada, V. de los A. Páez, and O. G. Martínez. 2015. Ploidy level and obligate apogamy in two populations of *Argyrochosma nivea* var. *tenera* (Pteridaceae). *Hoehnea* 42: 233–237.

Hörandl, E. 2010. The evolution of self-fertility in apomictic plants. *Sexual Plant Reproduction* 23: 73–86.

Hori, K., and N. Murakami. 2019. Origin of the *Diplazium hachijoense* complex (Athyriaceae). *PhytoKeys* 124: 57–76.

Hori, K., A. Tono, K. Fujimoto, J. Kato, A. Ebihara, Y. Watano, and N. Murakami. 2014. Reticulate evolution in the apogamous *Dryopteris varia* complex (Dryopteridaceae, subg. *Erythrovariae*, sect. *Variae*) and its related sexual species in Japan. *Journal of Plant Research* 127: 661–684.

Hori, K., Y. Watano, and N. Murakami. 2016. Hybrid origin of the apogamous fern *Dryopteris hondoensis* (Dryopteridaceae). *Acta Phytotaxonomica et Geobotanica* 67: 133–146.

Hori, K., X. Zhou, Y.-H. Yan, Y. Inoue, and N. Murakami. 2018. Evidence for maternal ability in hybridization of apogamous fern species: *Dryopteris tsushimense* K. Hori & N. Murak. and *D. subtsushimense* K. Hori & N. Murak. (Dryopteridaceae), new tetraploid apogamous pteridophytes of hybrid origin from Tsushima, Japan. *Acta Phytotaxonomica et Geobotanica* 69: 143–160.

Hornych, O., and L. Ekrt. 2017. Spore abortion index (SAI) as a promising tool of evaluation of spore fitness in ferns: an insight into sexual and apomictic species. *Plant Systematics and Evolution* 303: 497–507.

Hornych, O., L. Ekrt, F. Riedel, P. Koutecký, and J. Košnar. 2019. Asymmetric hybridization in Central European populations of the *Dryopteris carthusiana* group. *American Journal of Botany* 106: 1477–1486.

Hornych, O., W. L. Testo, E. B. Sessa, J. E. Watkins, C. E. Campany, J. Pittermann, and L. Ekrt. 2021. Insights into the evolutionary history and widespread occurrence of antheridiogen systems in ferns. *New Phytologist* 229: 607–619.

Huang, Y.-M., H.-M. Chiou, and W.-L. Chiou. 2004. Density affects gametophyte growth and sexual expression of *Osmunda cinnamomea* (Osmundaceae: Pteridophyta). *Annals of Botany* 94: 229–232.

Huang, Y.-M., H.-M. Chou, T.-H. Hsieh, J.-C. Wang, and W.-L. Chiou. 2006. Cryptic characteristics distinguish diploid and triploid varieties of *Pteris fauriei* (Pteridaceae). *Canadian Journal of Botany* 84: 261–268.

Huang, Y.-M., S.-Y. Hsu, M. H. Huang, and W.-L. Chiou. 2009. Reproductive biology of three Cheilanthoid ferns in Taiwan. *The International Journal of Plant Reproductive Biology* 1: 109–116.

Huang, Y.-M., S.-Y. Hsu, T.-H. Hsieh, and H.-M. Chou. 2011. Three *Pteris* species (Pteridaceae: Pteridophyta) reproduce by apogamy. *Botanical Studies* 52: 79–87.

Jaruwattanaphan, T., S. Matsumoto, and Y. Watano. 2013. Reconstructing hybrid speciation events in the *Pteris cretica* group (Pteridaceae) in Japan and adjacent regions. *Systematic Botany* 38: 15–27.

Kentner, E. K., and M. R. Mesler. 2000. Evidence for natural selection in a fern hybrid zone. *American Journal of Botany* 87: 1168–1174.

Korpelainen, H. 1994. Growth, sex determination and reproduction of *Dryopteris filix-mas* (L.) Schott gametophytes under varying nutritional conditions. *Botanical Journal of the Linnean Society* 114: 357–366.

Koutecký, P., T. Baďurová, M. Štech, J. Košnar, and J. Karásek. 2011. Hybridization between diploid *Centaurea pseudophrygia* and tetraploid *C. jacea* (Asteraceae): the role of mixed pollination, unreduced gametes, and mentor effects. *Biological Journal of the Linnean Society* 104: 93–106.

Krahulcová, A., S. Papoušková, and F. Krahulec. 2004. Reproduction mode in the allopolyploid facultatively apomictic hawkweed *Hieracium rubrum* (Asteraceae, H. subgen. *Pilosella*). *Hereditas* 141: 19–30.

Krahulcová, A., O. Rotreklová, and F. Krahulec. 2014. The detection, rate and manifestation of residual sexuality in apomictic populations of *Pilosella* (Asteraceae, Lactuceae). *Folia Geobotanica* 49: 239–258.

Laird, S., and E. Sheffield. 1986. Antheridia and archegonia of the apogamous fern *Pteris cretica*. *Annals of Botany* 57: 139–143.

Lepší, M., P. Koutecký, J. Nosková, P. Lepší, T. Urfus, and T. C. G. Rich. 2019. Versatility of reproductive modes and ploidy level interactions in *Sorbus* s.l. (Malinae, Rosaceae). *Botanical Journal of the Linnean Society* 191: 502–522.

Lin, S.-J., M. Kato, and K. Iwatsuki. 1992. Diploid and triploid offspring of triploid agamosporous fern *Dryopteris pacifica*. *Botanical Magazine Tokyo* 105: 443–452.

Liu, H.-M., R. J. Dyer, Z.-Y. Guo, Z. Meng, J.-H. Li, and H. Schneider. 2012. The evolutionary dynamics of apomixis in ferns: A case study from Polystichoid ferns. *Journal of Botany* 2012: 1–11.

Lloyd, R. M., and E. J. Klekowski. 1970. Spore germination and viability in Pteridophyta: Evolutionary significance of chlorophyllous spores. *Biotropica* 2: 129–137.

Ma, Y., W. Xie, X. Tian, W. Sun, Z. Wu, and R. Milne. 2014. Unidirectional hybridization and reproductive barriers between two heterostylous primrose species in north-west Yunnan, China. *Annals of Botany* 113: 763–775.

Manton, I. 1950. Problems of cytology and evolution in the Pteridophyta. Cambridge University Press, London.

Manton, I., S. K. Roy, and F. M. Jarrett. 1966. The cytotaxonomy of some members of the *Cheilanthes farinosa* complex in Africa and India. *Kew Bulletin* 18: 553–565.

Martínez, O. G., M. A. Hernández, and M. Ponce. 2017. Reproductive expression of cheilanthoid ferns (Pteridaceae) from South America. *Flora* 236–237: 126–131.

Mogie, M. 1992. The evolution of asexual reproduction in plants. Springer Netherlands.

Momose, S. 1967. Prothallia of the Japanese ferns (Filicales). University of Tokyo Press, Tokyo.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bio assays with Tabacco tissue cultures. *Physiologia Plantarum* 15: 473–497.

Näf, U. 1958. On the physiology of antheridium formation in the Bracken fern [*Pteridium aquilinum* (L) Kuhn]. *Physiologia Plantarum* 11: 728–746.

Näf, U. 1966. On dark-germination and antheridium formation in *Anemia phyllitidis*. *Physiologia Plantarum* 19: 1079–1088.

Näf, U., K. Nakanishi, and M. Endo. 1975. On the physiology and chemistry of fern antheridiogens. *Botanical Review* 41: 315–359.

Pangua, E., S. Pajarón, and L. G. Quintanilla. 2019. Fitness of an allopolyploid rupicolous fern compared with its diploid progenitors: from sporogenesis to sporophyte formation. *American Journal of Botany* 106: 984–995.

Park, C.-H., and M. Kato. 2003. Apomixis in the interspecific triploid hybrid fern *Cornopteris christenseniana* (Woodsiaceae). *Journal of Plant Research* 116: 93–103.

Patel, N., C.-X. Li, L.-B. Zhang, and D. S. Barrington. 2018. Biodiversity and apomixis: Insights from the East-Asian holly ferns in *Polystichum* section *Xiphopolystichum*. *Molecular Phylogenetics and Evolution* 127: 345–355.

Podio, M., L. A. Siena, D. Hojsgaard, J. Stein, C. L. Quarin, and J. P. A. Ortiz. 2012. Evaluation of meiotic abnormalities and pollen viability in aposporous and sexual tetraploid *Paspalum notatum* (Poaceae). *Plant Systematics and Evolution* 298: 1625–1633.

Poelt, J. 1960. *Dryopteris borreri* in Bavaria. *American Fern Journal* 50: 114–117.

Quintanilla, L. G., and A. Escudero. 2006. Spore fitness components do not differ between diploid and allotetraploid species of *Dryopteris* (Dryopteridaceae). *Annals of Botany* 98: 609–618.

Quintanilla, L. G., L. DeSoto, A. Jimenez, and M. Mendez. 2007. Do antheridiogens act via gametophyte size? A study of *Woodwardia radicans* (Blechnaceae). *American Journal of Botany* 94: 986–990.

R Development Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Regalado Gabancho, L., C. Prada, and J. M. Gabriel y Galán. 2010. Sexuality and apogamy in the Cuban *Asplenium auritum–monodon* complex (Aspleniaceae). *Plant Systematics and Evolution* 289: 137–146.

Reyes Jaramillo, I., S. L. Camargo Ricalde, and M. de los Á. Aquiahuatl Ramos. 2008. Mycorrhizal-like interaction between gametophytes and young sporophytes of the fern *Dryopteris muenchii* (Filicales) and its fungal endophyte. *Revista de Biología Tropical* 56: 1101–1107.

Rieseberg, L. H., and S. E. Carney. 1998. Plant hybridization. *New Phytologist* 140: 599–624.

Rotreklová, O., and A. Krahulcová. 2016. Estimating paternal efficiency in an agamic polyploid complex: pollen stainability and variation in pollen size related to reproduction mode, ploidy level and hybridogenous origin in *Pilosella* (Asteraceae). *Folia Geobotanica* 51: 175–186.

Sareen, B., A. Bhattacharya, M. Sharma, A. Sood, and P. S. Ahuja. 2014. A simple technique for tracking individual spore and gametophyte development in *Adiantum lunulatum* Burm. f. using modified extra thin alginate film technique. *Indian Journal of Experimental Biology* 52: 5.

Šarhanová, P., T. F. Sharbel, M. Sochor, R. J. Vašut, M. Dančák, and B. Trávníček. 2017. Hybridization drives evolution of apomicts in *Rubus* subgenus *Rubus*: evidence from microsatellite markers. *Annals of Botany* 120: 317–328.

Scheben, A., and D. Hojsgaard. 2020. Can we use gene-editing to induce apomixis in sexual plants? *Genes* 11: 781.

Schedlbauer, M. D., and E. J. Klekowski. 1972. Antheridogen activity in the fern *Ceratopteris thalictroides* (L.) Brongn. *Botanical Journal of the Linnean Society* 65: 399–413.

Schneller, J. J. 1981. Bemerkungen zur Biologie der Wurmfarngruppe. *Farnblätter* 7: 9–17.

Schneller, J. J. 1988. Spore bank, dark germination and gender determination in *Athyrium* and *Dryopteris*: results and implications for population biology of Pteridophyta. *Botanica Helvetica* 98: 77–86.

Schneller, J. J. 2008. Antheridiogens. *In* T. A. Ranker, and C. H. Haufler [eds.], Biology and evolution of ferns and lycophytes., 134–158. Cambridge University Press, Cambridge, UK.

Sessa, E. B., W. L. Testo, and J. E. Watkins. 2016. On the widespread capacity for, and functional significance of, extreme inbreeding in ferns. *New Phytologist* 211: 1108–1119.

Sigel, E. M. 2016. Genetic and genomic aspects of hybridization in ferns. *Journal of Systematics and Evolution* 54: 638–655.

Sigel, E. M., M. D. Windham, L. Huiet, G. Yatskievych, and K. M. Pryer. 2011. Species relationships and farina evolution in the cheilanthoid fern genus *Argyrochosma* (Pteridaceae). *Systematic Botany* 36: 554–564.

Smith, D. L., and P. M. Robinson. 1975. The effects of spore age on germination and gametophyte development in *Polypodium vulgare* L. *New Phytologist* 74: 101–108.

Steil, W. N. 1918. Studies of some new cases of apogamy in ferns. *Bulletin of the Torrey Botanical Club* 45: 93–108.

Tanaka, J., K. Yano, K. Aya, K. Hirano, S. Takehara, E. Koketsu, R. L. Ordonio, et al. 2014. Antheridiogen determines sex in ferns via a spatiotemporally split gibberellin synthesis pathway. *Science* 346: 469–473.

Testo, W. L., M. S. Grasso, and D. S. Barrington. 2014. Beyond antheridiogens: chemical competition between gametophytes of *Polypodium appalachianum* and *Polypodium virginianum*. *The Journal of the Torrey Botanical Society* 141: 302–312.

Testo, W. L., J. E. Watkins, and D. S. Barrington. 2015. Dynamics of asymmetrical hybridization in North American wood ferns: reconciling patterns of inheritance with gametophyte reproductive biology. *New Phytologist* 206: 785–795.

Tucker, M. R., A.-C. G. Araujo, N. A. Paech, V. Hecht, E. D. L. Schmidt, J.-B. Rossell, S. C. de Vries, and A. M. G. Koltunow. 2003. Sexual and apomictic reproduction in *Hieracium* subgenus *Pilosella* are closely interrelated developmental pathways. *The Plant Cell* 15: 1524–1537.

Tucker, M. R., and A. M. G. Koltunow. 2009. Sexual and asexual (apomictic) seed development in flowering plants: molecular, morphological and evolutionary relationships. *Functional Plant Biology* 36: 490–504.

Verma, S. C. 1977. Cytology and reproduction of *Cheilanthes farinosa* from Yemen. *Fern Gazette* 11: 325–328.

Voeller, B. R. 1964. Antheridiogens in ferns. Regulateurs naturels de la croissance vegetale., 665–684. Gif s/Yvette, Paris.

Wagner, W. H., and K. L. Chen. 1965. Abortion of spores and sporangia as a tool in the detection of *Dryopteris* hybrids. *American Fern Journal* 55: 9–29.

Walker, T. G. 1958. Hybridization in some species of *Pteris* L. *Evolution* 12: 82–92.

Walker, T. G. 1962. Cytology and evolution in the fern genus *Pteris* L. *Evolution* 16: 27–43.

Walker, T. G. 1985. Some aspects of agamospory in ferns—the Braithwaite system. *Proceedings of the Royal Society of Edinburgh* 86: 59–66.

Whittier, D. P. 1968. "Rate of gametophyte maturation in sexual and apogamous forms of *Pellaea glabella*." *American Fern Journal* 58: 12–19.

Whittier, D. P. 1970. The rate of gametophyte maturation in sexual and apogamous species of ferns. *Phytomorphology* 20: 30–35.

Whitton, J., C. J. Sears, E. J. Baack, and S. P. Otto. 2008. The dynamic nature of apomixis in the angiosperms. *International Journal of Plant Sciences* 169: 169–182.

Windham, M. D. 1983. The ferns of Elden Mountain, Arizona. *American Fern Journal* 73: 85.

Windham, M. D., P. G. Wolf, and T. A. Ranker. 1986. Factors affecting prolonged spore viability in herbarium collections of three species of *Pellaea*. *American Fern Journal* 76: 141–148.

Xiang, L., C. R. Werth, S. N. Emery, and D. E. McCauley. 2000. Population-specific gender-biased hybridization between *Dryopteris intermedia* and *D. carthusiana*: evidence from chloroplast DNA. *American Journal of Botany* 87: 1175–1180.

Yatskievych, G. 1993. Antheridiogen response in *Phanerophlebia* and related rern genera. *American Fern Journal* 83: 30–36.

Zanella, C. M., C. Palma-Silva, M. Goetze, and F. Bered. 2016. Hybridization between two sister species of Bromeliaceae: *Vriesea carinata* and *V. incurvata*. *Botanical Journal of the Linnean Society* 181: 491–504.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix S1. Samples used in this study. Vouchers are deposited in the University of South Bohemia, Czech Republic (CBFS), Tokyo Metropolitan University, Japan (MAK), Makino Botanical Garden, Japan (MBK) or spores were sourced directly from the Spore Exchange of the American Fern Society (AFS SE) and Carolina Biological Supply Company.

Appendix S2. Results of cultivation analyses over the course of 16 weeks.

Paper 4

Hornych O., Černochová L., Lisner A., Ekrt L. (2022): An experimental assessment of competitive interactions between sexual and apomictic fern gametophytes using Easy Leaf Area. – Applications in Plant Sciences 10: e11466.

APPLICATION ARTICLE

An experimental assessment of competitive interactions between sexual and apomictic fern gametophytes using Easy Leaf Area

Ondřej Hornych [ID] | Lucie Černochová | Aleš Lisner [ID] | Libor Ekrt [ID]

Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, České Budějovice, CZ-37005, Czech Republic

Correspondence

Ondřej Hornych, Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, České Budějovice, CZ-37005, Czech Republic.
Email: hornych.ondrej@gmail.com

This article is part of the special issue "Methodologies in Gametophyte Biology."

Abstract

Premise: Few studies have explored competition in fern gametophyte populations. One limiting factor is the tedious measurement of gametophyte size as a proxy for biomass in these small plants. Here, an alternative approach of estimating the number of green pixels from photos was employed to measure the competitive interactions among apomictic and sexual *Dryopteris* gametophytes.

Methods: We cultivated the gametophytes of two apomictic (diploid and triploid) and one sexual (tetraploid) *Dryopteris* species in monocultures and in two-species mixtures in the ratios 1 : 1 and 1 : 3. The total gametophyte cover of each population originating from 20 spores was assessed using Easy Leaf Area. Assessments were performed weekly between weeks 4 and 10 of cultivation. Additionally, during week 5, the cover of each species in each mixture was estimated separately.

Results: We identified a positive correlation between gametophyte size and ploidy level as well as sexual reproduction. The performance of the tested species in mixtures was dependent on the competitor species identity, indicating the importance of competition between gametophytes.

Discussion: The methods outlined can be used for a rapid assessment of fern gametophyte cover in large gametophyte populations. Ploidy level and reproduction type seem to play a major role in the competitive abilities of fern gametophytes, but more research is needed on this topic.

KEYWORDS

apomixis, competition, *Dryopteris*, monoculture, pteridophyte, spore, wood fern

The fern gametophyte represents a unique organism model that may be utilized for various research purposes. Unlike the gametophytes of other higher plants, fern gametophytes are spatially and nutritionally independent from the sporophyte (Haufler et al., 2016), although they are relatively small (millimeters or centimeters in size). Fern spores, from which gametophytes arise, remain viable for years or even decades in storage (Lloyd and Klekowski, 1970; Windham et al., 1986). They generally germinate within weeks in suitable conditions, and the gametophytes fully develop within months (Lloyd and Klekowski, 1970; Van Nguyen et al., 2020). The shape, size, and sexual expression of fern gametophytes reflect a multitude of abiotic and biotic factors (Korpelainen, 1994; Pajarón et al., 2015;

Pangua et al., 2019), which may be studied with a proper cultivation setup (Dyer, 1979).

The presence of fern gametophytes has been demonstrated to affect the size and sexual expression of other gametophytes in populations based on the resulting gametophyte density and the release of antheridiogens into the habitat. When sown at high densities, fern gametophytes tend to be small and male, or may even completely lack gametangia, while at lower densities, gametophytes are larger and female or hermaphroditic (Huang et al., 2004; DeSoto et al., 2008). However, at very low densities or in single-spore cultures, gametophyte growth may be retarded or abnormal (reviewed by Dyer, 1979). Antheridiogens are pheromones released into the environment by archegoniate (female) gametophytes (possessing a lateral meristem) and

absorbed exclusively by undifferentiated asexual gametophytes (Schneller et al., 1990). The receptive gametophytes respond by slowing their growth and producing abundant antheridia (male gametangia) at the expense of archegonia (Schneller, 2008). Most ferns seem to use antheridiogens (Hornych et al., 2021), the effects of which have been observed in natural populations (Tryon and Vitale, 1977). There is an ongoing discussion about whether antheridiogens primarily slow down growth, which prompts antheridial formation, or vice versa (Näf, 1956; Korpelainen, 1994; Quintanilla et al., 2007). Nevertheless, antheridiogens mediate interactions between gametophytes of different developmental stages or ages.

Comparably little is known about the interactions among the gametophytes of multiple species at the same stage/age occurring at densities permitting the formation of both types of gametangia. There are three possible outcomes of such interactions. First, the gametophytes in mixed-species populations may grow at the same rate as in monoculture. Second, overyielding may occur, leading to gametophytes of at least one species growing faster in the presence of another species, as has been observed in angiosperm sporophytes (Turnbull et al., 2013; Wright et al., 2017). Third, one or more species may underyield (grow smaller) due to competition for resources or chemical allelopathy (Rünk et al., 2004; Testo and Watkins, 2013; Cheng and Cheng, 2015). The effects may be combined; for example, one species may overyield while another underyields in a mixed population. Apart from the negative effects of allelopathy on gametophyte growth (Petersen and Fairbrothers, 1980; Wagner and Long, 1991; Testo et al., 2014), competitive interactions have rarely been studied in fern gametophytes (Testo and Watkins, 2013).

To address this lack of information, we cultivated three fern species of the *Dryopteris filix-mas* complex (Dryopteridaceae). *Dryopteris filix-mas* (L.) Schott is a sexually reproducing tetraploid with diploid gametophytes, while *D. affinis* (Newman) Kinahan and *D. borreri* (Newman) Kinahan are apomictic (gametophytes have the same ploidy level as sporophytes). We selected these very closely related species (Fraser-Jenkins, 2007) to avoid any major differences in developmental patterns; however, their differing ploidy levels (affecting cell size; Robinson et al., 2018; Zhang et al., 2019) and reproductive modes (affecting development speed; Whittier, 1968; Regalado Gabancho et al., 2010; Haufler et al., 2016) may play a role in these interactions.

Fern gametophyte area has been measured for various purposes, as mentioned above. Gametophyte area was previously estimated either indirectly by measuring width and/or length (Tryon and Vitale, 1977; Korpelainen, 1994; Huang et al., 2004) or directly using image processing software (Ganger and Sturey, 2012; Greer et al., 2012; Ganger et al., 2019), such as ImageJ (Quintanilla et al., 2007; DeSoto et al., 2008; Pajarón et al., 2015). Studies have typically measured hundreds or low thousands of gametophytes, and manually outlining each gametophyte individually can be extremely time consuming. A more expedient method would be beneficial for measuring larger quantities of gametophytes, i.e., tens of thousands.

Here, we ask the following questions: (1) Can the sizes of a large number of gametophytes be quickly and reliably assessed through a single analysis of the whole population? (2) Does gametophyte ploidy level and reproduction type affect the absolute and relative gametophyte sizes? (3) Do the gametophytes achieve different sizes in mixed communities and monocultures? (4) Are the relative differences between species consistent during their growth?

METHODS

Plants and cultivation conditions used

Fronds of three members of the fern genus *Dryopteris* Adans. (Dryopteridaceae) were collected in 2020 by Libor Ekrt from a garden cultivation (Telč, Czech Republic) and pressed into herbarium vouchers (Table 1). These closely related species (*D. affinis*, *D. borreri*, and *D. filix-mas*) all belong the *D. filix-mas* complex. The species range from diploid to tetraploid and reproduce either via apomixis (*D. affinis*: $2x$, *D. borreri*: $3x$) or sexually (*D. filix-mas*: $4x$). Based on the reproduction type, the spores have the same ploidy level as the sporophytes in the apomicts, but a reduced ploidy level ($4x \geq 2x$) in the sexual species (Table 1).

For the purpose of cultivation, the wells of ten 12-well plates were filled to approximately half capacity with 1% agar medium enriched with 25%-strength Murashige and Skoog inorganic nutrients (Murashige and Skoog, 1962). During the experiment, the plates were kept in a cultivation chamber (MLR-352 Climatic Test Chamber; PHC Europe B.V., Etten-Leur, the Netherlands) under a 12-h light/12-h dark regime at the lowest light setting (photosynthetic photon flux density: 1700 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and a temperature of 20°C.

TABLE 1 Herbarium specimens used for spores in this study (collected from garden cultivation) alongside species details and the place of origin.

Species	Voucher ID	Reproduction type	Origin	Ploidy level (sporophyte/gametophyte)
Dryopteris affinis (Newman) Kinahan	CBFS 9795	Apomict	Yorkshire Dales, United Kingdom	$2x/2x$
Dryopteris borreri (Newman) Kinahan	CBFS 9797	Apomict	Šumava, Czech Republic	$3x/3x$
Dryopteris filix-mas (L.) Schott	CBFS 9710	Sexual	Šumava, Czech Republic	$4x/2x$

Note: CBFS = University of South Bohemia.

Spore sowing and experimental design

Spores were transferred from dried fern fronds to microscope slides by lightly tapping the fronds. The slide was then observed under a microscope (100× magnification; Olympus CX31; Olympus, Tokyo, Japan) and a total of 20 non-aborted spores were transferred into each well of the 12-well plates using tweezers. The spores were attached to the tweezers from the side by static forces; they were not grabbed between the arms of the tweezers as that would have crushed the spores. The presence of the spore in the well was confirmed using a bifocal lens (Olympus S7X7). Sometimes, multiple spores became attached to the tweezers; these were then removed or redistributed evenly within the well. After each spore transfer, the tweezers were cleaned using a paper towel sprayed with ethanol to remove any unwanted spores, debris, or agar pieces. After the spores of one species were sown, the table and equipment were cleaned with ethanol, and the microscope slide was rinsed with water and dried before the next species was used. The plates were covered by a lid and sealed with a double layer of parafilm.

The 20 non-aborted spores in each well were randomly but evenly distributed within the central part of the well (ca. half of the total radius of 1.1 cm). Only the central part of the well was used to eliminate any effect of agar concavity at the edges of the well on the estimate of the gametophyte area. The spores within each well were either from only one species or from two species at a standardized ratio (1 : 1 or 1 : 3). Three species were used, meaning there were 12 possible combinations, each of which was represented once in every plate (Figure 1). To account for the possible influence of the position within the plate, each of the 10 plates had a unique position for every spore combination. Overall, 2400 spores were individually sown during the experiment. Despite the double layer of parafilm, the plates were somewhat ventilated, limiting water condensation. Water droplets have the potential to move spores around when falling onto the agar, which should be considered in alternative setups.

For all wells, an image was taken immediately after sowing. When two species were sown in one well, an additional image was taken before the second species was sown. Therefore, each two-species well had two images associated with it, one with the spores of one species, and the other with the spores of both species. These two images were combined for an accurate species identification for each spore. During week 5 (see below), the images with identified spores were used to identify the species of the individual gametophytes growing in the wells.

Observations and data processing

Each well of the ten 12-well plates was photographed weekly between weeks 4 and 10 using an Infinity 1 camera (Lumenera, Ottawa, Canada) attached to a bifocal lens at 6× magnification; the images were saved as TIFF files. We were unable to record the growth in plates K8, K9, and K10 during weeks 9 and 10 due to pandemic restrictions. Upon preliminary testing, some of the gametophyte area was not green enough to be recognized by Easy Leaf Area (Easlon and Bloom, 2014) (see below); therefore, every image obtained was altered using the Batch Image Manipulation Plugin (BIMP, version 2.5; Francesconi, 2021) for GNU Image Manipulation Program (GIMP, version 2.10; The GIMP Development Team, 2021) using two commands: (a) "change format and compression" → jpeg (necessary for the successful completion of the next step) and (b) "gimp-drawable-hue-saturation" → "hue-range-yellow" → "hue offset in degrees" = 10. This process shifts some of the yellow in the gametophyte images to green, making them more suitable for further analyses. The hue offset value was determined based on our preliminary tests. The most appropriate value for this parameter may differ in other setups based on lighting
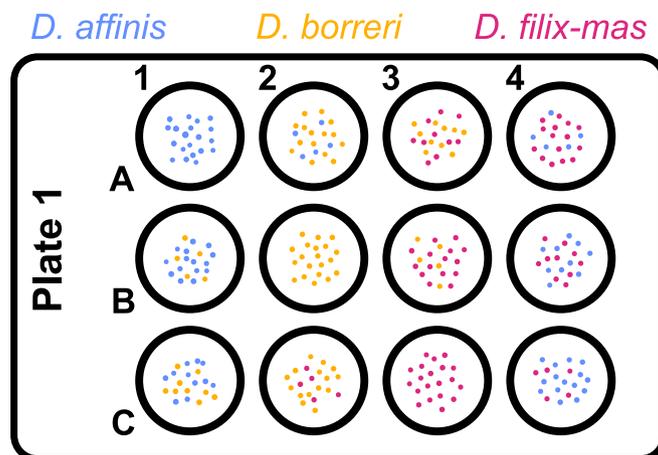
FIGURE 1 An example experimental setup of the gametophyte competition experiment, showing the *Dryopteris* species sown. The dots within the wells of the plate represent spores and eventually gametophytes, the color of which indicates the species. Each well contains 20 spores of up to two species in the ratios 20 : 0, 15 : 5, and 10 : 10.

conditions, tested species morphology (e.g., color), or any other relevant factors, and should be calibrated. The calibration process would involve testing the analysis software on unaltered images, observing any potential gametophyte cover not assigned to green pixels, and altering the hue to shift this area into green while preventing any assignment of non-gametophyte objects as green due to the alteration. One standard alteration setting should be applied to the entire data set unless there are valid reasons for multiple alteration settings being used.

The amount of green (in pixels) was obtained for each altered image using Easy Leaf Area. The parameters were set to values that covered most of the gametophyte mass, but did not include any non-gametophyte parts of the image (for an example, see Figure 2). Easy Leaf Area works with a calibration feature, in which a red object of known area is photographed alongside each sample in one image. The numbers of red and green pixels are then automatically compared to calculate the green area. We were unable to use this feature directly, however. We tried to stick a piece of paper with a 4-mm^2 red square under each well, but optical distortions prevented the scanning of the entire red square. Putting the red square directly inside the well disturbed the gametophytes and did not yield accurate results as the squares were randomly tilted due to the concave shape of the agar medium. We therefore photographed ten 100-mm^2 red squares separately. The images with red squares were analyzed, and the average number of red pixels was recorded. All images were taken at the lowest possible magnification (6×), making sure that the magnification wheel was moved until it could not go any further. Additionally, the processing speed (parameter in Easy Leaf Area affecting the processed image size) was always set to 2 as this setting alters the number of pixels analyzed.

Week 5 was the last week in which the gametophytes did not physically overlap; therefore, all the images taken at week 5 were processed further: (1) the species of each gametophyte was identified, (2) one species was deleted from the images by manual image alteration, and (3) the altered images were processed as outlined above (BIMP, GIMP, Easy Leaf Area), applying the same settings used for Easy Leaf Area. The new number of pixels was attributed to the non-deleted species, while the difference between the total pixel number (from the unaltered images) and the number of pixels from the altered images (with one species deleted) was attributed to the deleted species.

The images were taken from above; however, fern gametophytes may grow upward (perpendicular to the surface), especially at later stages of development, meaning the level of perpendicularity affects how the scanned green area reflects the real area of the gametophyte. To address this issue, the analyses outlined below were performed under the assumption that the perpendicularity of the gametophytes is similar at each stage of development, meaning the wells are directly comparable between images taken in the same week (proxy for development stage). Furthermore, fern species may differ in general
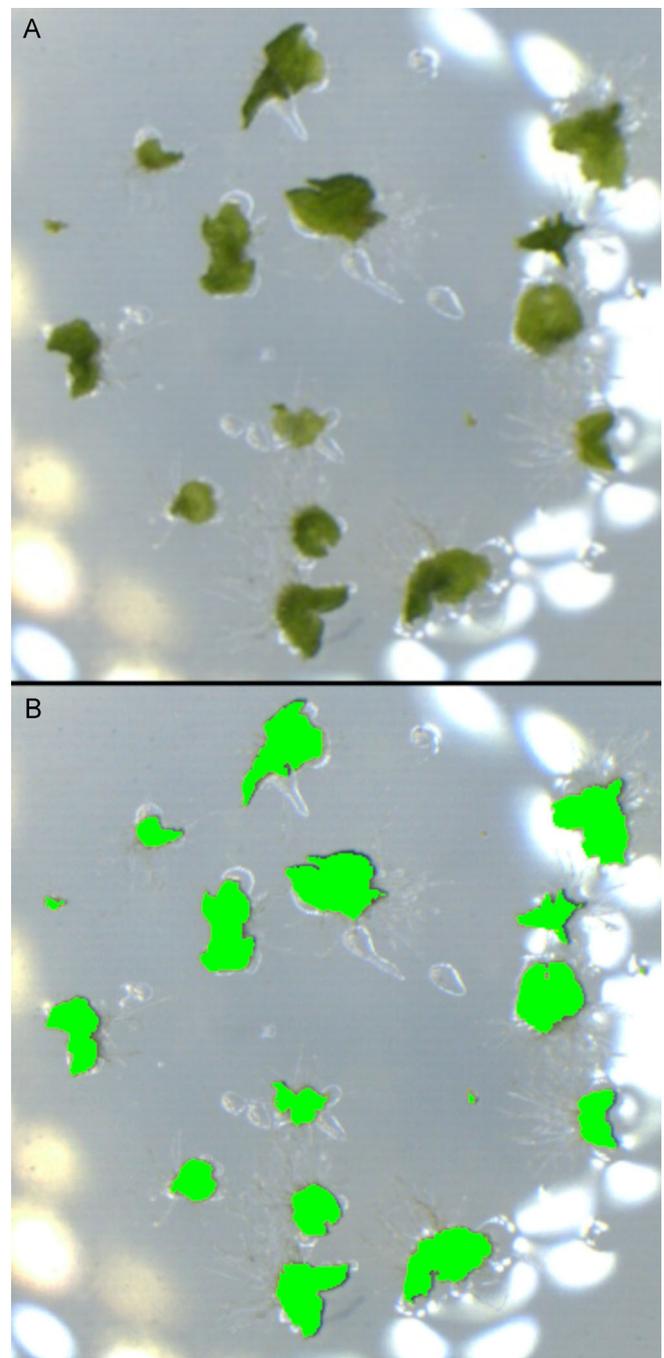
FIGURE 2 An example image of a gametophyte population (K5–4B) taken during week 5. (A) Image before processing with Easy Leaf Area (but with altered hue to accentuate green pixels). (B) Image processed with Easy Leaf Area, which highlighted and counted the number of green pixels.

morphology, which would similarly alter the results, and should be considered.

Comparison with ImageJ

To compare the accuracy of Easy Leaf Area as a measurement tool for fern gametophytes, 30 images of monocultures from

week 5 were also analyzed using ImageJ (Abràmoff et al., 2004), which was used in previous studies. This software calculates the area of a polygon. Each gametophyte in 30 wells was outlined using the polygon selection tool, approximating its shape. To increase the speed and accuracy of the estimates, the polygons were drawn using a Deco 01 (v2; XP-PEN, Chino Hills, California, USA) graphical tablet. The use of a graphical tablet also increases the level of comfort during the process, but is not necessary. The calibration was performed by outlining the 10 red squares used for the Easy Leaf Area calibration. The total gametophyte area in each well was divided by the average size of $1\,mm^2$ based on the calibration. The area estimates in square millimeters were compared between Easy Leaf Area and ImageJ by dividing the cover estimate obtained using the former by the estimate obtained using the latter.

Statistical analyses

Before the statistical analyses, the data were processed in the following ways: (1) The sizes in pixels were calibrated by dividing the number of pixels by 2190, representing the average number of pixels in $1\,mm^2$ based on 10 observations of the 100-mm^2 squares. (2) For every well, the theoretical area of each species was calculated by dividing the total area by 20 (total number of sown spores) and then multiplying this number by the number of spores of each species in the well. For the data from week 5, the real area of each species was divided by the theoretically calculated area. This ratio of the real to theoretical area of each species was then standardized to have a mean of 1 by dividing the ratio by its average. (3) Wells with fewer than 20 sown spores (approximately 4% of all wells) were removed from the statistical analyses. The data used for the statistical analyses can be found in Appendix S1.

The abiotic conditions experienced by each gametophyte may be influenced by the position of a well within the plate, specifically the number of bordering sides of each well (e.g., in Figure 1, position 4A has two bordering sides, while position 2B has no bordering sides). This problem should be solved by the experimental design. We confirmed that the number of bordering sides had no significant effect ($X_2^2 = 0.604$, $P = 0.739$) on gametophyte cover using generalized linear mixed-effects models (GLMMs) in the *lme4* package (Bates et al., 2015) for R (R Development Core Team, 2020), using a gamma error distribution and logarithmic link function. The identity of each plate was used as a random factor to control for the random variability between the plates, and this approach was used throughout all the following analyses. Based on the non-significant result, the well position within a plate was not included in further statistical analyses.

For the monocultures, the differences in cover between the individual species, observation period, species reproduction type, and ploidy level were assessed using GLMMs with a gamma error distribution and a logarithmic link function. The analysis was performed in two separate steps,

with the first model testing differences in observation period, individual species, and their interaction. The second model quantified the effect of the observation period, species reproduction type, ploidy level, and their mutual interactions. The models cannot be run in a single comprehensive model as species identity strongly overlaps with reproduction type and ploidy level, which does not allow for calculations of their interactions.

Similarly, GLMMs with a gamma error distribution and a logarithmic link function were used to quantify the gametophyte development over time and under different competitive conditions (including monocultures and mixtures), where the species cover was used as a response, and observation period, spore combinations, and their interaction were used as explanatory variables.

To explore how differing competitive environments affect the performance of the constituent species at week 5, the effect of different spore combinations (and thus different species proportions) on a standardized ratio of individual species cover (real to theoretical species cover) was assessed using a linear mixed-effects model (LMM). Pairwise differences between groups were calculated using the *emmeans* function in the R package *emmeans* (Lenth, 2021) and the *P* value was adjusted using the Tukey method. The analyses were performed separately within each species. Additionally, species monocultures were excluded from the analyses and only differences between mixtures were assessed to avoid circular reasoning because, specifically for week 5, monocultures were used for the calculation of the average individual of each species and this information was used in the assessment of the theoretical area (explained above). Consequently, monocultures also lack any variability, and the ratio always equals 1 (before standardization) because the theoretically calculated area equals the measured gametophyte area.

Please note that in all statistical analyses, a type I (sequential) sum of squares was used. Briefly, each model explains the variability of a response by first using explanatory variable A and then proceeds with explanatory variable B to account for the remaining unexplained variability. In this theoretical example, variable A is effectively a covariate for variable B. The order of the variables in the analyses follows their order in the text, and can also be found in Appendices S2 and S3. The statistical testing in all analyses was performed using a likelihood ratio test.

All statistical analyses were conducted in R software (R Development Core Team, 2020) and visualized in the *ggplot2* package (Wickham, 2016).

RESULTS

Monoculture

Of the 2400 spores sown, 2383 germinated, and the growth of these gametophytes was observed over the course of 10 weeks; the remaining 17 spores were missing from five

wells. In total, 312.72 cm^2 of gametophyte area was recorded. The area covered by 20 gametophytes in a single well ranged from 0.30 mm^2 (Well K2-1A, week 4) to 209.32 mm^2 (Well K3-4B, week 10).

The studied species displayed significantly different total coverage ($X_2^2 = 86.942$, $P < 0.001$) and growth rate (time: $X_1^2 = 218.102$, $P < 0.001$). The growth rate over time was consistent between the species, as the interaction between these variables was not statistically significant ($X_2^2 = 4.000$, $P = 0.129$). Overall, the growth of all three species was exponential at first (during weeks 4–7), but then slowed down, reaching a maximum at week 10 (Figure 3). The gametophytes began overlapping by week 6, and perpendicular growth became more prominent over time. The sexual species and the triploid were significantly larger that the apomicts and diploids, respectively (reproduction type: $X_1^2 = 56.289$, $P < 0.001$; ploidy level: $X_1^2 = 30.653$, $P < 0.001$). These effects were consistent throughout the observation period (time × reproduction type: $X_1^2 = 2.901$, $P = 0.089$; time × ploidy level: $X_1^2 = 1.198$, $P = 0.274$).

Interactions

The analysis of the entire observation period (all weeks) demonstrated that the total cover in each well is significantly affected by the date of observation (week; $X_6^2 = 1283.664$, $P < 0.001$) and spore combination ($X_{11}^2 = 332.413$, $P < 0.001$; Figure 3). Overall, the spore combinations resulting in the most cover generally included *D. filix-mas*, while the *D. affinis* monoculture had the least cover. The interaction of week and spore combination was not significant ($X_{66}^2 = 60.769$, $P = 0.659$), indicating that the relative differences between groups were consistent throughout the observation period.

A detailed analysis in week 5 revealed that the gametophytes of each species frequently under- or overperformed compared with the theoretical predictions (Figure 4). After standardization, *D. affinis* generally underperformed when in competition with stronger species (real to theoretical cover at five spores of *D. affinis* was 0.87) and was comparably larger when few (1.00 ratio at 15 spores) or no (1.35 ratio) spores of other species were present. Of the three species, *D. borreri* performed most consistently regardless of the number of spores present (ratios ranging from 0.95 at 15 spores to 1.09 at five spores) and had average growth in monocultures (1.04 ratio). The strongest competitor, *D. filix-mas*, relatively underperformed in monocultures (0.72 ratio) and fared best when it was rare (1.30 ratio at five spores). The different species competition alternations (species proportions) yielded different performances only when combined with *D. filix-mas* (*D. affinis*: $X_2^2 = 0.569$, $P = 0.753$; *D. borreri*: $X_2^2 = 1.012$, $P = 0.603$; *D. filix-mas*: $X_2^2 = 29.384$, $P < 0.001$; Figure 4, Appendix S3). The statistical analyses described above are summarized in Appendices S2 and S3.

Comparison with ImageJ

In the 30 images analyzed using both software, Easy Leaf Area estimated 86–114% of the gametophyte cover calculated using ImageJ. On average, the estimates by Easy Leaf Area were 3.9% larger. Easy Leaf Area generally overestimated images with larger gametophytes, while Image J overestimated the size of smaller gametophytes.

DISCUSSION

Monocultures and competitive interactions

The gametophytes of the three species in our study differed significantly in their growth capabilities, as expressed by
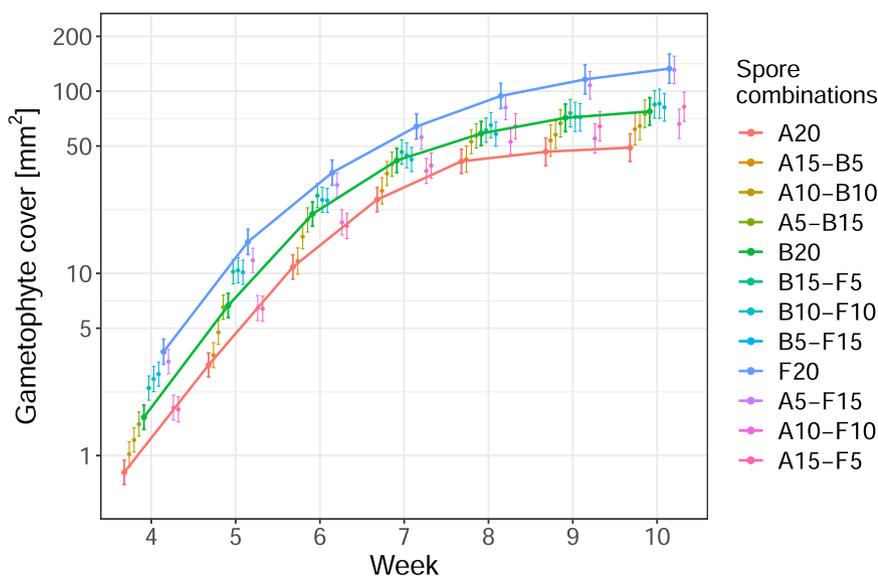
FIGURE 3 Gametophyte cover as a function of observation period for all 12 combinations of spore mixtures. Error bars indicate the standard error of the mean. For clarity, species monocultures are highlighted by line connections. The letters indicate the *Dryopteris* species used (A = *D. affinis*; B = *D. borreri*; F = *D. filix-mas*).
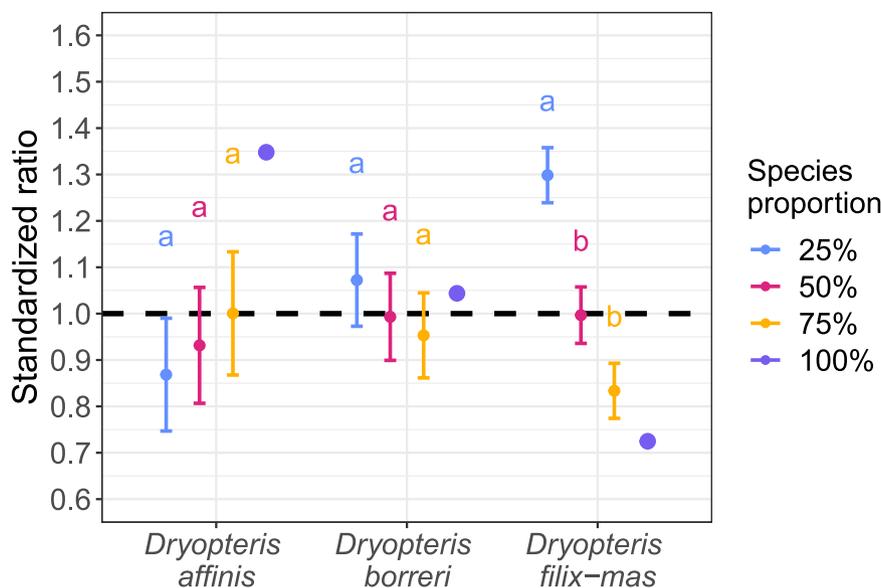
FIGURE 4 Standardized ratio of the real to theoretical cover for each species and for the three levels of species spore density mixtures (25%, 50%, or 75%) at week 5. For an easier comparison, monocultures (100%, 20 spores total) are passively displayed as points. The monocultures were excluded from the accompanying statistical analysis to avoid circular reasoning because they were used for the calculation of the standardized ratio of the mixtures (see details in the Methods). Each species spore density level sums to 20; the presented value is the number of spores of the targeted species. Error bars indicate standard error of the mean. Different letters denote statistically significant differences between species spore density groups for each species separately.

their total cover area in monocultures. The diploid gametophytes of the sexual tetraploid *D. filix-mas* grew largest. In comparison, the apomictic gametophytes were smaller, ordered by ploidy level, i.e., the triploid *D. borreri* was larger than the diploid *D. affinis*. The statistical analysis of the effects of reproductive type and ploidy level was limited by the absence of a hexaploid sexual species (with triploid gametophytes), which is not known in this group; nevertheless, significant differences were found.

Interestingly, the apomicts were smaller than the sexual species overall, which seems to go against the generally presumed faster growth of apomictic gametophytes (Whittier, 1968; Regalado Gabancho et al., 2010; Haufler et al., 2016). One possible explanation is that there may be weaker selective pressure for extensive lateral growth in the apomicts due to the lower requirements for sporophyte formation (no gametangia needed).

Ploidy level was positively correlated with gametophyte size, once reproductive mode was accounted for. This is possibly because fern spore size increases with ploidy level (Barrington et al., 1986, 2020; Ekrt and Koutecký, 2016) and larger spores may carry more resources for gametophyte establishment. Nevertheless, this effect was dwarfed by the differences between apomictic and sexually reproducing ferns in our selected species.

The difference in the gametophyte sizes in the monocultures was correlated with the distribution of the three cultivated species. The largest species, *D. filix-mas*, has a circumboreal distribution, with considerable coverage outside Europe (Hultén and Fries, 1986). The two apomicts are mostly confined to Europe, with *D. borreri* being more widespread than *D. affinis* (Fraser-Jenkins, 2007). It is

possible that the gametophyte size reflects the competitive ability of a species and, consequently, its abundance and distribution in nature. A similar correlation was found in the *D. carthusiana* (Vill.) H. P. Fuchs complex by Rünk et al. (2004) in Estonia. In their experiment, the three species of this complex differed in their competitive abilities against a grass, *Deschampsia cespitosa* (L.) P. Beauv. These differences reflected the regional species abundance except for *D. dilatata* (Hoffm.) A. Gray, which was competitively strong but comparably rare because it is at its distribution limits in Estonia (Rünk et al., 2004).

Their differing competitive abilities profoundly affected the performance of the tested species in mixed cultivations. One of the predictions stemming from the mass ratio hypothesis is that the dominant species has the most influence on the functioning of communities and the ecosystem (Grime, 1998; Diaz et al., 2007; Pakeman et al., 2011). In our tightly controlled environment (set number of individuals, stable conditions), the number of spores of the dominant species (*D. filix-mas*) greatly influenced the total productivity of the community, expressed as cover area. The performance of this species was significantly different based on its relative abundance (5, 10, or 15 spores in the mixture; Figure 4). The large size of *D. filix-mas* gametophytes meant they grew largest when in a minority because they do not suffer from as much strong intraspecific competition. The reverse tendency (stronger interspecific competition) was seen in the weakest competitor, *D. affinis*, which benefited from a lack of stronger competitors and did best in monocultures. The two effects may cancel each other out in *D. borreri*, as this species suffered from the competition of *D. filix-mas* but performed

better when exposed to *D. affinis*. Unfortunately, very few researchers previously focused on the general competitive interactions of ferns at the gametophytic level (Korpelainen, 1997; Testo et al., 2014); thus, our findings provide new insights into these relationships.

Methodological remarks

We recorded and analyzed the growth of gametophytes originating from 2383 spores using Easy Leaf Area, a fast and convenient tool for assessing the extent of area covered by green tissues. Several modifications from the original usage (Easlon and Bloom, 2014) were necessary. First, the calibration was performed externally due to optical distortions at such a small scale; thus, the level of magnification had to be carefully controlled. The images also had to be altered to shift some of the yellow color into green for the script to be able to detect the entirety of the gametophytes.

Furthermore, a couple of minor methodological issues emerged, of which researchers using this methodology should be aware. A small minority (17 of 2400, 0.7%) of spores were not detected after sowing; they likely did not stick to the agar properly and were blown away. For future studies, the number of spores on each dish should be checked after they have been sealed, and any lost spores should be replaced immediately. Extensive algal growth may also alter the results, and could be reduced by properly sterilizing equipment and work areas, as well as by using freshly prepared sterile plates and media. Nevertheless, if such growth appears, it may be manually deleted from the images (this, however, reduces overall analysis speed), or alternatively the careful choice of settings in Easy Leaf Area should limit or eliminate the inclusion of the algae. If algal growth cannot be sufficiently addressed, the observation should be discarded. This issue is specific to the use of software that analyzes the image as a whole (such as Easy Leaf Area), not one that requires individual gametophytes to be outlined before analysis. It is also important to consider that extensive algal growth might directly affect tested variables, depending on the research topic, i.e., algae could compete with gametophytes for a particular nutrient.

Given that fern gametophyte density affects sexual expression, germination, and growth (Dyer, 1979; Huang et al., 2004; DeSoto et al., 2008), spore sowing density should be chosen based on the research aims. In our study, the spores were sown close enough together to allow any potential interactions to manifest, but far enough apart to provide the gametophytes with space for growth. This trade-off approach resulted in the overlapping of gametophytes by week 6, in the second half of the experiment. The proper observation of gametophyte size at later stages of development would require lower sowing densities. In such cases, the fact that gametophyte development is abnormal in very low spore densities (reviewed by Dyer, 1979) should be considered.

When sowing fern spores at a given density/number, the issue of spore abortion should also be considered. Most fern species, and especially apomictic ones, abort some of their spores (Wagner and Chen, 1965; Hornych and Ekrt, 2017). Such spores are visibly malformed and will not germinate into gametophytes. If spores are sown individually, aborted spores should be avoided. Alternatively, if the spores are sown as a group, the size of the group should be altered to address the differences in spore abortion (higher abortion = more spore material used).

Comparison with other methods

Previously used methods of assessing gametophyte area include measuring the width and/or length (Tryon and Vitale, 1977; Korpelainen, 1994; Huang et al., 2004) of the plant. Such methods are expedient, but the results are not comparable for species with diverse gametophyte morphologies, such as is found in *Dryopteris* apomicts (Hornych, personal observation). A more modern approach is to outline and analyze the area of each individual gametophyte (Pajarón et al., 2015; Ganger et al., 2019). A diligent approach using this methodology can yield highly accurate results, but it takes considerably more time than simple two-dimensional measurements. We would recommend this method for a smaller number of gametophytes (up to hundreds). The methods outlined in the present study can be used to rapidly process a large number of gametophytes (thousands and up). Indeed, in our study, the processing of the 30 images in ImageJ took a similar amount of time as analyzing the entire data set using Easy Leaf Area. We therefore recommend using Easy Leaf Area when large numbers of individuals are used, which in turn balances potential inaccuracies. Nevertheless, the estimates from both Easy Leaf Area and ImageJ are similar. ImageJ overestimates the size of smaller gametophytes, likely due to the difficulty of accurately outlining small objects by hand, which could explain the overall 4% difference in cover to that which was calculated by Easy Leaf Area. A more meticulous approach will yield highly accurate results but take longer to perform.

Future directions

The outlined methodology could be employed for a variety of research topics. Here, we used these techniques to demonstrate the significance of competitive interactions between fern gametophytes, although further research is needed. The gametophytic phase of ferns is very sensitive to environmental pressures and requires more detailed study. This is especially true for gametophytes in their natural habitat. The total cover of gametophytes in natural populations may be correlated with various ecological factors, and likely differs based on the fern species present. The growth patterns of fern gametophytes may also be affected by their hybrid origin and reproductive mode; however, a proper analysis of these large evolutionary trends

would require hundreds of individuals from multiple taxa. A fast and efficient methodology, such as the one reported here, can process large quantities of samples in a relatively short amount of time.

The methodology presented in this study generates results comparable to those generated using more time-consuming methods. Nevertheless, certain limitations exist. Here we outline how they could be addressed. First, the use of 10 repetitions for each combination and 20 spores per well should theoretically offset possible errors stemming from the improper identification of gametophytes for the analyses at week 5; however, if a lower number of spores/ repetitions is to be used, or the accuracy of species identification is critical, molecular barcoding methods may be employed. The identification of fern gametophytes using plastid markers (such as *rbcL*, *matK*, and *trnH-psbA*) was successfully performed previously, although some related species pairs may be indistinguishable (de Groot et al., 2011; Nitta et al., 2017); therefore, the barcoding method should be tested on the species involved beforehand.

While the studied gametophytes were one cell thick and generally flat, they grew at various angles relative to the agar surface. Easy Leaf Area works with a binary assignment (i.e., either the pixel is green or not); however, some steps may be used to improve the accuracy of the results. One approach would be to alter the hue of images or the setting in Easy Leaf Area to yield pixel counts for various shades of green, which could then be translated into different cell densities; for example, one pixel of "dark green" could be equal to five pixels of "light green." Alternatively, new scripts could be prepared to specifically analyze the images, taking color value into consideration. Regardless, these methods would have to be carefully calibrated by measuring the dry biomass or the area of flattened gametophytes.

AUTHOR CONTRIBUTIONS

O.H. and L.E. conceived the study. L.E. collected spore material. O.H. and L.Č. prepared and recorded the cultivations. O.H., L.Č., and A.L. performed the data analysis. O.H and A.L wrote the first draft of the manuscript. All authors contributed to the writing of the manuscript and gave final approval for publication.

ACKNOWLEDGMENTS

DATA AVAILABILITY STATEMENT

The Supporting Information associated with this article was deposited to Zenodo and may be found online at https://doi. org/10.5281/zenodo.6338274.

ORCID

Ondřej Hornych http://orcid.org/0000-0002-5093-3103
Aleš Lisner http://orcid.org/0000-0003-4881-7362
Libor Ekrt http://orcid.org/0000-0003-3947-787X

REFERENCES

Abràmoff, M. D., P. J. Magalhães, and S. J. Ram. 2004. Image processing with ImageJ. *Biophotonics International* 11: 36–42.

Barrington, D. S., C. A. Paris, and T. A. Ranker. 1986. Systematic inferences from spore and stomate size in the ferns. *American Fern Journal* 76: 149–159.

Barrington, D. S., N. R. Patel, and M. W. Southgate. 2020. Inferring the impacts of evolutionary history and ecological constraints on spore size and shape in the ferns. *Applications in Plant Sciences* 8: e11339.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67: 1–48.

Cheng, F., and Z. Cheng. 2015. Research progress on the use of plant allelopathy in agriculture and the physiological and ecological mechanisms of allelopathy. *Frontiers in Plant Science* 6: 1020.

de Groot, G. A., H. J. During, J. W. Maas, H. Schneider, J. C. Vogel, and R. H. J. Erkens. 2011. Use of *rbcL* and *trnL-F* as a two-locus DNA barcode for identification of NW-European ferns: An ecological perspective. *PLoS ONE* 6: e16371.

DeSoto, L., L. G. Quintanilla, and M. Méndez. 2008. Environmental sex determination in ferns: Effects of nutrient availability and individual density in *Woodwardia radicans*. *Journal of Ecology* 96: 1319–1327.

Diaz, S., S. Lavorel, F. de Bello, F. Quetier, K. Grigulis, and T. M. Robson. 2007. Incorporating plant functional diversity effects in ecosystem service assessments. *Proceedings of the National Academy of Sciences, USA* 104: 20684–20689.

Dyer, A. F. 1979. The culture of fern gametophytes for experimental investigation. *In* A. F. Dyer [ed.], The experimental biology of ferns, 253–305. Academic Press, London, United Kingdom.

Easlon, H. M., and A. J. Bloom. 2014. Easy Leaf Area: Automated digital image analysis for rapid and accurate measurement of leaf area. *Applications in Plant Sciences* 2: 1400033.

Ekrt, L., and P. Koutecký. 2016. Between sexual and apomictic: Unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (Dryopteridaceae). *Annals of Botany* 117: 97–106.

Francesconi, A. 2021. BIMP. Website: https://alessandrofrancesconi.it/ projects/bimp/ [accessed 13 February 2022].

Fraser-Jenkins, C. R. 2007. The species and subspecies in the *Dryopteris affinis* group. *Fern Gazette* 18: 1–26.

Ganger, M., and T. Sturey. 2012. Antheridiogen concentration and spore size predict gametophyte size in *Ceratopteris richardii*. *Botany* 90: 175–179.

Ganger, M. T., R. Hiles, H. Hallowell, L. Cooper, N. McAllister, D. Youngdahl, J. Alfieri, and S. J. Ewing. 2019. A soil bacterium alters sex determination and rhizoid development in gametophytes of the fern *Ceratopteris richardii*. *AoB Plants* 11: plz012.

Greer, G. K., M. A. Dietrich, J. A. Devol, and A. Rebert. 2012. The effects of exogenous cytokinin on the morphology and gender expression of *Osmunda regalis* gametophytes. *American Fern Journal* 102: 32–46.

Grime, J. P. 1998. Benefits of plant diversity to ecosystems: Immediate, filter and founder effects. *Journal of Ecology* 86: 902–910.

Haufler, C. H., K. M. Pryer, E. Schuettpelz, E. B. Sessa, D. R. Farrar, R. Moran, J. J. Schneller, et al. 2016. Sex and the single gametophyte: Revising the homosporous vascular plant life cycle in light of contemporary research. *BioScience* 66: 928–937.

Hornych, O., and L. Ekrt. 2017. Spore abortion index (SAI) as a promising tool of evaluation of spore fitness in ferns: An insight into sexual and apomictic species. *Plant Systematics and Evolution* 303: 497–507.

Hornych, O., W. L. Testo, E. B. Sessa, J. E. Watkins, C. E. Campany, J. Pittermann, and L. Ekrt. 2021. Insights into the evolutionary history and widespread occurrence of antheridiogen systems in ferns. *New Phytologist* 229: 607–619.

Huang, Y.-M., H.-M. Chiou, and W.-L. Chiou. 2004. Density affects gametophyte growth and sexual expression of *Osmunda cinnamomea* (Osmundaceae: Pteridophyta). *Annals of Botany* 94: 229–232.

Hultén, E., and M. Fries. 1986. Atlas of North European vascular plants: North of the Tropic of Cancer. Koeltz Botanical Books, Königstein, Germany.

Korpelainen, H. 1994. Growth, sex determination and reproduction of *Dryopteris filix-mas* (L.) Schott gametophytes under varying nutritional conditions. *Botanical Journal of the Linnean Society* 114: 357–366.

Korpelainen, H. 1997. Comparison of gametophyte growth, sex determination and reproduction in three fern species from the tropics. *Nordic Journal of Botany* 17: 133–143.

Lenth, R. V. 2021. emmeans: Estimated Marginal Means, aka Least-Squares Means. Website: https://cran.r-project.org/web/packages/emmeans/index.html [accessed 13 February 2022].

Lloyd, R. M., and E. J. Klekowski. 1970. Spore germination and viability in Pteridophyta: Evolutionary significance of chlorophyllous spores. *Biotropica* 2: 129–137.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.

Näf, U. 1956. The demonstration of a factor concerned with the initiation of antheridia in polypodiaceous ferns. *Growth* 20: 91–105.

Nitta, J. H., J. Meyer, R. Taputuarai, and C. C. Davis. 2017. Life cycle matters: DNA barcoding reveals contrasting community structure between fern sporophytes and gametophytes. *Ecological Monographs* 87: 278–296.

Pajarón, S., E. Pangua, L. G. Quintanilla, and A. Jiménez. 2015. Influence of water availability on gender determination of gametophytes in a diploid–polyploid complex of a xerophytic fern genus. *AoB Plants* 7: plv047.

Pakeman, R. J., A. Eastwood, and A. Scobie. 2011. Leaf dry matter content as a predictor of grassland litter decomposition: A test of the 'mass ratio hypothesis'. *Plant and Soil* 342: 49–57.

Pangua, E., S. Pajarón, and L. G. Quintanilla. 2019. Fitness of an allopolyploid rupicolous fern compared with its diploid progenitors: From sporogenesis to sporophyte formation. *American Journal of Botany* 106: 984–995.

Petersen, R. L., and D. E. Fairbrothers. 1980. Reciprocal allelopathy between the gametophytes of *Osmunda cinnamomea* and *Dryopteris intermedia*. *American Fern Journal* 70: 73–78.

Quintanilla, L. G., L. DeSoto, A. Jimenez, and M. Mendez. 2007. Do antheridiogens act via gametophyte size? A study of *Woodwardia radicans* (Blechnaceae). *American Journal of Botany* 94: 986–990.

R Development Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Website: http://www.R-project.org/ [accessed 13 February 2022].

Regalado Gabancho, L., C. Prada, and J. M. Gabriel y Galán. 2010. Sexuality and apogamy in the Cuban *Asplenium auritum–monodon* complex (Aspleniaceae). *Plant Systematics and Evolution* 289: 137–146.

Robinson, D. O., J. E. Coate, A. Singh, L. Hong, M. Bush, J. J. Doyle, and A. H. K. Roeder. 2018. Ploidy and size at multiple scales in the *Arabidopsis* sepal. *The Plant Cell* 30: 2308–2329.

Rünk, K., M. Moora, and M. Zobel. 2004. Do different competitive abilities of three fern species explain their different regional abundances? *Journal of Vegetation Science* 15: 351–356.

Schneller, J. J. 2008. Antheridiogens. *In* T. A. Ranker and C. H. Haufler [eds.], Biology and evolution of ferns and lycophytes, 134–158. Cambridge University Press, Cambridge, United Kingdom.

Schneller, J. J., C. H. Haufler, and T. A. Ranker. 1990. Antheridiogen and natural gametophyte populations. *American Fern Journal* 80: 143–152.

Testo, W. L., and J. E. Watkins. 2013. Understanding mechanisms of rarity in pteridophytes: Competition and climate change threaten the rare fern *Asplenium scolopendrium* var. *americanum* (Aspleniaceae). *American Journal of Botany* 100: 2261–2270.

Testo, W. L., M. S. Grasso, and D. S. Barrington. 2014. Beyond antheridiogens: Chemical competition between gametophytes of *Polypodium appalachianum* and *Polypodium virginianum*. *Journal of the Torrey Botanical Society* 141: 302–312.

The GIMP Development Team. 2019. GIMP. Website: https://www.gimp.org [accessed 13 February 2022].

Tryon, R. M., and G. Vitale. 1977. Evidence for antheridogen production and its mediation of a mating system in natural populations of fern gametophytes. *Botanical Journal of the Linnean Society* 74: 243–249.

Turnbull, L. A., J. M. Levine, M. Loreau, and A. Hector. 2013. Coexistence, niches and biodiversity effects on ecosystem functioning. *Ecology Letters* 16: 116–127.

Van Nguyen, Q., H. T. Khuat, Y.-N. T. Nguyen, D. T. Vu, T.-H. Bui, and K.-H. Boo. 2020. *Drynaria bonii* spore culture: Optimization of culture conditions and analysis of gametophyte and sporophyte development. *Plant Biotechnology Reports* 14: 575–584.

Wagner, W. H., and K. L. Chen. 1965. Abortion of spores and sporangia as a tool in the detection of *Dryopteris* hybrids. *American Fern Journal* 55: 9–29.

Wagner, H. B., and K. E. Long. 1991. Allelopathic effects of *Osmunda cinnamomea* on three species of *Dryopteris*. *American Fern Journal* 81: 134–138.

Whittier, D. P. 1968. Rate of gametophyte maturation in sexual and apogamous forms of *Pellaea glabella*. *American Fern Journal* 58: 12–19.

Wickham, H. 2016. ggplot2: Elegant graphics for data analysis. Springer, New York, New York, USA.

Windham, M. D., P. G. Wolf, and T. A. Ranker. 1986. Factors affecting prolonged spore viability in herbarium collections of three species of *Pellaea*. *American Fern Journal* 76: 141–148.

Wright, A. J., D. A. Wardle, R. Callaway, and A. Gaxiola. 2017. The overlooked role of facilitation in biodiversity experiments. *Trends in Ecology & Evolution* 32: 383–390.

Zhang, Y., B. Wang, S. Qi, M. Dong, Z. Wang, Y. Li, S. Chen, et al. 2019. Ploidy and hybridity effects on leaf size, cell size and related genes expression in triploids, diploids and their parents in *Populus*. *Planta* 249: 635–646.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix S1. The data set for the cultivation experiment using three representatives of the genus *Dryopteris*. The theoretical number of pixels was calculated as the total number of pixels divided by the total spore number (usually 20) and multiplied by the number of spores of a given species. The standardized ratio refers to the real : theoretical ratio, standardized to a mean of 1 (monocultures excluded) using division by the mean value.

Appendix S2. Results of the generalized linear mixed-effects models, where fern species cover was used as a response to the observation period and fern species identity in the monocultures; the observation period, reproduction type, and ploidy level in the monocultures; and the observational period and spore combinations. A type I (sequential) sum of squares was used in the analyses.

Appendix S3. Results of the linear mixed-effects models for week 5, in which a standardized ratio of real : theoretical cover for each species was used as a response variable and species spore combinations were used as predictors. A type I (sequential) sum of squares was used in the analyses. The individual contrasts between the three levels of species spore combinations (5, 10, or 15 spores) within each species were quantified using the Tukey post-hoc method.

Paper 5

Hornych O., Testo W., Sessa E., Watkins J., Campany C., Pittermann J. & Ekrt L. (2021): Insights into the evolutionary history and widespread occurrence of antheridiogen systems in ferns. – New Phytologist 229: 607–619.

Insights into the evolutionary history and widespread occurrence of antheridiogen systems in ferns

Ondřej Hornych[1] (iD)**, Weston L. Testo[2]** (iD)**, Emily B. Sessa[2]** (iD)**, James E. Watkins Jr.[3]** (iD)**, Courtney E. Campany[4]** (iD)**, Jarmila Pittermann[5]** (iD) **and Libor Ekrt[1]** (iD)

[1]Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, České Budějovice, CZ 37005, Czech Republic; [2]Department of Biology, University of Florida, Box 118525, Gainesville, FL 32611, USA; [3]Department of Biology, Colgate University, 13 Oak Drive Hamilton, Hamilton, NY 13346, USA; [4]Department of Biology, Shepherd University, PO Box 5000, Shepherdstown, WV 25443, USA; [5]Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, CA 95060, USA

Author for correspondence:
Ondřej Hornych
Tel: +420 606 931 888
Email: hornych.ondrej@gmail.com

Summary

- Sex expression of homosporous ferns is controlled by multiple factors, one being the antheridiogen system. Antheridiogens are pheromones released by sexually mature female fern gametophytes, turning nearby asexual gametophytes precociously male. Nevertheless, not all species respond. It is still unknown how many fern species use antheridiogens, how the antheridiogen system evolved, and whether it is affected by polyploidy and/or apomixis.
- We tested the response of 68 fern species to antheridiogens in cultivation. These results were combined with a comprehensive review of literature to form the largest dataset of antheridiogen interactions to date. Analyzed species also were coded as apomictic or sexual and diploid or polyploid.
- Our final dataset contains a total of 498 interactions involving 208 species (*c.* 2% of all ferns). About 65% of studied species respond to antheridiogen. Multiple antheridiogen types were delimited and their evolution is discussed. Antheridiogen responsiveness was not significantly affected by apomixis or polyploidy.
- Antheridiogens are widely used by ferns to direct sex expression. The antheridiogen system likely evolved multiple times and provides homosporous ferns with the benefits often associated with heterospory, such as increased rates of outcrossing. Despite expectations, antheridiogens may be beneficial to polyploids and apomicts.

Introduction

Homospory (the production of a single spore type at meiosis) is presumed to be the ancestral state in land plants, yet the majority of extant species are heterosporous (producing two types of spores, typically male and female, at meiosis) and heterospory evolved a minimum of 11 times (Bateman & DiMichele, 1994). Heterospory promotes genetic diversity by limiting inbreeding (Qiu *et al.*, 2012). In contrast, gametophytes of homosporous plants can be bisexual and are theoretically capable of gametophytic selfing, that is the fusion of two gametes originating from a single gametophyte via mitosis (Haufler *et al.*, 2016). As the gametophyte grows from a spore originating via a single meiotic event, the sporophyte arising from gametophytic selfing is completely homozygous (Klekowski & Lloyd, 1968). Nevertheless, contemporary homosporous lineages maintain their genetic diversity by mechanisms that reduce the rate of gametophytic selfing. Some bryophyte gametophytes have their sex determined via sex chromosomes (Renner *et al.*, 2017), whereas fern gametophytes often use a dynamic system controlling sex expression via pheromones called antheridiogens (Schneller, 2008).

Walter Döpp first discovered antheridiogen (hereafter abbreviated AG) in 1950, originally named 'A-substanz'. During his experiments with gametophyte cultivation, Döpp noted that reusing agar media previously used to cultivate *Pteridium aquilinum* gametophytes caused precocious formation of antheridia in young gametophytes of *Dryopteris filix-mas* (Döpp, 1950). This effect was confirmed by Näf in 1956 and attributed to a pheromone exuded by older gametophytes that was later named antheridiogen (Näf *et al.*, 1975). Since the discovery of AG, evidence of the utilization of the pheromone has been documented in some (but not all) fern species that have been tested across phylogenetically disparate lineages (Schneller, 2008).

Available evidence suggests that AG production and response varies considerably among fern taxa and that the system involves complex inter- and intraspecific interactions. This has been evident since Döpp's initial discovery of AG as his report involved taxa belonging to two different families. Later studies revealed that AGs often have a gibberellin-like structure (Yamane, 1998) and indicated that various types of AGs occur across the fern clade (Schneller *et al.*, 1990). Generally, AGs have been classified either by the species producing them (e.g. A$_{An}$ for AG released

from *Anemia phyllitidis*) or in broad groups/types according to the taxa that they affect. Three main types of AGs typically are recognized under the second classification scheme (Schneller *et al.*, 1990). First, A or A_{Pt} type is used widely by many species throughout the order Polypodiales, notably by *P. aquilinum* and *Onoclea sensibilis*. Second, B or A_{An} type is used only within the order Schizaeales, notably by *Anemia* and *Lygodium*. Interestingly, gibberellins known from seed plants can evoke the same response as the A_{An} type (Voeller, 1964; Weinberg & Voeller, 1969). Finally, C or A_{Ce} type is used exclusively by the genus *Ceratopteris*. Several other types have been described by a limited number of studies, for example in *Asplenium ruta-muraria* by Schneller & Hess (1995).

Although several distinct types of AGs were described, the primary function of all AGs is the stimulation of precocious formation of antheridia. When a gametophyte of an AG-responsive species grows in the absence of this pheromone, it first develops archegonia (i.e. becomes female; Döpp, 1950). However, right before the gametophyte reaches the archegoniate phase, it begins exuding AGs into its environment (Näf *et al.*, 1975). At the same time, the gametophyte loses the ability to respond to AGs (Näf, 1958). Younger or slow-growing asexual gametophytes in the immediate surroundings of the first gametophyte respond to the AGs by halting growth and forming antheridia (i.e. becoming male). The population ends up composed of a few larger female gametophytes and many smaller male gametophytes (Tryon & Vitale, 1977). As fern sperm are flagellated and need to swim through water to reach archegonia, a greater abundance of sperm due to the AG system may help overcome the limitations of dry environments (Schneller & Hess, 1995). Likewise, AG leads to a greater number of unisexual gametophytes, therefore limiting self-fertilization and facilitating outcrossing, the exchange of gametes between gametophytes, and therefore maintaining heterozygosity in fern populations (Schedlbauer & Klekowski, 1972). Through the AG system, homosporous ferns gain these advantages, which are usually afforded to heterosporous plants because of their pre-determined sexes and consequent inability to undergo the extreme form of selfing found in homosporous plants (Bateman & DiMichele, 1994). Additionally, larger gametophytes may be able to pheromonally suppress the ability of smaller gametophytes to bear sporophytes, thus reducing competition. However, smaller gametophytes may use the system to contribute to the next generation despite being unable to form archegonia or support young sporophytes owing to unfavorable conditions (Willson, 1981).

Generally, fern spores require light to germinate, but AG was found to replace the need for light in spores cultivated in complete darkness (Raghavan, 1989). In nature, spores buried under a thin layer of soil or detritus affected by AG may form tiny gametophytes and reach the surface or use their limited resources to form a small number of antheridia, skipping the archegonial phase (Schneller, 1988). The sperm from those gametophytes then can reach female gametophytes aboveground (Haufler & Welling, 1994). Therefore, AG enables the mobilization of the genetic and sperm-producing potential of spores buried underground. The concentrations of AG needed to stimulate the

precocious formation of antheridia and germination in darkness may differ (Schraudolf, 1962; Weinberg & Voeller, 1969; Endo *et al.*, 1972). If the two effects, germination in darkness and precocious antheridium formation, are tightly correlated, dark germination could be used to test the response to AGs in multiple species, as was done by Weinberg & Voeller (1969). However, most authors comparing the two effects of AGs within one study have only focused on a few species (Yamane *et al.*, 1987; Chiou & Farrar, 1997; Chiou *et al.*, 1998) and a thorough review is necessary.

In theory, some fern species may gain very little but lose a lot by responding to AGs. For example, neopolyploid species (*sensu* Vida, 1976; herein after referred to as polyploid), having more than two sets of chromosomes and therefore the potential to 'buffer' against the deleterious effects of gametophytic selfing, may reproduce by self-fertilization and still retain genetic variation (Klekowski & Baker, 1966; Hickok, 1978). So, polyploids should tend to self-fertilize more than diploids (Masuyama, 1979; Soltis & Soltis, 2000; Sessa *et al.*, 2016). As the AG system limits self-fertilization, polyploid species may be more likely to stop using the pheromone, potentially allowing all polyploid gametophytes to bear sporophytes and thus avoid any negative adverse effects. However, no comparison of AG response between diploids and polyploids has been conducted until now. A more extreme case of AGs as a potential liability exists in apomictic ferns. Apomictic gametophytes form sporophytes apogamously from a somatic cell, without the need for fertilization. This renders any extra sperm present in a population as a response to AGs presumably useless. Nevertheless, the ability to suppress surrounding gametophytes may be potentially advantageous from the standpoint of reducing competition. The limited number of tested apomicts were found either to respond to AGs (*Bommeria pedata*, Haufler & Gastony, 1978; *Dryopteris affinis*, Schneller, 1981) or ignore AGs (*Cyrtomium* spp., Yatskievych, 1993). However, a thorough study of AG response in apomictic ferns has not yet been conducted.

Despite the apparently widespread occurrence of AG systems in ferns and their potentially large evolutionary significance via their effects on population structure and mating behavior, our understanding of their evolution and distribution across the fern phylogeny remains limited. Several authors have put together lists of all ferns tested for AG response (Näf *et al.*, 1975; Raghavan, 1989; Schneller, 2008) but we are unaware of any attempt to evaluate AG systems in a broader evolutionary context (with the exception of Greer *et al.*, 2009) which incorporated only the handful of species responding to gibberellins). To determine how widespread the involvement of AGs is among ferns, we combined results of our cultivation experiments with a meta-analysis of all published results of similar assays available to us, including 208 species in total. Using this large dataset, we address the following questions: How many fern species have been tested for AG response and how many of those respond? How many different types of AGs appear to exist and what is their evolutionary history? How tightly are the two effects of antheridium induction and germination in darkness correlated? How are AG production and response correlated with ploidy level and reproductive mode?

Materials and Methods

Cultivation

Frond material with mature sporangia of 69 fern species from 19 families was collected from wild or cultivated plants (Supporting Information Table S1) from tropical and temperate regions. Fronds were allowed to air-dry in paper envelopes to facilitate spore release.

Spores were sown on 1% agar supplemented with standard Bold's medium (Bold, 1957) modified with Nitsch's micronutrients (Nitsch, 1951) in 100×25 mm Petri plates. Surface sterilization of spores was not performed, and no fungal contamination was observed within the test period. For all cultivation experiments (with or without mature gametophytes), spores were sown at an approximate density of 25 spores per 100×25 mm Petri plate (ThermoFisher Scientific, Waltham, MA, USA) by dispersing them through pinholes from glassine envelopes. Cultures were kept at 25°C and exposed to a 12 h : 12 h, light : dark photoperiod achieved with fluorescent grow bulbs (65 µmol m^{-2} s^{-1}) in growth chambers (BioChambers, Winnipeg, MB, Canada and Percival Scientific, Perry, IA, USA).

In order to test the potential influence of antheridiogen (AG), spores of each species were sown in the presence of either a single conspecific or a single *Pteridium aquilinum* archegoniate (mature) gametophyte. Since the discovery of AGs in this species (Döpp, 1950), *P. aquilinum* commonly has been utilized as a source of AGs and a 'positive control' to test whether a given species is capable of responding to them (e.g. Yatskievych, 1993). Spores from up to four sporophytes per species were tested and each combination (paired with conspecific or *P. aquilinum*) was replicated three times. As a control, spores of each species also were sown without any mature gametophytes present. All plates were checked under a stereoscope for the presence of gametangia (Fig. 1) once a week for 12 wk. A species was considered as responding to either *P. aquilinum* or conspecific AGs if (1) archegonia were formed only in the absence of influencing gametophytes and (2) antheridia were formed in the presence of influencing gametophytes.

Antheridiogen response meta-analysis

In order to provide a broader sampling of taxa, we combined the data from our experiments with the results of previous studies (88 papers; Table S2) to create a dataset of all known AG production and responsiveness across ferns (Table S3). For each tested (target) species, we scored the following: the types of response tested ('antheridium formation' or 'dark germination'), the outcome of the response ('positive' or 'negative'; implicit evidence sometimes was considered), and the source species used to induce the response (e.g. the same species, a congener, or a control AG source such as *P. aquilinum*). Some species were tested with multiple source species. In cases where different responses were recorded from the same species combination, we recorded each as a separate data point. For some data points obtained from the literature, assays for responses were carried out not with exogenous AGs produced from gametophytes, but with gibberellins (which are known to produce an AG-like effect in some responsive species; Näf *et al.*, 1975). In these cases, 'gibberellin' was provided in place of the inducing species. In addition, we also scored ploidy level (diploid or polyploid) and reproductive mode (sexual or apomictic) for each species (Table S4). Ploidy data were obtained from floristic treatments, monographs and published chromosome counts; in some cases, data were not available. The reproductive mode of each taxon was obtained from Liu *et al.* (2012). Only species that are obligately apomictic were scored as apomictic.

The dataset enabled us to score each taxon as AG responsive or unresponsive (Table S4). Each species was considered as responsive to AG if at least one of these combinations yielded positive results. Five species were excluded from all additional analyses because they were either tested only against a type of AG inappropriate for the species (*Cibotium barometz* – gibberellin + *Pteridium aquilinum*, *Cyathea australis* – gibberellin + *P. aquilinum*, *Pentarrhizidium orientale* – gibberellin, *Radiovittaria stipitata* – *P. aquilinum*) or because conflicting data made it impossible to clearly label the species as responsive or not (*Phlebodium aureum*; Näf, 1956; Voeller, 1964; Weinberg & Voeller, 1969; Gemmrich, 1986; Chiou & Farrar, 1997). The first four species mentioned were removed as they are
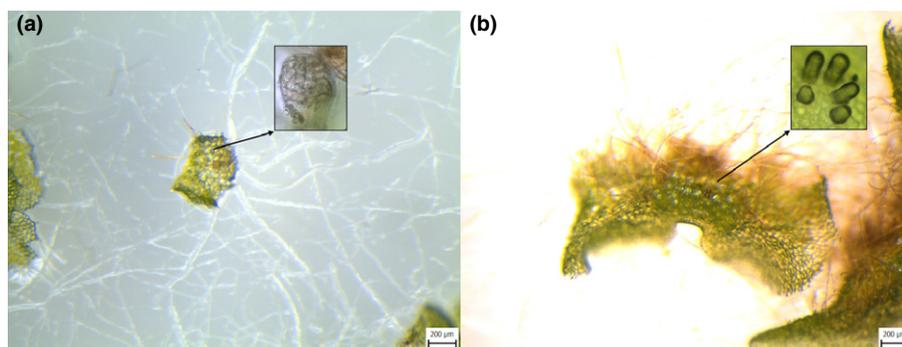
Fig. 1 Gametophytes of *Asplenium ruta-muraria* with gametangia at magnification used for sex determination in this study with details at higher magnification presented in rectangles: (a) male gametophyte bearing antheridia and (b) female gametophyte bearing archegonia.

almost certainly false negatives and further testing is needed for a proper assessment of all five excluded taxa.

Additionally, our dataset allowed for re-evaluating previously described AG groups/types. There are two conditions for each type to be unique. First, each taxon produces and/or reacts to only one AG type. If, for instance, one species reacted to two potential types, the types were merged into one. Second, taxa do not have to react to every single AG source within a type. As they represent a wide array of different chemicals, we do not consider the 'gibberellin' group to be AGs for the purposes of type delimitation.

In order to examine whether precocious antheridium formation and dark germination are tightly linked, we used our dataset to find any potential correlation. Species tested for both effects were evaluated as consistent (either affected under light and dark conditions or never) or inconsistent (affected only under one condition). Species tested for only one effect or against an inappropriate AG type (usually gibberellins failing to affect members of Polypodiales) were excluded from this comparison.

The correlation of AG production and response with species attributes (e.g. apomict/sexual, diploid/polyploid) was evaluated using chi-square tests performed in R v.3.4.3 (R Core Team, 2017). Species whose ploidy level could not be determined were excluded from the diploid/polyploid comparison.

Results

Cultivation

A total of 68 species were cultivated and tested for AG response (Table 1). Of those, 56 species were tested with a conspecific AG source, and 25 reacted. Additionally, 44 species were tested using *P. aquilinum* as the source, and 22 reacted. Six species from the genera *Asplenium*, *Ctenitis*, *Cyathea* and *Pityrogramma* responded to conspecific but not to *P. aquilinum* AG. The opposite case, reacting only to *P. aquilinum* but not to conspecific AG, occurred for *Odontosoria* and *Phlebodium*.

AG meta-analysis

Datasets The final dataset (Table S3) included a total of 208 species from 26 families, involved in a total of 498 pairings, either with a conspecific or another taxon (Figs 2, 3). After the exclusion of five species (see the Materials and Methods section), 64.5% of the 203 taxa responded to AGs (Fig. 4). Interestingly, three species (*Cyrtomium fortunei*, *C. macrophyllum* and *Polystichum lonchitis*) seemed to produce AG but did not react to any tested AG source. Tested representatives of five families (Culcitaceae, Equisetaceae, Hymenophyllaceae, Lomariopsidaceae and Osmundaceae) did not appear to produce or respond to AG at all (Fig. 2).

Antheridiogen types From our dataset, we identified two main AG types, corresponding to the *Pteridium* and *Anemia* types affecting Polypodiales and Schizaeales, respectively (Fig. 3a). In total, 64.6% of the tested representatives of Polypodiales responded to AG (usually from *P. aquilinum*; Fig. 3b) and

response to gibberellin was extremely rare (Fig. 3c). All representatives of Schizaeales responded to some form of AG (Fig. 4) and to supplemented gibberellins (Fig. 3c), if tested. Using our definition (in Methods), we also identified several different potential minor AG types, affecting only a single species or genus. Based on our definition, many species were considered as having their own type only because they have not been tested against any other species (*Davallia fejeensis*, *Elaphoglossum latifolium*, *Gymnocarpium disjunctum*, *Oleandra articulata*, *Parapolystichum excultum*, *Polypodium cambricum*, *Sadleria* spp., *Woodwardia radicans*) or were cross-tested within a small group of species (*Cheilanthes* spp.). These taxa all belong to the order Polypodiales. *Thelypteris ovata* and *Hemionitis palmata* only failed to respond to gibberellins and *Pteris vittata*, respectively, but their congener responded to *P. aquilinum*. Three species of *Asplenium* failed to react to *P. aquilinum* (*Asplenium auritum*, *Asplenium serratum*) and gibberellins (*Asplenium ruta-muraria*), but they are not phylogenetically closely related and other *Asplenium* species (*A. cuneifolium*, *A. septentrionale*) respond to *Pteridium*-type AG. *Pityrogramma calomelanos* successfully influenced itself and two species of *Onychium* but failed to react to *P. aquilinum*. Related *Pityrogramma* species also did not respond to *Pteris vittata* AG. These results indicate that a distinct AG system may be operating within *Pityrogramma*. Likewise, *Vittaria* spp. gemmae responded to exudates of long-lived congeneric gametophytes and supplemented gibberellins by forming antheridia. *Pteridium aquilinum* AG failed to induce the same response. This would indicate an AG system similar to the Anemia type. *Ceratopteris* spp. were affected only by conspecific AG but failed to respond to *Anemia* spp., *P. aquilinum* and *Pteris vittata*. *Ceratopteris* AG also failed to influence *Bommeria* species responsive to *P. aquilinum*. It is uncertain whether *Ceratopteris* AG would be needed in higher concentrations for any effect to occur, or perhaps it is distinct or different enough to be unable to affect the few species tested but still within the *Pteridium* type. Outside of Polypodiales, three tree fern (Cyatheales) species (*Cibotium menziesii*, *Cyathea microdonta*, *Cyathea multiflora*) responded to conspecific AG but not to *P. aquilinum*. Related species (*Cibotium barometz*, *Cyathea australis*) also failed to respond to gibberellins, indicating that tree ferns may utilize chemically and phylogenetically different AGs belonging to one or multiple types.

Dark germination Data on dark germination were obtained for 53 taxa. Data were sufficient (see Methods) to evaluate 32 taxa (20.4% of all taxa with determined AG response). In this subset, 26 taxa (81.3%) germinated in darkness. In three cases, the dark germination response was different than the observed antheridium induction response: *Ceratopteris thalictroides* and *Thelypteris ovata* did not germinate in darkness despite being influenced under light, and *Polypodium cambricum* germinated in darkness despite not responding to AG under light.

Reproductive types and polyploidy Overall, 12 (6%) of the taxa sampled were obligately apomictic. Apomictic and sexual taxa had similar (i.e. not significantly different) response rates to AGs of 66.7% and 64.4%, respectively ($\chi^2 < 10^{-6}$; df = 1; $P = 1$;

Table 1 Overview of the response of 68 tested species to the cultivation experiment.

Tested species	Family	Response to self[1]	Response to *Pteridium aquilinum*[1]
Adiantum radicans	Pteridaceae	Yes	Yes
Asplenium adiantum-nigrum	Aspleniaceae	Not tested	Yes
Asplenium auritum	Aspleniaceae	Yes	No
Asplenium cuneifolium	Aspleniaceae	Not tested	Yes
Asplenium ruta-muraria	Aspleniaceae	Not tested	No
Asplenium scolopendrium	Aspleniaceae	Not tested	No
Asplenium septentrionale	Aspleniaceae	Not tested	Yes
Asplenium serratum	Aspleniaceae	Yes	No
Blechnum occidentale	Blechnaceae	Yes	Yes
Blechnum polypodioides	Blechnaceae	Yes	Yes
Bolbitis portoricensis	Dryopteridaceae	No	No
Campyloneurum aphanophlebium	Polypodiaceae	No	No
Campyloneurum brevifolium	Polypodiaceae	Yes	Not tested
Christella dentata	Thelypteridaceae	Yes	Yes
Cibotium menziesii	Cibotiaceae	Yes	No
Ctenitis sloanei	Dryopteridaceae	No	Not tested
Cyathea microdonta	Cyatheaceae	Yes	No
Cyathea multiflora	Cyatheaceae	Yes	No
Davallia fejeensis	Davalliaceae	Yes	Not tested
Diplazium striatastrum	Athyriaceae	No	No
Draconopteris draconoptera	Tectariaceae	No	Not tested
Dryopteris carthusiana	Dryopteridaceae	Not tested	Yes
Dryopteris caucasica	Dryopteridaceae	Not tested	Yes
Dryopteris dilatata	Dryopteridaceae	Not tested	No
Dryopteris expansa	Dryopteridaceae	Not tested	Yes
Dryopteris filix-mas	Dryopteridaceae	Not tested	Yes
Dryopteris oreades	Dryopteridaceae	Not tested	Yes
Elaphoglossum latifolium	Dryopteridaceae	Yes	Not tested
Elaphoglossum peltatum	Dryopteridaceae	No	No
Equisetum arvense	Equisetaceae	No	No
Equisetum fluviatile	Equisetaceae	No	No
Equisetum palustre	Equisetaceae	No	Not tested
Equisetum sylvaticum	Equisetaceae	No	Not tested
Goniopteris curta	Thelypteridaceae	No	Not tested
Goniopteris nicaraguensis	Thelypteridaceae	Yes	Yes
Hypoderris brauniana	Tectariaceae	Yes	Yes
Lomariopsis japurensis	Lomariopsidaceae	No	Not tested
Lomariopsis vestita	Lomariopsidaceae	No	No
Lygodium japonicum	Lygodiaceae	Yes	Not tested
Lygodium microphyllum	Lygodiacea	Yes	Not tested
Macrothelypteris torresiana	Thelypteridaceae	No	No
Meniscium lingulatum	Thelypteridaceae	Yes	Yes
Mickelia nicotianifolia	Dryopteridaceae	No	Not tested
Microgramma lycopodioides	Polypodiaceae	No	Not tested
Zealandia pustulata	Polypodiaceae	No	No
Nephrolepis biserrata	Nephrolepidaceae	No	No
Odontosoria c.f. gymnogrammoides	Lindsaeaceae	No	Yes
Oleandra articulata	Oleandraceae	Yes	Not tested
Olfersia cervina	Dryopteridaceae	No	No
Osmunda claytoniana	Osmundaceae	No	Not tested
Osmunda regalis	Osmundaceae	No	Not tested
Osmundastrum cinnamomeum	Osmundaceae	No	Not tested

Table 1 (Continued).

Tested species	Family	Response to self[1]	Response to *Pteridium aquilinum*[1]
Parapolystichum excultum	Dryopteridaceae	Yes	Not tested
Pecluma pectinata	Polypodiaceae	No	Not tested
Phlebodium pseudoaureum	Polypodiaceae	No	Yes
Pityrogramma calomelanos	Pteridaceae	Yes	No
Pleopeltis furfuracea	Polypodiaceae	No	Not tested
Polybotrya osmundacea	Dryopteridaceae	No	Not tested
Polystichum munitum	Dryopteridaceae	No	No
Polystichum setiferum	Dryopteridaceae	Not tested	Yes
Pteris propinqua	Pteridaceae	Yes	Yes
Saccoloma elegans	Saccolomataceae	Yes	Yes
Saccoloma inaequale	Saccolomataceae	Yes	Not tested
Salpichlaena volubilis	Blechnaceae	No	No
Serpocaulon triseriale	Polypodiaceae	Yes	Yes
Tectaria heracleifolia	Tectariaceae	No	Not tested
Thelypteris kunthii	Thelypteridaceae	Yes	Yes
Trichomanes diversifrons	Hymenophyllaceae	No	Not tested

[1]Taxa that responded formed antheridia first when exposed to an archegoniate conspecific or *Pteridium aquilinum* gametophyte, despite forming archegonia first under control conditions.

Fig. 4). Of the 208 taxa included in the dataset, ploidy level and AG response were determined for 172 taxa. The 100 diploid and 72 polyploid taxa had nearly equal response rates of 67.0% and 68.1%, respectively ($\chi^2 = 0$; df = 1; $P = 1$; Fig. 4).

Discussion

Antheridiogen data synthesis and meta-analysis

Combining our results with published data from the literature, we present an updated list of 208 fern species from 26 families that have been tested for antheridiogen (AG) response or production (Table S4). Unlike previous major reviews on the topic (Näf et al., 1975; Raghavan, 1989; Schneller, 2008), we have recorded the response of each species to all tested AG sources (Table S3). A recent estimate puts the number of fern species at 10 578 (PPG 1, 2016), meaning that 2% of all known fern species have now been tested for AG activity. This is a substantial increase from the < 1% tested that was estimated by Kirkpatrick & Soltis (1992). However, the vast majority of fern diversity remains unstudied. Athyriaceae and Cyatheaceae deserve special attention as these are species-rich families with only three species tested each.

About two-thirds (64.5%) of all tested species responded positively to some type of AG. Additionally, three species produced AG but did not react to the pheromone. Clearly, AGs play a major role in the lives of fern gametophytes. Nevertheless, the real percentage of fern species responding may be different. Responsive species may be over-represented in our dataset, as negative results are less likely to be published. However, some species may be incorrectly labelled as nonresponsive if they failed to respond to some of the model AG sources. For example,

(no. positive, no. tested, no. species in family)

○ Untested
● AG response absent
● AG response present

Polypodiaceae (11, 27, 1625)
Davalliaceae (1, 2, 65)
Oleandraceae (1, 1, 15)
Tectariaceae (2, 5, 250)
Lomariopsidaceae (0, 2, 69)
Nephrolepidaceae (2, 3, 19)
Dryopteridaceae (18, 39, 2115)
Hypodematiaceae (–, –, 22)
Didymochlaenaceae (–, –, 1)
Thelypteridaceae (6, 10, 1034)
Athyriaceae (2, 3, 650)
Blechnaceae (11, 12, 265)
Onocleaceae (2, 2, 5)
Woodsiaceae (1, 1, 39)
Aspleniaceae (8, 10, 730)
Hemidictyaceae (–, –, 1)
Desmophlebiaceae (–, –, 2)
Diplaziopsidaceae (–, –, 4)
Rhachidosoraceae (–, –, 8)
Cystopteridaceae (4, 5, 37)
Dennstaedtiaceae (3, 4, 265)
Pteridaceae (39, 46, 1211)
Lindsaeaceae (1, 1, 234)
Lonchitidaceae (–, –, 2)
Cystodiaceae (–, –, 1)
Saccolomataceae (2, 2, 18)
Cyatheaceae (2, 2, 643)
Dicksoniaceae (–, –, 35)
Metaxyaceae (–, –, 6)
Cibotiaceae (1, 1, 9)
Plagiogyriaceae (–, –, 15)
Culcitaceae (0, 1, 2)
Loxsomataceae (–, –, 2)
Thyrsopteridaceae (–, –, 1)
Marsileaceae (–, –, 61)
Salviniaceae (–, –, 21)
Anemiaceae (8, 8, 115)
Schizaeaceae (–, –, 35)
Lygodiaceae (7, 7, 40)
Gleicheniaceae (–, –, 157)
Matoniaceae (–, –, 4)
Dipteridaceae (–, –, 11)
Hymenophyllaceae (0, 1, 434)
Osmundaceae (0, 4, 18)
Marattiaceae (–, –, 111)
Psilotaceae (–, –, 17)
Ophioglossaceae (–, –, 112)
Equisetaceae (0, 4, 15)

Eupolypods
Polypodiales
Leptosporangiates

Fig. 2 Fern phylogeny (with relationships based on PPG 1, 2016) indicating the families tested for antheridiogen (AG) response. The number of responsive, tested and total species in a family also is given.

Pentarrhizidium orientale failed to react to gibberellins (Weinberg & Voeller, 1969). Labelling the species as nonresponsive based only on this result could be misleading (the species was excluded as a false negative) as the closely related *Onoclea sensibilis* reacts to

AG of ≥ 27 other species, but not to gibberellins. Not all cases can be as clear as that of *Pentarrhizidium orientale* and AG systems are too complex to accurately assign species as nonresponsive based on a limited number of pairings.
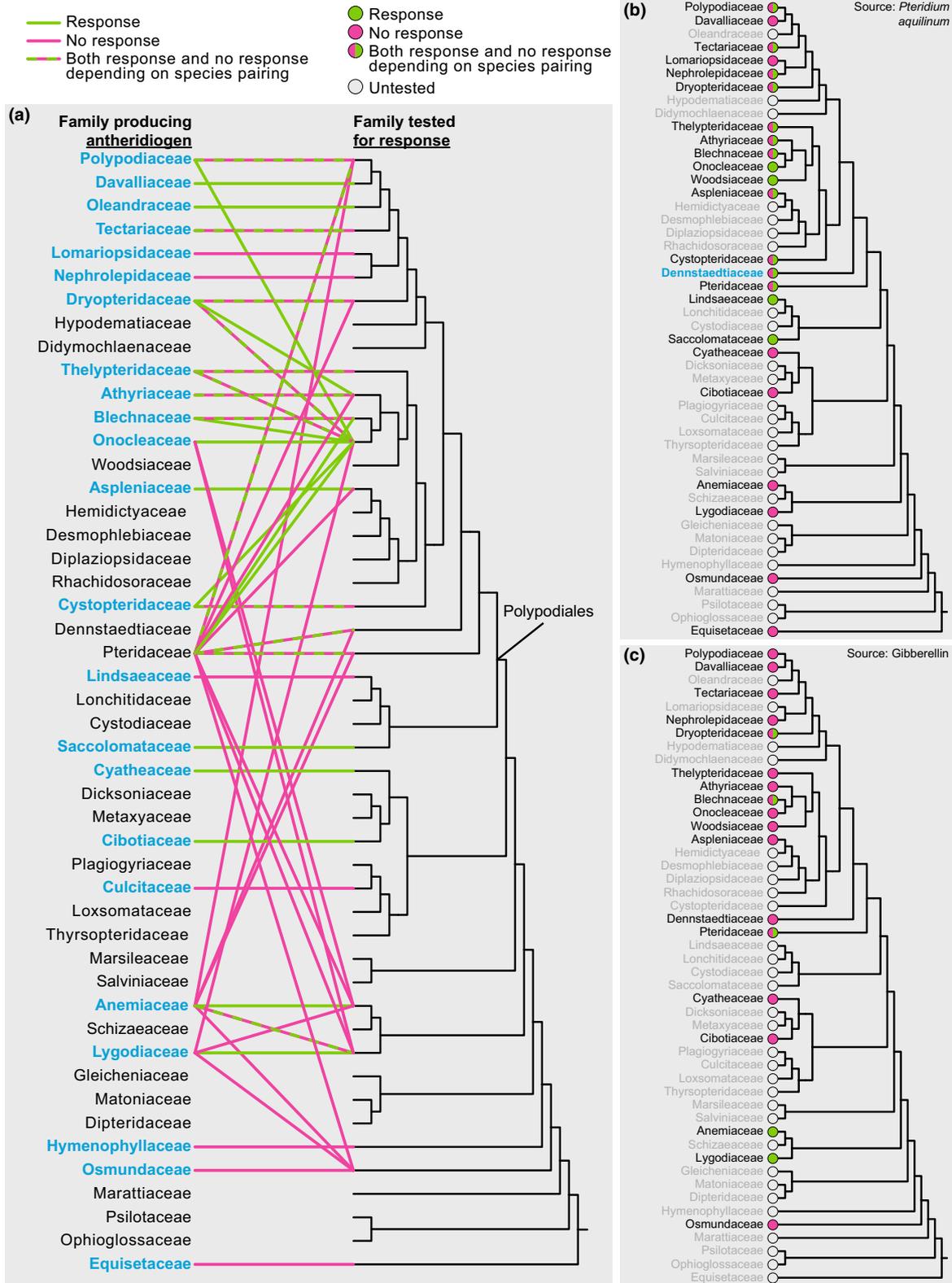
Fig. 3 Overview of antheridiogen interactions (response or no response) between tested fern taxa on a family level (phylogeny tree based on PPG 1, 2016): (a) interactions between all families, excluding *Pteridium aquilinum* (Dennstaedtiaceae) as antheridiogen producer (families tested labelled blue); (b) response of taxa to *P. aquilinum* (Dennstaedtiaceae, labelled blue); and (c) response to gibberellins.
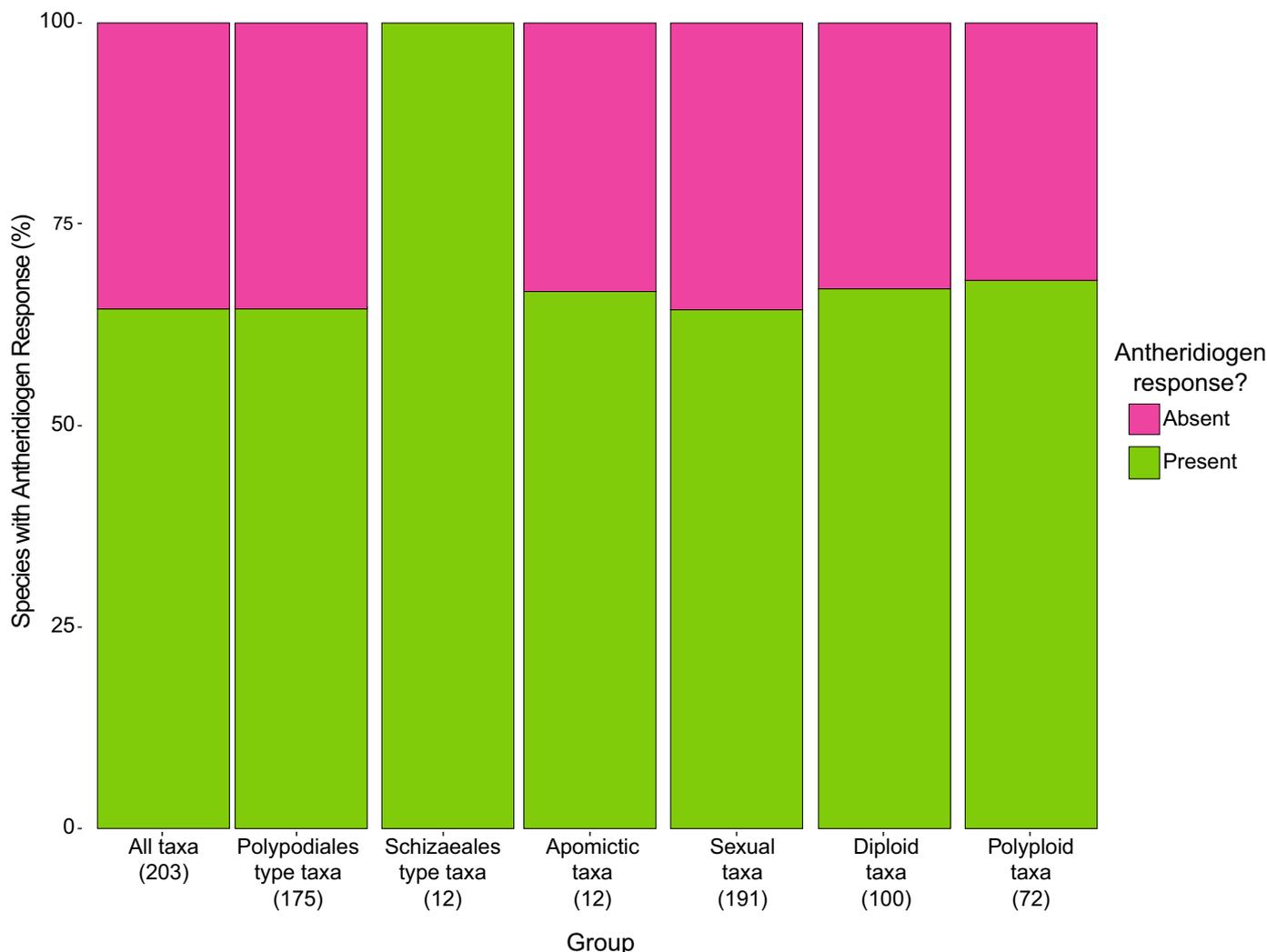
Fig. 4 Overview of the percentage of fern taxa reacting to antheridiogens. The percentage of all studied taxa is listed alongside those for various subgroups based on antheridiogen type (Polypodiales-type AG (AGPo)/ Schizaeales-type AG (AGSc); others not listed), ploidy level (diploid/polyploid) and reproductive types (apomictic/sexually reproducing).

Antheridiogen types

Our dataset clearly demonstrates two major AG types, one affecting the order Polypodiales and the other affecting Schizaeales. Among the many minor types described, most would likely fall within the main Polypodiales type, if more pairings are conducted. Some of these minor types (e.g. *Asplenium, Pityrogramma, Vittaria*) are supported with inconclusive evidence and require further study. *Ceratopteris* is generally considered to have its own AG type (Schneller *et al.*, 1990) and this is supported by the most convincing evidence, such as the lack of response to multiple other species and no germination in darkness. However, we would like to caution against an unambiguous distinction of the *Ceratopteris* type until more pairings are done with other Polypodiales species, especially from Pteridaceae. Some sort of AG system also operates in tree ferns (Cyatheales) that seems distinct from the two major AG types. However, we have insufficient data on the chemical nature and the extent of influence of this system. Tree ferns clearly deserve more study.

In accordance with our merger of many minor types to the larger ones, we would like to suggest the following naming convention: The types should be named after the broadest group under their influence; for example, Polypodiales-type AG, Schizaeales-type AG, Cyatheales-type AG (for a possible unified tree fern AG type) and Ceratopteris-type AG (for the potential *Ceratopteris* type). To allow for easier reading and understanding we advise abbreviating the types as AGPo, AGSc, AGCy and AGCe, respectively, avoiding the usually applied subscript.

Evolution of antheridiogens

Pheromonal control of sex expression via AGs is widespread among leptosporangiate ferns and has likely evolved multiple times. To understand the evolution of AGs, phylogeny must be considered. For the purpose of this analysis, we presume that only

three main types (one being a unified Cyatheales type) described above are distinct. All pairings involving *Equisetum* indicate that it has no AG system, suggesting that AGs evolved within the ferns after the divergence of *Equisetum*. No other eusporangiate ferns have yet been tested, but studies of sexual expression in Osmundaceae, which are sister to all other leptosporangiate ferns, indicate a potential pheromonal control different from AG (Hollingsworth *et al.*, 2012). Phylogenetically, the three types of AG system we recognize could represent three separate origins. The first true AG system is that found in the order Schizaeales, chemically based on gibberellins (Fig. 3c). The origin of this AG type is uncertain until denser sampling of non-polypod leptosporangiates is achieved. Nevertheless, it could be either that the order represents an independent acquisition of AGs, or that AGs were present in the common ancestor of the entire group (Schizaeales through Polypodiales), and the system was later lost in water ferns and its chemical nature was changed considerably in other groups. Therefore, it seems more likely that the Schizaeales type system evolved independently within that order. The second origin, or perhaps multiple origins, appeared in Cyatheales. However, our knowledge in this group is scarce and further research is required. The third origin of AG, the Polypodiales type, could have evolved right at the origin of Polypodiales, potentially as a key innovation of this highly diverse lineage. Our results indicate the presence of AG activity in *Saccoloma* and Lindsaeaceae (Fig. 3a,b). Antheridiogens are certainly well-established within Pteridaceae, as four of its five subfamilies, including the Cryptogrammoideae, which are sister to the other four (Schuettpelz *et al.*, 2007), have species responsive to AG, and members of other families in Polypodiales react to Pteridaceae AG (Fig. 3a). Further studies of *Saccoloma*, *Cystodium*, *Lonchitis* and Lindsaeaceae will be critical for establishing the origins of AGs within Polypodiales, and studies of non-leptosporangiates are necessary to understand the evolution of antheridiogens across all ferns.

Although we advocate for a broader AG type concept, it is important to note that AGs likely diversify considerably within each type. A considerable number of various distinct chemical entities were described within Schizaeales (Nakanishi *et al.*, 1971; Endo *et al.*, 1972; Nester *et al.*, 1987; Yamane *et al.*, 1988; Yamauchi *et al.*, 1996; Wynne *et al.*, 1998). In this order, all species reacted positively to congeners, if tested. However, the compatibility between the two families Anemiaceae and Lygodiaceae was limited. No chemical compound was fully described within Polypodiales, but the lack of compatibility across some families is reminiscent of what is seen in Schizaeales. For example, members of Pteridaceae are capable of inducing a response in members of Onocleaceae and Blechnaceae, but not Aspleniaceae (Fig. 3a). This phenomenon could be caused by the lack of selective pressure to conserve the chemical structure of AG. The chemical compounds may diversify in each lineage, being less capable of affecting evolutionarily distant species. A possible result would be the evolution of new types, for example in *Ceratopteris*.

Ceratopteris is of particular interest when considering the evolution of ferns in association with AG systems. This genus is the only representative of homosporous aquatic ferns. Its species also form the largest spores of all homosporous ferns (Tryon & Lugardon, 1991) and it may have its own unique AG type. In theory, plants in aquatic environments benefit from propagules with higher energy reserves to speed up the life cycle and help survive in a carbon-dioxide-poor environment (Petersen & Burd, 2017). A greater abundance of male gametes also is beneficial to increase the chance of mating in water. Heterospory, in which a few large megaspores and many small microspores are produced, fits perfectly into this environment. Unsurprisingly, the known cases of heterospory in ferns involve the true water ferns (Salviniales) and *Pteris platyzomoides*, a unique species growing in seasonally waterlogged habitats (Tryon, 1964). *Ceratopteris* represents an alternative solution to this problem. Large spores provide the energy reserves, and the AG system guarantees an overabundance of male gametes in populations whereas single spores can still grow into bisexuals and colonize new habitats. In *Ceratopteris*, the AG system does not just substitute the genetic variation aspect of heterospory, but also confers the benefits of heterospory in aquatic environments. It is possible that the ancestors of Salviniales developed heterospory, in part, due to a lack of AG system in their lineage. In turn, *Ceratopteris* might have become fully heterosporous were it not for AGs.

Germination of spores in darkness

In addition to stimulating precocious formation of antheridia, AGs also enable germination of spores in darkness. Of the 32 species evaluated, 80% reacted to AG, compared to the 64% overall reaction. This higher response rate is likely caused by the over-representation of Schizaeales (which all react) in the dark germination subset. Furthermore, 29 of 32 species (90.6%) were consistent in their response. The first exception was *Polypodium cambricum* germinating in darkness, but without induced antheridia in illuminated cultures (Welling & Haufler, 1993). However, some members of Polypodiaceae are known to respond only to high concentrations of AG (Chiou & Farrar, 1997) and it is possible that the concentration of AG used by Welling & Haufler (1993) was insufficient to promote antheridium formation in illuminated cultures. The second exception, *Thelypteris ovata*, failed to germinate in darkness despite responding to AG in light (Nester-Hudson *et al.*, 1997). However, germination percentages were checked only 7 d after sowing, a duration equal to the time needed for germination in light for the species. As dark germination may take longer than germination under normally illuminated conditions (Weinberg & Voeller, 1969), it is possible that AG-induced dark germination would have been observed at a later point. Finally, *Ceratopteris thalictroides* does not induce germination in darkness (Schedlbauer, 1976). Spores of *Ceratopteris* generally do not germinate in darkness, although some exceptions have been reported (Scott & Hickok, 1987). From our data, this is the only clear example of AG being capable of inducing antheridiogenesis but not germination in darkness. With the notable exception in *Ceratopteris*, germination in darkness seems to be a reliable indicator of antheridiogen response in fern species. If done properly, assays of dark germination could be

used as this method is less demanding than its light counterpart and lends itself to mass analysis of the many species yet to be studied.

Antheridiogens in apomictic ferns

Apomictic ferns, which produce sporophytes spontaneously from gametophytes without fertilization (Grusz, 2016), can produce and respond to AG. Usage of AG in apomicts presents an interesting evolutionary dilemma. On the one hand, apomictic gametophytes that respond to AGs and produce antheridia may be wasting valuable resources to do so, and any subsequent slowed growth might limit their ability to form sporophytes apomictically. On the other, an appealing possibility is that production of AGs may confer a competitive advantage to apomictic fern gametophytes over co-occurring sexual taxa, as some apomictic gametophytes grow faster than their sexual competitors (Whittier, 1968; Haufler & Gastony, 1978). Theoretically, a disturbance revealing a new niche for ferns could be colonized by fast-growing apomictic gametophytes that would suppress sexual gametophytes of similar age and any latecomers. Response to AG in apomicts would then be irrelevant, as older gametophytes producing AG themselves are insensitive to it (Näf, 1958). Provoking dark germination and subsequent antheridium formation in subterranean spores would have the added effect of depleting the spore bank, thus limiting potential future competition.

Like sexual species, about two thirds of apomictic species respond to AG. To date, assessment of AG responsiveness in apomict-containing lineages is too limited to draw broad conclusions, and evaluation of their responses needs to be tested in a phylogenetic context. However, we present several possible explanations for the similar response between apomictic and sexually reproducing taxa. First, apomicts arise from sexual ancestors (Grusz, 2016) and AG response is therefore inherited from them, and thus may be evolutionarily conserved within some lineages. In *Cheilanthes* and *Cystopteris*, the AG-responsiveness of an allotetraploid species was reported as the average of its diploid progenitors, suggesting a legacy of AG activity in descendants (Haufler & Ranker, 1985; Pajarón *et al.*, 2015). Many apomicts start as hybrids (Grusz *et al.*, 2009; Liu *et al.*, 2012; Ekrt & Koutecký, 2016) and likely follow a similar pattern. Yatskievych (1993) found that two apomictic *Cyrtomium* species retained the ability to produce AG but did not react to it, thus keeping the advantage, but losing the liability. The simple inheritance of AG systems from ancestors may be the most parsimonious explanation of the equal usage of AGs among sexual and apomictic taxa. Nevertheless, the presence of apomictic species that have lost sensitivity to AG despite being able to synthesize it indicates that adaptive pressures affect the use of the AG system in apomicts.

Second, apomicts may adapt not by losing the ability to respond to AGs, but instead by increasing the needed concentration of the pheromone. Once the species is insensitive enough that common competitors and their typical AG output cannot influence it, there would be no selective pressures to reduce sensitivity further. Schneller (1981) reported AG effects to be weaker in apomictic members of the *Dryopteris affinis* group compared

to sexually reproducing *D. filix-mas*. In the same genus, sexually reproducing *D. carthusiana* reacts to AG of *Pteridium aquilinum*, but not to congeneric species that it competes with (Testo *et al.*, 2015). Thus, in laboratory experiments, unusually high concentrations or slightly different sources of AG may result in a positive response in otherwise insensitive species.

Finally, response to AG may be adaptive for apomicts. As mentioned above, apomicts may use AGs to suppress sexual competitors. Furthermore, related apomictic and sexually reproducing ferns may hybridize to form semi-fertile hybrids, which then reproduce via apomixis (Ekrt & Koutecký, 2016). This peculiar merger likely happens via fertilization of an egg from a sexual species by an apomict's sperm, as most apomicts do not form archegonia (Döpp, 1959; Whittier, 1968; Yatskievych, 1993, but see Hori & Murakami, 2019). This way, sporophyte-bearing apomictic gametophytes not only reduce future competition by suppressing conspecific gametophytes, but the suppressed apomictic gametophytes also flood sexual gametophytes that made it to the archegoniate phase with interspecific sperm. Likewise, a sexual archegoniate gametophyte growing on top of an apomictic spore bank may be forced to hybridize this way. In both cases, sexually reproducing gametophytes are either denied fully functioning sporophytic offspring or end up propagating the genes of the apomict. However, owing to the lack of testing in field conditions, we cannot be sure how important this competition between apomicts and sexually reproducing species really is.

Antheridiogens and polyploidy

Polyploidy plays an important role in fern evolution (Vida, 1976; Wood *et al.*, 2009; Liu *et al.*, 2019). Most ferns use a mixed-mating system (Haufler *et al.*, 2016), forming sporophytes by self-fertilization (gametophytic selfing) or by exchanging gametes with other gametophytes (sporophytic selfing or outcrossing). The use of AG promotes the latter option as unisexual gametophytes are more likely to occur. However, polyploid ferns should in theory be more tolerant of forming progeny by self-fertilization on bisexual gametophytes (Masuyama, 1979; Soltis & Soltis, 2000; Pangua *et al.*, 2003; Flinn, 2006; Testo *et al.*, 2015; Sessa *et al.*, 2016). As sensitivity towards AGs limits the ability to form bisexual gametophytes, polyploids might be more likely to abandon the use of AGs. That way, each polyploid gametophyte can self-fertilize and take advantage of their inherent genetic diversity (Hickok, 1978). However, the ratio of responsive diploids and polyploids is nearly identical (Fig. 4). As in apomicts, the optimal strategy for a predominantly selfing species may be to exude AGs but not react to them. This strategy has been described in the tetraploid *D. carthusiana* (Testo *et al.*, 2015). As mentioned above, removing the inherited sensitivity towards AG may be a long and difficult process, but polyploids are often evolutionarily young. Alternatively, Schneller & Hess (1995) suggest that AGs in tetraploid *Asplenium ruta-muraria* may be used to increase the quantity of available sperm in their environments, where water is a factor limiting fertilization (such as in the rock walls that *A. ruta-muraria* typically inhabits). The gametophytic

community in such a habitat might be founded by a single sporophyte, so the end goal is not increased genetic diversity but an increased chance of fertilization. Finally, polyploids may still benefit from the outcrossing supported by AGs and the positives of retaining their AG sensitivity outweigh the potential negatives of selfing with little genetic risk.

Conclusion

This comprehensive meta-analysis of 88 published papers together with new data from cultivation experiments has focused on the occurrence of antheridiogens in ferns, especially from the perspective of phylogeny, dark germination, mating modes and ploidies. The meta-analysis shows that the AG system is widespread among ferns. About two-thirds (64.5%) of all tested species responded positively to AGs. This finding demonstrates their far-reaching importance, likely related to consequences that affect many aspects of fern reproduction. This unique system of sex determination and ensuing population demographic control deserves more interest. Seventy years after the discovery of AGs by Walter Döpp (Döpp, 1950) the vast majority of fern species (98%) remains unexplored. We suggest that large, species-rich families such as Athyriaceae and Cyatheaceae, with barely any species tested, should be the subject of future inquiries, as should the non-leptosporangiate fern lineages. Several AG types are well-established by now, but others still require thorough testing to determine their scope, distinctness, and features.

Despite expectations, the majority (66.7%) of apomictic species surveyed to date respond to AG. The consequences of this may play an important role in survival and competition among fern gametophytes in nature as well as interactions between apomictic and sexually reproducing taxa. Our study also suggests that there is no difference between diploids (67.0%) and polyploids (68.1%) in response to AGs, so the pheromonal system may be advantageous even to species capable of being predominantly selfing. Finally, there is a strong correlation between germination in darkness and precocious antheridium formation in light. Testing for dark germination can be done through more expedient methods with binary results. These methods may be key to mass testing AG response in many of the yet unstudied species. We are now beginning to understand how AGs operate on the molecular level (Valledor *et al.*, 2014; Ganger *et al.*, 2015; Attalah *et al.*, 2018; Chen *et al.*, 2019) but many questions about their distribution and evolution remain unanswered. Hopefully, our comprehensive dataset can provide a starting point for fern researchers to learn whether their species of interest use this intriguing system of pheromonal control over sexual determination.

Author contributions

OH, WLT, JEW and LE designed the study; OH, WLT, CEC and JP conducted the cultivation experiments; OH compiled the meta-analysis list; and OH, WLT and EBS analyzed the data. All authors contributed to the writing of the manuscript.

ORCID

Courtney E. Campany https://orcid.org/0000-0003-2986-434X
Libor Ekrt https://orcid.org/0000-0003-3947-787X
Ondřej Hornych https://orcid.org/0000-0002-5093-3103
Jarmila Pittermann https://orcid.org/0000-0003-1880-1888
Emily B. Sessa https://orcid.org/0000-0002-6496-5536
Weston L. Testo https://orcid.org/0000-0003-3194-5763
James E. Watkins Jr. https://orcid.org/0000-0002-4789-2056

References

Attalah NM, Viter O, Gaiti F, Tanurdzic M, Banks JA. 2018. Sex determination in *Ceratopteris richardii* is accompanied by transcriptome changes that drive epigenetic reprogramming of the young gametophyte. *G3: Genes Genomes, Genetics* 8: 2205–2214.

Bateman RM, DiMichele WA. 1944. Heterospory: the most iterative key innovation in the evolutionary history of the plant kingdom. *Biological Reviews* 69: 345–417.

Bold HC. 1957. *Morphology of plants*. New York, NY, USA: Harper & Row.

Chen X, Chen Z, Huang W, Fu H, Wang Q, Wang Y, Cao J. 2019. Proteomic analysis of gametophytic sex expression in the fern *Ceratopteris thalictroides*. *PLoS ONE* 14: e0221470.

Chiou W-L, Farrar DR. 1997. Antheridiogen production and response in Polypodiaceae species. *American Journal of Botany* 84: 633–640.

Chiou W-L, Farrar DR, Ranker TA. 1998. Gametophyte morphology and reproductive biology in *Elaphoglossum*. *Canadian Journal of Botany* 76: 1967–1977.

Döpp W. 1950. Eine die Antheridienbildung bei Farnen fördernde Substanz in den Prothallien von *Pteridium aquilinum* (L.) Kuhn. *Berichte der Deutschen Botanischen Gesellschaft* 63: 139–147.

Döpp W. 1959. Über eine hemmende und eine fördernde Substanz bei der Antheridienbildung in den Prothallien von *Pteridium aquilinum*. *Berichte der Deutschen Botanischen Gesellschaft* 72: 11–24.

Ekrt L, Koutecký P. 2016. Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (*Dryopteridaceae*). *Annals of Botany* 117: 97–106.

Endo M, Nakanishi K, Näf U, McKeon W, Walker R. 1972. Isolation of the antheridiogen in *Anemia phyllitidis*. *Physiologia Plantarum* 26: 183–185.

Flinn KM. 2006. Reproductive biology of three fern species may contribute to differential colonization success in post-agricultural forests. *American Journal of Botany* 93: 1289–1294.

Ganger MT, Giouard JA, Smith HA, Bahny B, Ewing SJ. 2015. Antheridiogen and abscisic acid affect conversion and ANI1. *Botany-Botanique* 93: 109–116.

Gemmrich AR. 1986. Antheridiogenesis in the Fern *Pteris vittata* II) hormonal control of antheridium formation. *Journal of Plant Physiology* 125: 157–166.

Greer GK, Dietrich MA, Stewart S, Devol J, Rebert A. 2009. Morphological functions of gibberellins in leptosporangiate fern gametophytes: insights into the evolution of form and gender expression. *Botanical Journal of the Linnean Society* 159: 599–615.

Grusz AL. 2016. A current perspective on apomixis in ferns. *Journal of Systematics and Evolution* 54: 656–665.

Grusz AL, Windham MD, Pryer KM. 2009. Deciphering the origins of apomictic polyploids in the *Cheilanthes yavapensis* complex (Pteridaceae). *American Journal of Botany* 96: 1636–1645.

Haufler CH, Gastony GJ. 1978. Antheridiogen and the breeding system in the fern genus *Bommeria*. *Canadian Journal of Botany* 56: 1594–1601.

Haufler CH, Pryer KM, Schuettpelz E, Sessa EB, Farrar DR, Moran R, Schneller JJ, Watkins JE Jr, Windham MD. 2016. Sex and the single gametophyte: Revising the homosporous vascular plant life cycle in light of contemporary research. *BioScience* 66: 928–937.

Haufler CH, Ranker TA. 1985. Differential antheridiogen response and evolutionary mechanisms in *Cystopteris*. *American Journal of Botany* 72: 659–665.

Haufler CH, Welling CB. 1994. Antheridiogen, dark spore germination, and outcrossing mechanisms in *Bommeria* (Adiantaceae). *American Journal of Botany* 80: 616–621.

Hickok LG. 1978. Homeologous chromosome pairing and restricted segregation in the fern *Ceratopteris*. *American Journal of Botany* 65: 516–521.

Hollingsworth SN, Andres EA, Greer GK. 2012. Pheromonal interactions among gametophytes of *Osmundastrum cinnamomeum* and the origins of antheridiogen systems in leptosporangiate ferns. *International Journal of Plant Sciences* 173: 382–390.

Hori K, Murakami N. 2019. Origin of the *Diplazium hachijoense* complex (Athyriaceae). *PhytoKeys* 124: 57–76.

Kirkpatrick REB, Soltis PS. 1992. Antheridiogen production and response in *Gymnocarpium dryopteris* ssp. *disjunctum*. *Plant Species Biology* 7: 1–9.

Klekowski EJ, Baker HG. 1966. Evolutionary significance of polyploidy in the Pteridophyta. *Science* 153: 305–307.

Klekowski EJ, Lloyd RM. 1968. Reproductive biology of the Pteridophyta 1. General considerations and a study of *Onoclea sensibilis* L. *Botanical Journal of the Linnean Society* 60: 315–324.

Liu H-M, Dyer RJ, Guo Z-Y, Meng Z, Li J-H, Schneider H. 2012. The evolutionary dynamics of apomixis in ferns: A case study from polystichoid ferns. *Journal of Botany* 2012: 1–11.

Liu H-M, Ekrt L, Koutecký P, Pellicer J, Hidalgo O, Marquardt J, Pustahija F, Ebihara A, Siljak-Yakovlev S, Gibby M et al. 2019. Polyploidy does not control all: lineage-specific average chromosome length constraints genome size evolution in ferns. *Journal of Systematics and Evolution* 57: 418–430.

Masuyama S. 1979. Reproductive biology of the fern *Phegopteris decursive-pinnata*. I. The dissimilar mating systems of diploids and tetraploids. *Botanical magazine, Tokyo* 92: 275–289.

Näf U. 1956. The demonstration of a factor concerned with the initiation of antheridia in polypodiaceous ferns. *Growth* 20: 91–105.

Näf U. 1958. On the physiology of antheridium formation in the Bracken Fern [*Pteridium aquilinum* (L) Kuhn]. *Physiologia Plantarum* 11: 728–746.

Näf U, Nakanishi K, Endo M. 1975. On the physiology and chemistry of fern antheridiogens. *Botanical Reviews* 41: 315–359.

Nakanishi K, Endo M, Näf U, Johnson LR. 1971. Structure of the antheridium-inducing factor of the fern *Anemia phyllitidis*. *Journal of the American Chemical Society* 93: 5579–5581.

Nester JE, Veysey S, Coolbaugh RC. 1987. Partial characterization of an antheridiogen of *Anemia mexicana*: Comparison with the antheridiogen of *A. phyllitidis*. *Planta* 170: 26–33.

Nester-Hudson JE, Ladas C, McClurd A. 1997. Gametophyte development and antheridiogen activity in *Thelypteris ovata* var. *lindheimeri*. *American Fern Journal* 87: 131–142.

Nitsch JA. 1951. Growth and development in vitro of excised ovaries. *American Journal of Botany* 38: 566–577.

Pajarón S, Pangua E, Quintanilla LG, Jiménez A. 2015. Influence of water availability on gender determination of gametophytes in a diploid–polyploid complex of a xerophytic fern genus. *Aob Plants* 7: plv047.

Pangua E, Quintanilla LG, Sancho A, Pajarón S. 2003. A comparative study of the gametophytic generation in the *Polystichum aculeatum* group (Pteridophyta). *International Journal of Plant Sciences* 164: 295–303.

Petersen KB, Burd M. 2017. Why did heterospory evolve? *Biological Reviews* 92: 1739–1754.

PPG1 (The Pteridophyte Phylogeny Group). 2016. A community-derived classification for extant lycophytes and ferns. *Journal of Systematics and Evolution* 54: 563–603.

Qiu Y-L, Taylor AB, McManus HA. 2012. Evolution of the life cycle in land plants. *Journal of Systematics and Evolution* 50: 171–194.

Raghavan V. 1989. *Developmental biology of fern gametophytes*. Cambridge, UK: Cambridge University Press.

R Core Team. 2017. *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Renner SS, Heinrichs J, Sousa A. 2017. The sex chromosomes of bryophytes: Recent insights, open questions, and reinvestigations of *Frullania dilatata* and *Plagiochila asplenioides*. *Journal of Systematics and Evolution* 55: 333–339.

Schedlbauer MD. 1976. Specificity of the antheridiogen from *Ceratopteris thalictroides* (L.) Brongn. *Plant Physiology* 57: 666–669.

Schedlbauer MD, Klekowski EJ. 1972. Antheridogen activity in the fern *Ceratopteris thalictroides* (L.) Brongn. *Botanical Journal of the Linnean Society* 65: 399–413.

Schneller JJ. 1981. *Bemerkungen zur Biologie der Wurmfarngruppe*. *Farnblätter* 7: 9–17.

Schneller JJ. 1988. Spore bank, dark germination and gender determination in *Athyrium* and *Dryopteris*: results and implications for population biology of Pteridophyta. *Botanica Helvetica* 98: 77–86.

Schneller JJ. 2008. Antheridiogens. In: Ranker TA, Haufler CH, eds. *Biology and evolution of ferns and lycophytes*. Cambridge, UK: Cambridge University Press, 134–158.

Schneller JJ, Haufler CH, Ranker TA. 1990. Antheridiogen and natural gametophyte populations. *American Fern Journal* 80: 143–152.

Schneller JJ, Hess A. 1995. Antheridiogen system in the fern *Asplenium ruta-muraria* (Aspleniaceae: Pteridophyta). *Fern Gazette* 15: 64–70.

Schraudolf H. 1962. Die Wirkung von Phytohormonen auf Keimung und Entwicklung von Farnprotballien. 1. Auslosung der Antheridienbildung mid Dunkelkeiniung bei Schizaeaceen durch Gibberellinsiiure. *Biologisches Zentralblatt* 81: 731–740.

Schuettpelz E, Schneider H, Huiet L, Windham MD, Pryer KM. 2007. A molecular phylogeny of the fern family Pteridaceae: Assessing overall relationships and the affinities of previously unsampled genera. *Molecular Phylogenetics and Evolution* 44: 1172–1185.

Scott RJ, Hickok LG. 1991. Inheritance and characterization of a dark-germinating, light-inhibited mutant in the fern *Ceratopteris richardii*. *Canadian Journal of Botany* 69: 2616–2619.

Sessa EB, Testo WL, Watkins JE Jr. 2016. On the widespread capacity for, and functional significance of, extreme inbreeding in ferns. *New Phytologist* 211: 1108–1119.

Soltis PS, Soltis DE. 2000. The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences, USA* 97: 7051–7057.

Testo WL, Watkins JE, Barrington DS. 2015. Dynamics of asymmetrical hybridization in North American wood ferns: reconciling patterns of inheritance with gametophyte reproductive biology. *New Phytologist* 206: 785–795.

Tryon AF. 1964. *Platyzoma*-A Queensland fern with incipient heterospory. *American Journal of Botany* 51: 939–942.

Tryon AF, Lugardon B. 1991. *Spores of the Pteridophyta*. New York, NY, USA: Springer.

Tryon RM, Vitale G. 1977. Evidence for antheridogen production and its mediation of a mating system in natural populations of fern gametophytes. *Botanical Journal of the Linnean Society* 74: 243–249.

Valledor L, Menéndez V, Canal MJ, Revilla A, Fernández H. 2014. Proteomic approaches to sexual development mediated by antheridiogen in the fern *Blechnum spicant* L. *Proteomics* 14: 2061–2071.

Vida G. 1976. The role of polyploidy in evolution. In: Novák VJA, Pacltová B, eds. *Evolutionary Biology*. Prague, Czechoslovakia: Czechoslovak Academy of Science, 267–294.

Voeller BR. 1964. Antheridiogens in ferns. In: *Regulateurs naturels de la croissance vegetale*. Paris, France: Centre National de la Recherche Scientifique, 665–684.

Weinberg ES, Voeller BR. 1969. External factors inducing germination of fern spores. *American Fern Journal* 59: 153–167.

Welling CB, Haufler CH. 1993. Antheridiogen and its puzzling effects on *Polypodium australe*. *American Journal of Botany* 8: 113.

Whittier DP. 1968. Rate of gametophyte maturation in sexual and apogamous forms of *Pellaea glabella*. *American Fern Journal* **58**: 12–19.

Willson MF. 1981. Sex expression in fern gametophytes: Some evolutionary possibilities. *Journal of Theoretical Biology* **93**: 403–409.

Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH. 2009. The frequency of polyploid speciation in vascular plants. *Proceedings of the National Academy of Sciences, USA* **106**: 13875–13879.

Wynne G, Mander LN, Goto N, Yamane H, Omori T. 1998. Gibberelin A117 methyl ester, a new antheridiogen from *Lygodium circinnatum*. *Phytochemistry* **49**: 1837–1840.

Yamane H. 1998. Fern antheridiogens. *International Review of Cytology* **184**: 1–32.

Yamane H, Nohara K, Takahashi N, Schraudolf H. 1987. Identification of antheridic acid as an antheridiogen in *Anemia rotundifolia* and *Anemia flexuosa*. *Plant and Cell Physiology* **28**: 1203–1207.

Yamane H, Satoh Y, Nohara K, Makayama M, Murofushi N, Takahashi N, Takeno K, Furuya M, Furber M, Mander LN. 1988. The methyl ester of a new gibberellin, GA73: The principal antheridiogen in *Lygodium japonicum*. *Tetrahedron Letters* **29**: 3959–3962.

Yamauchi T, Oyama N, Yamane H, Murofushi N, Schraudolf H, Pour M, Furber M, Mander LN. 1996. Identification of antheridiogen in *Lygodium circinatum* and *Lygodium flexuosum*. *Plant Physiology* **111**: 741–745.

Yatskievych G. 1993. Antheridiogen response in *Phanerophlebia* and related fern genera. *American Fern Journal* **83**: 30–36.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Tables S1 List of taxa cultivated in this study.

Table S2 List of literature used to compile interactions dataset.

Table S3 List of antheridiogen interactions between fern taxa (meta-analysis + cultivation results).

Table S4 List of all taxa (meta-analysis + cultivation results) determined as responsive or not to antheridiogens with additional information for each taxon.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.

About *New Phytologist*

- *New Phytologist* is an electronic (online-only) journal owned by the New Phytologist Foundation, a **not-for-profit organization** dedicated to the promotion of plant science, facilitating projects from symposia to free access for our Tansley reviews and Tansley insights.

- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication 'as ready' via *Early View* – our average time to decision is <26 days. There are **no page or colour charges** and a PDF version will be provided for each article.

- The journal is available online at Wiley Online Library. Visit **www.newphytologist.com** to search the articles and register for table of contents email alerts.

- If you have any questions, do get in touch with Central Office (np-centraloffice@lancaster.ac.uk) or, if it is more convenient, our USA Office (np-usaoffice@lancaster.ac.uk)

- For submission instructions, subscription and all the latest information visit **www.newphytologist.com**

Paper 6

Hornych O., Černochová L., Košnar J. & Ekrt L. (2022): Biotic interactions between the gametophytes of wall rue (*Asplenium ruta-muraria*) and other fern species. – International Journal of Plant Sciences 183(1): 10–17.

BIOTIC INTERACTIONS BETWEEN THE GAMETOPHYTES OF WALL RUE (*ASPLENIUM RUTA-MURARIA*) AND OTHER FERN SPECIES

O. Hornych,[1],* L. Černochová,* J. Košnar,* and L. Ekrt*

*Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, České Budějovice, CZ 37005, Czech Republic

Editor: Jennifer J. Weber

Premise of research. The gametophytes of ferns are nutritionally independent of the sporophytes and are potentially hermaphroditic. The sexual expression of fern gametophytes is based on environmental cues. To prevent excessive self-fertilization, fern gametophytes employ strategies to increase mating between gametophytes. One of these strategies relies on antheridiogens, pheromones released by older gametophytes and absorbed by younger gametophytes. There are multiple distinct antheridiogen types, some of which are poorly understood and in need of further examination. A still-unresolved antheridiogen type was described in *Asplenium ruta-muraria*.

Methodology. We employed cultivation experiments using spores of 12 fern species to assess the extent and uniqueness of the antheridiogen released by *A. ruta-muraria*. We tested antheridiogen interactions between representatives of three well-established antheridiogen types and *A. ruta-muraria* to assess their uniqueness. Furthermore, the effect of potentially antheridiogen-releasing gametophytes of *A. ruta-muraria* on multiple *Asplenium* species was examined. Germination in darkness in response to antheridiogens was also tested.

Pivotal results. The younger gametophytes of *A. ruta-muraria* did not respond to the presence of older conspecific gametophytes in a way that could be attributed to antheridiogens. No antheridiogen interactions between *A. ruta-muraria* and any other species were observed. Nevertheless, the exudates of older *A. ruta-muraria* gametophytes may affect the development of younger conspecific and interspecific gametophytes.

Conclusions. On the basis of its interaction with representatives of known antheridiogen types and the lack of germination in darkness, we conclude that our sample of *A. ruta-muraria* does not use antheridiogens. This discrepancy between our experiment and the initial publication describing antheridiogens in *A. ruta-muraria* may have been caused by intraspecific genetic variability within the species. The studied individual of *A. ruta-muraria* may be able to affect the growth of other gametophytes by other means, possibly via allelopathy, although this aspect of gametophyte interaction is poorly understood.

Keywords: allelopathy, antheridiogen, archegonia, Aspleniaceae, cultivation, facilitation.

Online enhancements: supplemental tables.

Introduction

Ferns, along with lycophytes, are unique among the extant land plants in having both life stages, gametophytic and sporophytic, independently of each other (Haufler et al. 2016; Pinson et al. 2017). However, the life stages are quite different from each other. Fern sporophytes are larger, while gametophytes are smaller and anatomically simple. The sexual expression of fern gametophytes is based on environmental cues such as nutrients and the presence of other gametophytes (Korpelainen

1998; DeSoto et al. 2008). As most ferns are also homosporous, each gametophyte can form both male and female gametangia (Raghavan 1989). Both types of gametangia can be present on a single hermaphroditic individual. A hermaphrodite is capable of self-fertilization and gametophytic selfing, enabling the formation of a sporophyte from a single spore. However, such a sporophyte is completely homozygous (Klekowski and Lloyd 1968; Haufler et al. 2016).

Because of this negative aspect of gametophytic selfing, fern gametophytes employ strategies to promote the opposite type of mating, outcrossing, when convenient. This dual nature of fern mating allows isolated gametophytes (i.e., by long-distance dispersal) to still reproduce by selfing, while gametophytes in populations benefit from the genetic potential of their surroundings

[1] Author for correspondence; email: hornych.ondrej@gmail.com.

Correction: This article was reposted on December 16, 2021, to replace a version that originally appeared online October 6, 2021, and contained an incorrect image for figure 1. This version of the article contains the figure that was intended for final publication.

(Wubs et al. 2010; Peredo et al. 2013; Sessa et al. 2016). These strategies seem to be effective, as most population genetic studies of ferns indicate high rates of gametophytic outcrossing among ferns (Soltis and Soltis 1992; Pelosi and Sessa 2021). The simplest strategy facilitating outcrossing is sequential sexual expression. Several different types of sexual ontogenies, the most common being the male-into-hermaphrodite sequence, were described by Klekowski (1969). In the simplest terms, fern gametophytes generally become unisexual first and hermaphroditic later, with notable exceptions (Banks 1997).

Another prominent strategy promoting outcrossing relies on the use of antheridiogens (Hornych et al. 2021). Antheridiogens are pheromones released to the environment by gametophytes with a lateral meristem (usually female or hermaphroditic; Näf et al. 1975; Raghavan 1989). The chemical message is absorbed by premeristic asexual gametophytes, which respond with precocious and abundant formation of antheridia (male gametangia; Döpp 1950; Schneller 2008) at the expense of archegonia (female gametangia). Antheridiogens increase the prevalence of unisexual gametophytes and boost the amount of available sperm. Both effects lead to a higher incidence of outcrossing. The antheridiogen system allows fern gametophytes to accurately identify the presence of potential mates. The system is potent enough to substantially affect outcrossing rates (Ranker 1987; Schneller et al. 1990) but remains flexible by allowing males to turn into hermaphrodites when the females are removed from the population (Cheruiyot and Schwartz 2007; Ganger et al. 2015, forthcoming). Because of their effectiveness, most fern species likely use antheridiogens (Hornych et al. 2021).

Apart from the precocious formation of antheridia, antheridiogens also enable spores to germinate in darkness, bypassing the usual requirement of light (Weinberg and Voeller 1969; Haufler and Welling 1994). Such germinated spores grow into small belowground gametophytes with a few antheridia (Schneller et al. 1990). The sperm released from these antheridia then navigate toward open archegonia (Schneller 1998; Lopez-Smith and Renzaglia 2008) and may fertilize the antheridiogen-releasing gametophytes aboveground. Thus, antheridiogens effectively allow ferns to mine the otherwise dormant spore bank for genetic diversity (Schneller 1988).

Antheridiogens are not species specific and can be effective even between members of two different families; nevertheless, there are several types of antheridiogens (Näf et al. 1975; Schneller et al. 1990; Schneller 2008). Each type of antheridiogen may be viewed as a group of chemical entities produced by and affecting a specific group of species. According to the definition of Hornych et al. (2021), antheridiogens produced by any species do not have to be able to affect all the species using the same antheridiogen type, but no species may respond to two types of antheridiogens; otherwise, the types are merged. On the basis of this definition, three or four types are well supported, and many additional types may exist but need further evidence (Hornych et al. 2021).

One of these still-unresolved antheridiogen types is reported in *Asplenium ruta-muraria* (wall rue; Schneller 1995) from the family Aspleniaceae. This small calcicole fern species inhabits the crevices of walls and rocks. Schneller (1995) initially described antheridiogens in *A. ruta-muraria* as unique on the basis of the species' lack of response to gibberellins, which are similar to Schizaeales-type antheridiogens (AGSc, antheridiogen B) in

chemistry and effect. Indeed, all chemically described antheridiogens are structurally related to gibberellins (Yamane 1998). Eventually, a lack of response to *Pteridium aquilinum*, the model species of the Polypodiales type (AGPo, antheridiogen A), was mentioned in his review of antheridiogens (Schneller 2008). We know very little about the chemistry and structure of the most common Polypodiales-type antheridiogen (Döpp 1950; Näf et al. 1975; Fernandez et al. 1999). The *Asplenium* type possibly could have evolved from the Polypodiales-type antheridiogen. A similar approach to Schneller's was taken by Schedlbauer (1974), who supported the uniqueness of the *Ceratopteris*-type antheridiogen (AGCe, antheridiogen C) by cultivating *Ceratopteris thalictroides* alongside representatives of the other two types. While the features of *Ceratopteris* antheridiogens were studied thoroughly (e.g., Banks 1997; Ganger and Sturey 2012; Atallah et al. 2018), to our knowledge, *A. ruta-muraria* was not studied any further and is in need of confirmation.

The aim of this study was to assess the potentially unique antheridiogen system of *A. ruta-muraria*. Like Schedlbauer (1974), we cultivated the spores of multiple fern species, including representatives of three established antheridiogen types and various related and unrelated members of Aspleniaceae. Specifically, the effect of older meristic gametophytes on the sexual expression of younger gametophytes was observed. This study attempted to answer the following questions: (1) Does *A. ruta-muraria* use an antheridiogen system? (2) If so, is the antheridiogen of *A. ruta-muraria* different from other common antheridiogen types? (3) Do exudates of *A. ruta-muraria* affect gametophytes in a way not related to antheridiogens?

Material and Methods

Fertile fronds of 10 fern taxa, including *Asplenium ruta-muraria*, were obtained from personal herbaria and deposited in the CBFS herbarium (table 1). Additionally, spores of two taxa were obtained from the Spore Exchange of the American Fern Society (*Anemia phyllitidis*) and the Carolina Biological Supply Company (*Ceratopteris richardii*).

Before the experiment, the viability of five 1% agar medium types for cultivating *A. ruta-muraria* was assessed. Among them, Murashige and Skoog (MS) medium (Murashige and Skoog 1962) at a 25% concentration, MS medium at a 10% concentration, and Mohr's medium (Mohr 1956) were suitable. Dyer's medium (Mohr medium with antimycotics added; Dyer 1979) and full-strength MS medium induced slower and irregular growth. The 25% MS was eventually chosen as the most appropriate for all other experiments. Spores were sown on 12 well plates, allowing for four species combinations in three replications (triplets) in each well plate. Spores were sown either on their own (control) or in the presence of an archegoniate gametophyte (treatment) that was transplanted into the well 1 wk before spore sowing (fig. 1). Generally, one triplet was used for each treatment, and each treatment triplet was associated with its own control triplet in the same well plate to account for potential differences among well plates. Because of the focus on *A. ruta-muraria*, this species was cultivated in two additional triplets (two control, two treatment in total) in a single well plate. Cultivated gametophytes were kept in a growth chamber (MLR-352 Climatic Test Chamber, PHC Europe) under a 12 h:12 h light-to-dark regimen at 20°C.

Table 1
Voucher Information for the Samples Used in This Study

Species	Locality/source	GPS coordinates (lat., long.; WGS 84)	Collection date	Collector
Anemia phyllitidis	Spore Exchange of the American Fern Society		September 2019	B. Aikins
Asplenium adiantum-nigrum	ESP; Deba, along a forest path in a forest ca 2.1 km east-northeast of the church in the town of Deba	43°17′45.0″N, 002°19′38.0″W	June 23, 2019	L. Ekrt
Asplenium fontanum	CZE; Hrádek u Znojma, wall surrounding the church at the southwest edge of town	48°46′18.0″N, 016°16′02.0″E	September 22, 2019	L. Ekrt
Asplenium onopteris	GRC; Glossa, island of Skopelos, slope above road ca 1.5 km east-northeast of the town center	39°10′45.1″N, 023°38′07.1″E	August 16, 2019	K. Vejvodová
Asplenium ruta-muraria	CZE; Kutná Hora, wall in town	49°56′45.3″N, 015°15′22.3″E	August 3, 2020	O. Hornych
Asplenium septentrionale	CZE; Kutná Hora, rocky outcrop in Vrchlice Valley	49°56′09.9″N, 015°15′41.7″E	August 1, 2020	O. Hornych
Asplenium trichomanes subsp. *trichomanes*	CZE; Dolní Třebonín, rocky outcrop in Vltava Valley	48°52′25.7″N, 014°21′53.8″E	July 29, 2020	O. Hornych; L. Černochová
Athyrium filix-femina	CZE; Hlavňov, near the cave "Kovárna"; in cultivation in Telč, Czechia, spores collected in 2019	50°33′45.3″N, 016°16′14.1″E	2010	L. Ekrt
Ceratopteris richardii	Carolina Biological Supply Company		Ordered May 2020	
Dryopteris filix-mas	CZE; Stožec, ca. 750 m east of the Stožec peak; in cultivation in Telč, Czechia, spores collected in 2019	48°52′N, 013°49′E	2005	L. Ekrt
Onoclea sensibilis	CZE; Hejnice, Ferdinandov, ca. 570 m southwest of the crossroad "Ferdinandov" by the arboretum	50°52′09.1″N, 015°09′57.2″E	July 5, 2020	K. Vejvodová
Pteridium aquilinum	CZE; Tři Studně, the southeast edge of the Sykovec pond	49°36′24.5″N, 016°02′09.4″E	September 2020	O. Hornych

Note. All herbarium vouchers were deposited in the CBFS herbarium in České Budějovice, Czech Republic. ESP = Spain; CZE = Czech Republic; GRC = Greece.
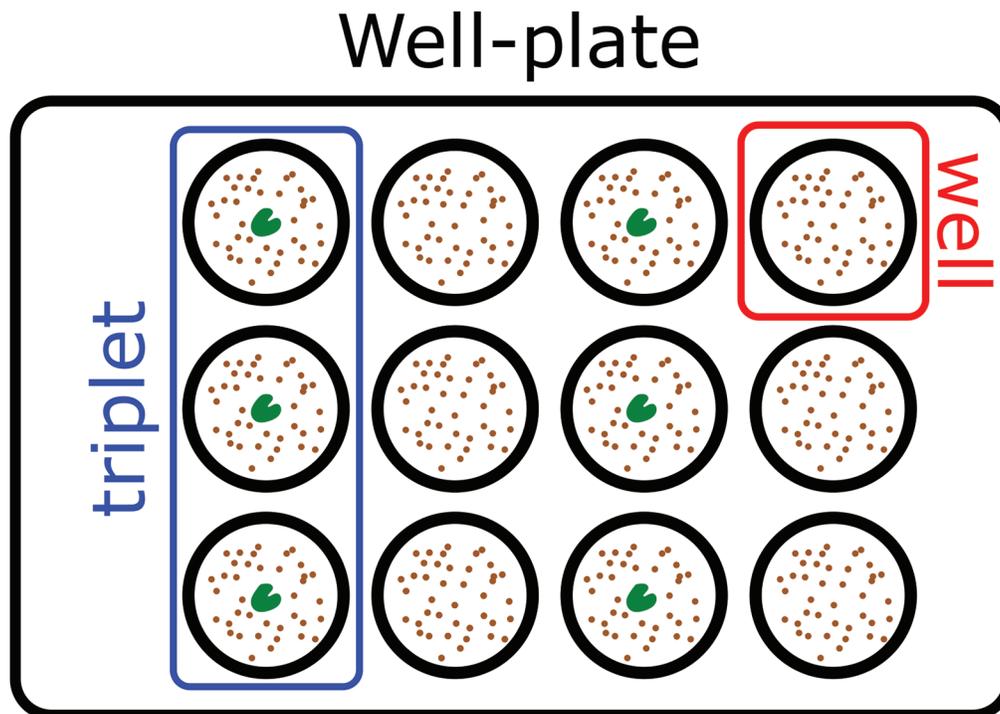


Fig. 1 Overview of the experimental setup used in this study. The spores of each species were cultivated in at least one affected triplet (three wells with an older gametophyte present in each) and one control triplet (no older gametophyte).

Three experiments were performed (1) to test the relation of the *A. ruta-muraria* antheridiogen to other known antheridiogen types, (2) to test whether spores of *A. ruta-muraria* germinate in darkness in the presence of conspecific gametophytes, and (3) to test the possible influence of the *A. ruta-muraria* antheridiogen on various Aspleniaceae and other Polypodiales. All species cultivated in the presence of adult gametophytes were also sown without any influence in separate wells for control purposes (fig. 1).

In experiment 1 (summarized in table 2), *A. ruta-muraria* spores were cultivated in the presence of three antheridiogen types, AGCe, AGPo, and AGSc. We released antheridiogens by adding mature gametophytes of the model species, *C. richardii*, *Pteridium aquilinum*, and *A. phyllitidis*. As a control, spores of the model species were sown in the presence of their antheridiogen type or without any influence. *Onoclea sensibilis* was used as the responding species of the AGPo type instead of *P. aquilinum*, as is standard practice; for examples, see Chiou and Farrar (1997) or Testo et al. (2015). Additionally, spores of *C. richardii* and *O. sensibilis* were cultivated in the presence of an *A. ruta-muraria* gametophyte.

In experiment 2, spores of *A. ruta-muraria* were cultivated under either no or a conspecific gametophytic influence in complete darkness; the well plate was covered in aluminum foil and kept in a closed box.

In experiment 3, spores of eight species (*Asplenium adiantum-nigrum*, *Asplenium fontanum*, *Asplenium onopteris*, *A. ruta-muraria*, *Asplenium septentrionale*, *Asplenium trichomanes* subsp. *trichomanes*, *Athyrium filix-femina*, and *Dryopteris filix-mas*) were grown in the presence of a mature *A. ruta-muraria* gametophyte and in control wells (no gametophytic influence).

Well plates were checked for gametophyte development biweekly from week 4 on. We extracted 5–10 gametophytes from each well and stained them with acetocarmine for 30 min. Acetocarmine stains chromosomes red and makes antheridia prominent and easily distinguishable. Each gametophyte was scored as asexual, male, female, or hermaphroditic under a light microscope (Olympus CX31). The observation period generally ended once female gametangia were found in two consecutive biweekly assessments of a triplet pair (treatment and control) within a well plate or when the well became depleted, which may distort further results. Sometimes we extended the observation period to observe long-term trends. In experiment 2, the well plate was uncovered after 1 and 2 mo, and 50 spores were checked for rhizoid emergence as a sign of germination each time. After one additional month under the standard light-dark regimen, 50 spores were checked again as a positive control.

To compare the results, we used two metrics. The first metric, first confirmed females, was defined as the week in which female gametangia appeared within a triplet (not necessarily in all wells)

for the first time in two consecutive biweekly assessments. The second metric, male ratio, was defined as the ratio of gametophytes bearing antheridia (including hermaphrodites) to the sum of gametophytes bearing antheridia and gametophytes bearing archegonia within a triplet. Each hermaphrodite gametophyte was counted twice in the denominator. For both metrics, if only one gametophyte was found for either gametangium type per triplet, it was ignored to account for possible contamination. Both metrics were compared between treatment spores and control spores. The results were mapped onto a phylogenetic tree based on those of PPG I (2016; family level) and Xu et al. (2020; Aspleniaceae).

Results

All three cultivation experiments were successful, although three triplets (*Pteridium aquilinum* affecting *Asplenium ruta-muraria*, its control triplet, and one of the two triplets of *A. ruta-muraria* affecting conspecific spores) developed very poorly and were excluded. Asexual, male, female, and hermaphroditic gametophytes were observed, although hermaphrodites other than those of *Ceratopteris richardii* were rare. Differences between well plates were observed, so comparisons between treatment and control triplets were made only within well plates whenever possible. The results are summarized in tables A1 and A2 (available online).

Experiment 1

In experiment 1, the effect of exudates released by *Asplenium ruta-muraria* was compared with that of three known antheridiogen types. Within the most common antheridiogen type, AGPo, *Pteridium aquilinum* markedly affected younger gametophytes of *Onoclea sensibilis*; by week 6, all treatment gametophytes were male, while the control was all female. The results of *P. aquilinum* affecting *A. ruta-muraria* were excluded, but *A. ruta-muraria* failed to affect *O. sensibilis*; both treatment and control triplets were mostly female.

The antheridiogen released by *Anemia phyllitidis*, AGSc, affected conspecific gametophytes. Although the first confirmed females appeared 2 wk earlier in the treatment triplet (week 10 compared with week 12), the treatment triplet had a considerably higher male ratio during weeks 8 (0.625 compared with asexual), 10 (0.84 compared with 0.15), and 12 (0.66 compared with 0.4). The male ratio evened out by week 14 (0.81 compared with 0.86) as a result of the depletion of the control triplet, distorting the results. The gametophytes of *A. ruta-muraria* were unaffected by AGSc. The first confirmed females appeared by week 16 regardless of treatment.

Table 2

Overview of Experiment 1, Comparing *Asplenium ruta-muraria* Antheridiogen with Other Known Antheridiogen Types: Schizaeales Type (AGSc; *Anemia phyllitidis*), *Ceratopteris* Type (AGCe; *Ceratopteris richardii*), Polypodiales Type (AGPo; *Pteridium aquilinum*, Producing Species; *Onoclea sensibilis*, Responsive Species)

Species sown	A. ruta-muraria	A. phyllitidis	C. richardii	P. aquilinum
A. ruta-muraria	X	X	X	X
A. phyllitidis		X		
C. richardii	X		X	
O. sensibilis	X			X

Note.   Cultivated combinations are denoted by an X.

Gametophytes of *Ceratopteris richardii* were affected by AGCe early on; the overall number of males was higher in the treatment triplet compared with the control in weeks 4 (average male number of 0.67 compared with 0.2) and 6 (average male number of 0.4 compared with 0). Both the treatment and the control were almost completely all hermaphroditic from week 8, possibly because of a detachment of releasing gametophytes from the medium. Interestingly, in *A. ruta-muraria* affected by AGCe, the first confirmed females appeared 2 wk earlier (week 12 compared with week 14); no other effects were observed. Gametophytes of *C. richardii* responded slightly erratically to *A. ruta-muraria*, forming more males by week 4 but more females by weeks 6 and 8 compared with the mostly hermaphroditic control.

The conspecific influence of *A. ruta-muraria* did not match any of the three observed antheridiogen patterns (fig. 2). The gametophytes of *A. ruta-muraria* generally start as male, and eventually females and hermaphrodites appear. Although one treatment triplet was excluded, the results overall showed a slowdown in development by about 5 wk. The treatment triplet included very few male gametophytes during weeks 4 to 8 (average of ca. 12% male over the course of three observation periods). The first confirmed females appeared in the treatment triplet by week 18, compared with weeks 12 and 14 in the two control triplets. Once the shift was accounted for, the develop-

mental patterns of the treatment were similar to those of the control, although the effect seemed stronger early on (eight extra weeks needed to be mostly male), but treatment gametophytes caught up sooner (five extra weeks needed for the first confirmed females).

Experiment 2

Experiment 2 clearly demonstrated that exudates of *Asplenium ruta-muraria* did not stimulate germination in conspecific spores. No germination was found in either the treatment or the control triplets by months 1 and 2. After one additional month in light, all observed spores germinated.

Experiment 3

In experiment 3, the effect of exudates released by *Asplenium ruta-muraria* on various Polypodiales species was assessed (fig. 3). The exudates of *A. ruta-muraria* accelerated the change from males to females in *Asplenium adiantum-nigrum*. The first confirmed females appeared by week 10 in the treatment triplet, compared with week 14, when they likely would have been found in the control (observations were terminated by week 12). Overall, the developmental pattern was similar for the treatment and the
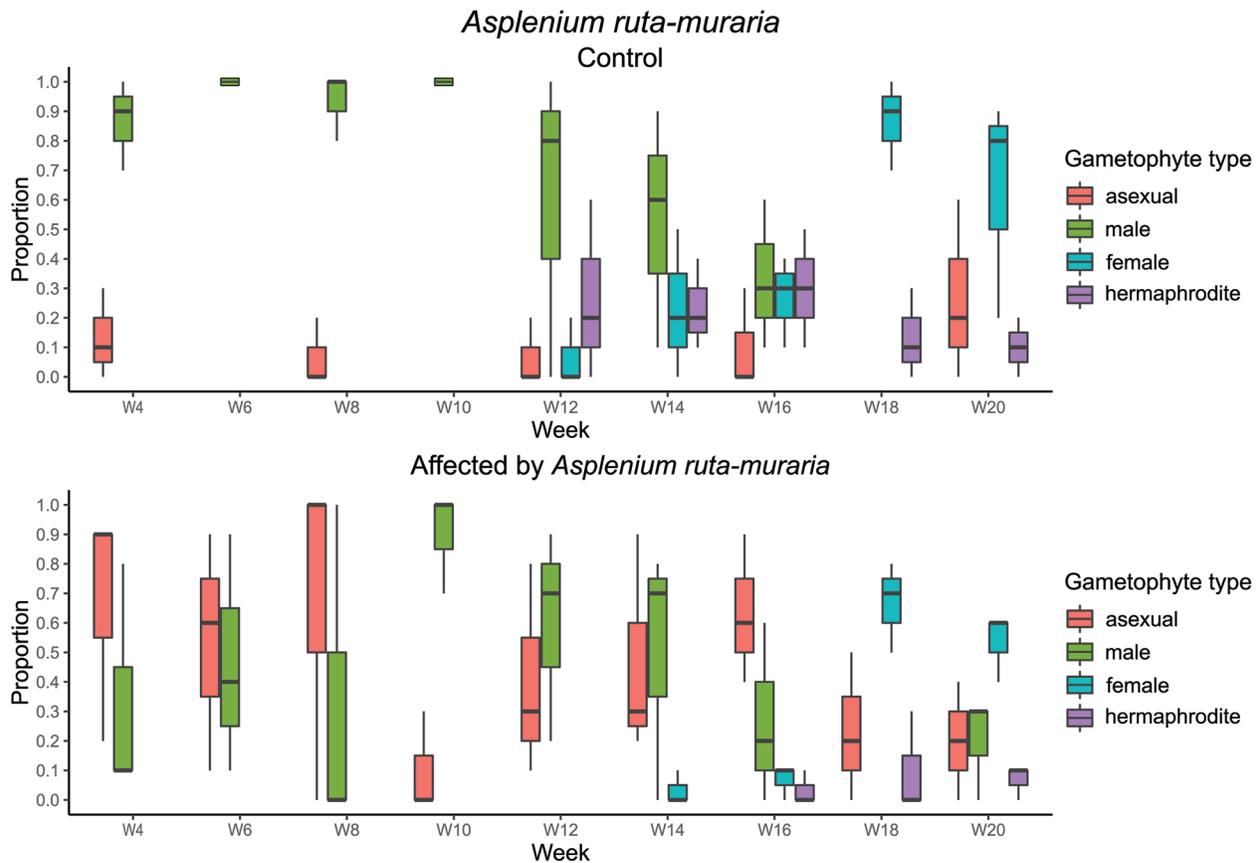
Fig. 2 Sexual expression pattern of *Asplenium ruta-muraria* gametophytes in a control environment (no added gametophyte) or under the influence of a female conspecific gametophyte over the course of 20 wk (measured biweekly). The gametophytes affected by a female *A. ruta-muraria* developed archegonia (female gametangia) later.
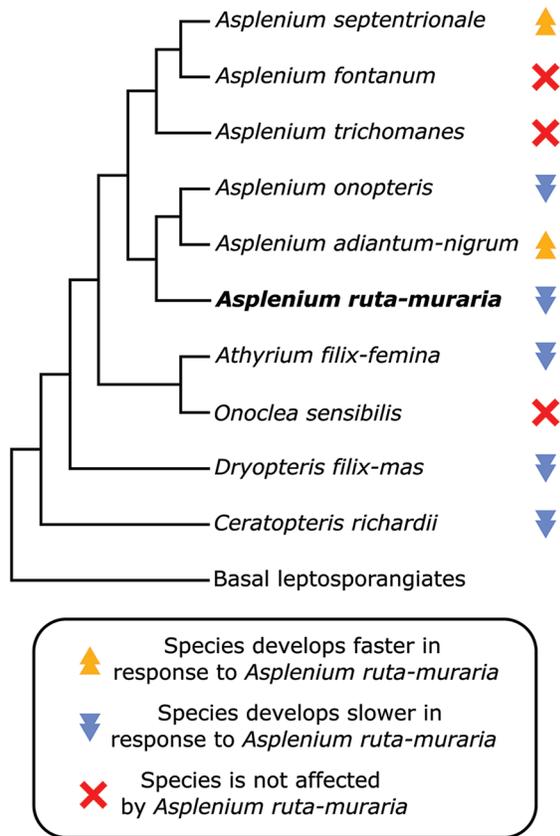
Fig. 3 Phylogenetic tree (based on PPG I 2016; Xu et al. 2020) of the tested fern taxa and their response to exudates of older gametophytes of *Asplenium ruta-muraria*. The speed of development is based on the slower or faster emergence of archegonia (female gametangia) compared with the control (no older gametophytes present).

control once the 4-wk shift was accounted for. In *Asplenium fontanum*, the first confirmed females appeared by week 8 in both the treatment and the control triplets. Males were found only rarely in the treatment triplet, but this species grew poorly, and the results may not be fully representative. Gametophytes of *Asplenium onopteris* started as males, but the treatment triplet developed females 2 wk later than the control (likely week 12 compared with week 10; observations were terminated by week 10). Interestingly, the control triplet of *Asplenium septentrionale* developed only males in large quantities during 14 wk of observation. The first confirmed females appeared in the treatment triplet by week 14. It is possible that the development of *A. septentrionale* was accelerated, as it was for *A. adiantum-nigrum*. The development of *Asplenium trichomanes* was not markedly affected in the treatment triplet. The first confirmed females appeared by week 8 in both triplets.

Two additional species outside Aspleniaceae were tested. The exudates of *A. ruta-muraria* slowed down the development of *Athyrium filix-femina* by 2 wk; the first confirmed females appeared by week 8 in the treatment triplet and by week 6 in the control triplet. Similarly, the first females appeared in the *Dryopteris filix-mas* treatment triplet during week 8, compared with week 6 in the control.

Discussion

This study has attempted to confirm and further describe the antheridiogen system of *Asplenium ruta-muraria* described by Schneller (1995). The original author viewed antheridiogens as advantageous for this epilithic plant, as rocks and walls are spatially complex niches and an increased number of sperm may be necessary for fertilization in those environments. Schneller (1995) further described the antheridiogens of *A. ruta-muraria* as distinct from other types of antheridiogens, specifically AGSc, represented by gibberellins, and AGPo, represented by *Pteridium aquilinum* (Schneller 2008; mentioned in the text).

However, it is clear from experiments 1 and 2 that our specimen of *A. ruta-muraria* produces neither a unique antheridiogen system nor one like any of the three antheridiogens tested. *Asplenium ruta-muraria* did not respond to conspecific exudates with precocious and more abundant formation of antheridia or with spore germination in darkness. Furthermore, some young gametophytes eventually developed into females despite the presence of a mature potentially antheridiogen-releasing female. The only observable effect was the slower onset of archegonia production. Nevertheless, Schneller (1995) presented compelling evidence of an antheridiogen system functioning in his specimen of *A. ruta-muraria*. We present a possible explanation for the incongruity of our and Schneller's results. It is possible that there is intraspecific variation in antheridiogen use. Such variation has been observed in *Hemionitis palmata* by Ranker (1987). In that study, two populations were observed. Both representatives of population J responded to antheridiogens, while only one of three members of population Oax did (Ranker 1987; Schneller et al. 1990). The difference in antheridiogen sensitivity was correlated with the levels of genetic load, indicating that the results of these cultivation experiments were accurate. Similarly, Chiou and Farrar (1997) observed precocious antheridial formation in response to *P. aquilinum* antheridiogens in *Phlebodium aureum* but no germination in darkness after the same antheridiogen exposure in this species, despite these two effects being highly correlated (Hornych et al. 2021). These results would indicate that different threshold levels of antheridiogens are necessary for either antheridial formation or germination in darkness, but ultimately, *P. aureum* responds to *Pteridium* antheridiogens. However, previous studies described *P. aureum* as unresponsive to *Pteridium* (Näf 1956; Voeller 1964). When the results for *A. ruta-muraria*, *H. palmata*, and *P. aureum* are considered, it is possible that antheridiogen sensitivity varies among lineages of the same species or that the sensitivity may be so low that slightly different cultivation conditions could significantly affect the outcome. Nevertheless, it is evident that fern species should be more thoroughly studied using individuals from separate populations before we assume that all members of that species use antheridiogens. It is possible that some lineages may lose antheridiogen sensitivity or production capacity. For *A. ruta-muraria*, the loss of antheridiogens may not have consequences significant enough to eliminate antheridiogen-insensitive individuals. As explored by Hornych et al. (2021), the loss of antheridiogens may even be beneficial for this tetraploid species, for example, because of potentially faster sporophyte formation by selfing with little genetic cost due to tetraploidy.

The patterns of gametophyte development observed in our study differ somewhat from those of other published reports.

Asplenium ruta-muraria populations are believed to develop hermaphrodites first and unisexual gametophytes later (Pangua et al. 1994; Schneller 1995). In contrast, our studied *A. ruta-muraria* formed males first, and archegonia emerged later. Hermaphrodites played a relatively minor role but were more abundant than in any other *Asplenium* cultivated in this study. Such a male-first strategy was observed by Schneller (1995) among gametophytes affected by antheridiogens; however, in that case no females developed. In *Asplenium fontanum*, both males and females are described as appearing simultaneously, with the females eventually becoming hermaphroditic (Herrero et al. 2002). During our cultivation, *A. fontanum* was mostly female, but the observations were terminated prematurely because of poor growth. The development of *Asplenium trichomanes* subsp. *quadrivalens* (tetraploid) observed by Pangua et al. (1994) follows the female-to-hermaphrodite pattern, with a limited number of males appearing later on. In our study, *A. trichomanes* started as male, but archegonia-bearing gametophytes quickly outnumbered the males. However, our studied specimen was the nominal diploid variant *A. trichomanes* subsp. *trichomanes*, which may have affected gametophyte development. Overall, when we consider the published reports of *Asplenium* (Pangua et al. 1994; Herrero et al. 2002; Regalado Gabancho et al. 2010), our studied plants formed more males and fewer hermaphrodites than may have been expected. Culture conditions, for example, the nutrient medium, may have caused these discrepancies, but we were unable to see any major differences in early gametophyte development between the MS 25% (used in our study) and Mohr's medium (used by Schneller 1995). In turn, Mohr's medium has the same concentration of nutrients as Dyer's medium (used by Pangua et al. 1994) and differs only in the absence of antimycotics. Therefore, we cannot exclude the possibility of nutrient conditions affecting our results, but we do not assume that that was the case. Another possible explanation for our results may be the differing densities of gametophytes. Overall, spores sown at higher densities reduce the incidence of female and hermaphroditic gametophytes (DeSoto et al. 2008). In extremes, only males and asexual gametophytes may be found (Huang et al. 2004). Our cultivations may have been sown at a marginally higher density, leading to more males and fewer hermaphrodites. Nevertheless, the sowing density was appropriately low enough to allow for the formation of both gametangia types. However, because of the binary nature of the antheridiogen response (germination in darkness, lack of archegonia), we do not believe that this density effect could invalidate our conclusions about the lack of antheridiogens in the studied sample of *A. ruta-muraria*.

The exudates of *A. ruta-muraria* suppressed or facilitated the gametangial development of most of the Polypodiales species (fig. 3). However, as neither of the two effects were particularly pronounced, it is possible that the results may have been the outcome of random chance. Nevertheless, there are biological explanations for both effects. Allelopathy, the chemical suppression of other individuals (Cheng and Cheng 2015), is known among ferns and was observed among gametophytes (Petersen and Fairbrothers 1980; Testo et al. 2014) or between a sporophyte and a gametophyte (Wagner and Long 1991). The suppression of growth by older gametophytes may possibly be caused by allelopathic compounds released into the medium. Facilitation has not yet been observed among fern gametophytes and ferns in general. Nevertheless, there are various described ways by which one angiosperm species may cause faster growth of another species (Beckage and Gross 2006; Turnbull et al. 2013; Wright et al. 2017), some of which (i.e., pathogen resistance, abiotic environment change) may be applicable to ferns. The concepts of allelopathy and facilitation have barely been explored among fern gametophytes; however, our study indicates that these effects may play an underappreciated role in fern reproduction and should be examined in future research.

Conclusions

We were unable to confirm the presence of a unique antheridiogen system in *Asplenium ruta-muraria*. Our tested individual of this species decidedly did not employ any system resembling antheridiogens as observed in Schizaeales and Polypodiales. The exudates of female gametophytes did not preclude the formation of archegonia among younger gametophytes or stimulate germination in darkness. Further tests of additional samples from different geographical regions are needed. Nevertheless, the exudates of *A. ruta-muraria* may have suppressive or facilitative effects on younger gametophytes of various fern species. The mechanics and properties of these effects have been poorly explored, may have evolutionary significance, and deserve further study in ferns.

Acknowledgments

Literature Cited

Atallah NM, O Vitek, F Gaiti, M Tanurdzic, JA Banks 2018 Sex determination in *Ceratopteris richardii* is accompanied by transcriptome changes that drive epigenetic reprogramming of the young gametophyte. G3 8:2205–2214.

Banks JA 1997 Sex determination in the fern *Ceratopteris*. Trends Plant Sci 2:175–180.

Beckage B, LJ Gross 2006 Overyielding and species diversity: what should we expect? New Phytol 172:140–148.

Cheng F, Z Cheng 2015 Research progress on the use of plant allelopathy in agriculture and the physiological and ecological mechanisms of allelopathy. Front Plant Sci 6:1020.

Cheruiyot DJ, BW Schwartz 2007 Conversion of male gametophytes to hermaphrodites in the fern *Ceratopteris richardii*. Bios 78:58–61.

Chiou W-L, DR Farrar 1997 Antheridiogen production and response in Polypodiaceae species. Am J Bot 84:633–640.

DeSoto L, LG Quintanilla, M Méndez 2008 Environmental sex determination in ferns: effects of nutrient availability and individual density in *Woodwardia radicans*. J Ecol 96:1319–1327.

Döpp W 1950 Eine die antheridienbildung bei farnen fördernde substanz in den Prothallien von *Pteridium aquilinum* (L.) Kuhn. Ber Dtsch Bot Ges 63:139–147.

Dyer AF 1979 The culture of fern gametophytes for experimental investigation. Pages 253–305 *in* AF Dyer, ed. The experimental biology of ferns. Academic Press, London.

Fernandez H, AM Bertrand, MI Sierra, R Sanchez-Tames 1999 An apolar GA-like compound responsible for the antheridiogen activity in *Blechnum spicant*. Plant Growth Regul 28:143–433.

Ganger MT, JA Girouard, HM Smith, BA Bahny, SJ Ewing 2015 Antheridiogen and abscisic acid affect conversion and *ANI1* expression in *Ceratopteris richardii* gametophytes. Botany 93:109–116.

Ganger MT, T Sturey 2012 Antheridiogen concentration and spore size predict gametophyte size in *Ceratopteris richardii*. Botany 90:175–179.

Ganger MT, S Wilczynski, M Bronder Forthcoming Male to hermaphrodite sex change in gametophytes of the fern *Ceratopteris richardii* is affected by hermaphrodite density, male age, and male size. Botany.

Haufler CH, KM Pryer, E Schuettpelz, EB Sessa, DR Farrar, R Moran, JJ Schneller, JE Watkins, MD Windham 2016 Sex and the single gametophyte: revising the homosporous vascular plant life cycle in light of contemporary research. BioScience 66:928–937.

Haufler CH, CB Welling 1994 Antheridiogen, dark spore germination, and outcrossing mechanisms in *Bommeria* (Adiantaceae). Am J Bot 81:616–621.

Herrero A, C Prada, S Pajarón 2002 Gametophyte morphology and gametangial ontogeny of *Asplenium foreziense* and related taxa (Aspleniaceae: Pteridophyta). Bot J Linn Soc 139:87–98.

Hornych O, WL Testo, EB Sessa, JE Watkins, CE Campany, J Pittermann, L Ekrt 2021 Insights into the evolutionary history and widespread occurrence of antheridiogen systems in ferns. New Phytol 229:607–619.

Huang Y-M, H-M Chiou, W-L Chiou 2004 Density affects gametophyte growth and sexual expression of *Osmunda cinnamomea* (Osmundaceae: Pteridophyta). Ann Bot 94:229–232.

Klekowski EJ 1969 Reproductive biology of the Pteridophyta. II. Theoretical considerations. Bot J Linn Soc 62:347–359.

Klekowski EJ, RM Lloyd 1968 Reproductive biology of the Pteridophyta. I. General considerations and a study of *Onoclea sensibilis* L. Bot J Linn Soc 60:315–324.

Korpelainen H 1998 Labile sex expression in plants. Biol Rev 73:157–180.

Lopez-Smith R, K Renzaglia 2008 Sperm cell architecture, insemination, and fertilization in the model fern, *Ceratopteris richardii*. Sex Plant Reprod 21:153–167.

Mohr H 1956 Die abhängigkeit des Protonemawachstums un der Protonemapolarität bei farnen vom Licht. Planta 47:127–158.

Murashige T, F Skoog 1962 A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497.

Näf U 1956 The demonstration of a factor concerned with the initiation of antheridia in polypodiaceous ferns. Growth 20:91–105.

Näf U, K Nakanishi, M Endo 1975 On the physiology and chemistry of fern antheridiogens. Bot Rev 41:315–359.

Pangua E, S Lindsay, A Dyer 1994 Spore germination and gametophyte development in three species of *Asplenium*. Ann Bot 73:587–593.

Pelosi JA, EB Sessa 2021 From genomes to populations: a meta-analysis and review of fern population genetics. Int J Plant Sci 182:325–343.

Peredo EL, M Méndez-Couz, MA Revilla, H Fernández 2013 Mating system in *Blechnum spicant* and *Dryopteris affinis* ssp. *affinis* correlates with genetic variability. Am J Bot 103:27–39.

Petersen RL, DE Fairbrothers 1980 Reciprocal allelopathy between the gametophytes of *Osmunda cinnamomea* and *Dryopteris intermedia*. Am Fern J 70:73–78.

Pinson JB, SM Chambers, JH Nitta, L-Y Kuo, EB Sessa 2017 The separation of generations: biology and biogeography of long-lived sporophyteless fern gametophytes. Int J Plant Sci 178:1–18.

PPG I (Pteridophyte Phylogeny Group I) 2016 A community-derived classification for extant lycophytes and ferns: PPG I. J Syst Evol 54:563–603.

Raghavan V 1989 Developmental biology of fern gametophytes. Cambridge University Press, Cambridge.

Ranker TA 1987 Experimental systematics and population biology of the fern genera *Hemionitis* and *Gymnopteris* with reference to *Bommeria*. PhD diss. University of Kansas, Lawrence.

Regalado Gabancho L, C Prada, JM Gabriel y Galán 2010 Sexuality and apogamy in the Cuban *Asplenium auritum-monodon* complex (Aspleniaceae). Plant Syst Evol 289:137–146.

Schedlbauer MD 1974 Biological specificity of the antheridogen. from *Ceratopteris thalictroides* (L.) Brongn. Planta 116:39–43.

Schneller JJ 1988 Spore bank, dark germination and gender determination in *Athyrium* and *Dryopteris*: results and implications for population biology of Pteridophyta. Bot Helv 98:77–86.

——— 1995 Antheridiogen system in the fern *Asplenium rutamuraria* (Aspleniaceae: Pteridophyta). Fern Gaz 15:64–70.

——— 1998 How much genetic variation in fern populations is stored in the spore banks? a study of *Athyrium filix-femina* (L.) Roth. Bot J Linn Soc 127:195–206.

——— 2008 Antheridiogens. Pages 134–158 *in* TA Ranker, CH Haufler, eds. Biology and evolution of ferns and lycophytes. Cambridge University Press, Cambridge.

Schneller JJ, CH Haufler, TA Ranker 1990 Antheridiogen and natural gametophyte populations. Am Fern J 80:143–152.

Sessa EB, WL Testo, JE Watkins 2016 On the widespread capacity for, and functional significance of, extreme inbreeding in ferns. New Phytol 211:1108–1119.

Soltis DE, PS Soltis 1992 The distribution of selfing rates in homosporous ferns. Am J Bot 79:97–100.

Testo WL, MS Grasso, DS Barrington 2014 Beyond antheridiogens: chemical competition between gametophytes of *Polypodium appalachianum* and *Polypodium virginianum*. J Torrey Bot Soc 141:302–312.

Testo WL, JE Watkins, DS Barrington 2015 Dynamics of asymmetrical hybridization in North American wood ferns: reconciling patterns of inheritance with gametophyte reproductive biology. New Phytol 206:785–795.

Turnbull LA, JM Levine, M Loreau, A Hector 2013 Coexistence, niches and biodiversity effects on ecosystem functioning. Ecol Lett 16:116–127.

Voeller BR 1964 Antheridiogens in ferns. Pages 665–684 *in* Centre National de la Recherche Scientifique, ed. Regulateurs naturels de la croissance vegetale. Centre National de la Recherche Scientifique, Paris.

Wagner HB, KE Long 1991 Allelopathic effects of *Osmunda cinnamomea* on three species of *Dryopteris*. Am Fern J 81:134–138.

Weinberg ES, BR Voeller 1969 External factors inducing germination of fern spores. Am Fern J 59:153–167.

Wright AJ, DA Wardle, R Callaway, A Gaxiola 2017 The overlooked role of facilitation in biodiversity experiments. Trends Ecol Evol 32:383–390.

Wubs ERJ, GA de Groot, HJ During, JC Vogel, M Grundmann, P Bremer, H Schneider 2010 Mixed mating system in the fern *Asplenium scolopendrium*: implications for colonization potential. Ann Bot 106:583–590.

Xu K, L Zhang, CJ Rothfels, AR Smith, R Viane, D Lorence, KR Wood, et al 2020 A global plastid phylogeny of the fern genus *Asplenium* (Aspleniaceae). Cladistics 36:22–71.

Yamane H 1998 Fern antheridiogens. Pages 1–32 *in* K Jeon, ed. International review of cytology, vol 184. Academic Press, San Diego, CA.

Paper 7

Liu H-M., Ekrt L., Koutecký P., Pellicer J., Hidalgo O., Marquardt J., Pustahija F., Ebihara A., Siljak-Yakovlev S., Gibby M., Leitch I. & Schneider H. (2019): Polyploidy does not control all: lineage-specific average chromosome length constraints genome size evolution in ferns. – Journal of Systematics and Evolution 57: 418–430.

Research Article

Polyploidy does not control all: Lineage-specific average chromosome length constrains genome size evolution in ferns

Hong-Mei Liu[1], Libor Ekrt[2], Petr Koutecky[2], Jaume Pellicer[3], Oriane Hidalgo[3,4,5], Jeannine Marquardt[4,5,6], Fatima Pustahija[7], Atsushi Ebihara[8], Sonja Siljak-Yakovlev[9], Mary Gibby[10], Ilia Leitch[3], and Harald Schneider[11]* (ID)

[1]Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun 666303, China
[2]Department of Botany, Faculty of Science, University of South Bohemia, Ceske Budejovice 370 05, Czech Republic
[3]Royal Botanic Gardens, Kew, Richmond TW9 3DS, UK
[4]Laboratori de Botànica, Unitat associada al CSIC, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona 08028, Spain
[5]Department of Life Sciences, Natural History Museum, London SW7 5BD, UK
[6]Botanischer Garten und Botanisches Museum Berlin Dahlem & Freie Universität Berlin, Berlin 14195, Germany
[7]Faculty of Forestry, University of Sarajevo, Sarajevo 71000, Bosnia and Herzegovina
[8]Department of Botany, National Museum of Nature and Science, Tsukuba 305-0005, Japan
[9]Laboratoire d'Ecologie, Systématique et Evolution, Université Paris-Sud, UPS-CNRS-AgroParisTech, Orsay France
[10]Royal Botanic Garden Edinburgh, Edinburgh EH3 5LR, UK
[11]Center for Integrative Conservation, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun 666303, China
*Author for correspondence. E-mail: harald@xtbg.ac.cn
Received 9 March 2019; Accepted 19 June 2019; Article first published online 26 June 2019

Abstract Recent studies investigating the evolution of genome size diversity in ferns have shown that they have a distinctive genome profile compared with other land plants. Ferns are typically characterized by possessing medium-sized genomes, although a few lineages have evolved very large genomes. Ferns are different from other vascular plant lineages as they are the only group to show evidence for a correlation between genome size and chromosome number. In this study, we aim to explore whether the evolution of fern genome sizes is not only shaped by chromosome number changes arising from polyploidy but also by constraints on the average amount of DNA per chromosome. We selected the genus *Asplenium* L. as a model genus to study the question because of the unique combination of a highly conserved base chromosome number and a high frequency of polyploidy. New genome size data for *Asplenium* taxa were combined with existing data and analyzed within a phylogenetic framework. Genome size varied substantially between diploid species, resulting in overlapping genome sizes among diploid and tetraploid spleenworts. The observed additive pattern indicates the absence of genome downsizing following polyploidy. The genome size of diploids varied non-randomly and we found evidence for clade-specific trends towards larger or smaller genomes. The 578-fold range of fern genome sizes have arisen not only from repeated cycles of polyploidy but also through clade-specific constraints governing accumulation and/or elimination of DNA.

Key words: chromosome number, DNA-C value, evolutionary constraint, evolvability, genome evolution, land plant, macroevolution, plant diversity, whole genome duplication.

1 Introduction

In recent years, increasing attention has focused on the contrasting genomic profiles and evolutionary processes underlying different lineages of land plants, and the consequences of these differences on their diversification and evolutionary trajectories (Leitch & Leitch, 2012, 2013; Scarpino et al., 2014; Puttick et al., 2015; Carta & Peruzzi, 2016; van de Peer et al., 2017; Landis et al., 2018). One notable example is seen in the frequency of whole genome duplications (WGD; i.e., polyploidy) and the subsequent genomic response in ferns compared with angiosperms (flowering plants). Polyploidy is certainly an important evolutionary process in both these lineages given the abundance of polyploids reported in extant species (Wood et al., 2009), and yet it is becoming clear that there might be differences in the post-polyploidization processes because diploidization is arguably not linked with genome downsizing in ferns (e.g., Barker, 2009; Barker & Wolf, 2010; Haufler, 2014; Henry et al., 2014; Wolf et al.,

2015; Clark et al., 2016; Dauphin et al., 2016), in contrast to angiosperms (Leitch & Bennett, 2004; Soltis et al., 2015; Wendel, 2015). Thus, although ferns and flowering plants could share some of the pathways involved in diploidizing the genome, such as methylation (Takuno et al., 2016), they differ in their response to the additional chromosomes and DNA arising from WGD (Clark et al., 2016). This difference could partly explain why: (i) the distribution of genome sizes in angiosperms is skewed to small genomes (median = 1.7 *pg*/1C) but is more normally distributed with medium-sized genomes in ferns (median = 11.4 *pg*/1C) (Leitch & Leitch, 2013; Suda et al., 2014; Pellicer et al., 2018); (ii) ferns are typically characterized by higher chromosome numbers (mean 2*n* = 121.0; 2*n* range = 18–1440) compared with angiosperms (mean 2*n* = 15.99; 2*n* range = 4–640) (Clark et al., 2016); and (iii) genome size and chromosome number (2*n*) are often correlated in ferns but not in angiosperms (Barker, 2009, 2013; Haufler, 2014; Clark et al., 2016). Such differences at the molecular level could also contribute to the higher contribution of hybrid speciation to extant fern diversity (Wood et al., 2009; Mayrose et al., 2011; Soltis et al., 2015) and evolutionary potential (Vanneste et al., 2015; Clark et al., 2016) compared with other land plant lineages. Nevertheless, these broad-scale differences in the response to WGD do not exclude a role for other genomic processes, such as changes in repeat composition and dynamics, impacting the evolution of particular fern lineages (Dodsworth et al., 2015; Wolf et al., 2015). For example, genome size variation in royal ferns (Osmundaceae) is not correlated with chromosome number (Schneider et al., 2015), and the genome size of the apomictic fern *Asplenium monanthes L.* complex and relatives cannot be explained solely by polyploidy (Dyer et al., 2013). In this context, it is also notable that an analysis of the repeat content for six polypod fern species suggested that fern genomes might carry a large proportion of DNA transposons and simple repeats but a small proportion of satellite DNA compared to seed plants (Wolf et al., 2015). However, the recent whole genome sequences of the heterosporous ferns *Azolla Lam.* and *Salvinia Seg.* showed a more similar pattern to seed plants with a much higher proportion of retrotransposons compared with DNA transposons and satellite repeats (Li et al., 2018).

Overall, such observations suggest that genome size evolution in ferns is not only shaped by repeated cycles of polyploidy but also by other mechanisms. Whereas some evidence has been put forward to suggest that chromosome sizes might be constrained within some angiosperm species (Hudakova et al., 2002; Li et al., 2011; Schubert & Vu, 2016), less attention has been given to comparisons among species and in other plant groups. In lineages with highly conserved chromosome numbers, such as Aspleniaceae, if chromosome sizes are indeed constrained within certain boundaries, then this might restrict the variation in monoploid genome size (i.e., the amount of DNA in one set of monoploid chromosomes, 2C-value/*x* ploidy level; Greihuber et al., 2005) and the average amount of DNA per chromosome (2C-value/2*n* chromosome number). Past research on the karyotype of ferns suggested conservation of small chromosome sizes, but the critical review by Duncan & Smith (1978) stressed the limitations of chromosome light-microscopy for making and analyzing chromosome preparations. In turn, evolutionary constraints on chromosome length are predicted to pose limits on genome size variation in the absence of polyploidy. Such conservation in karyotype structure has not yet been shown for

ferns despite it being consistent with the reported correlation between chromosome number and genome size (e.g., Bainard et al., 2011; Clark et al., 2016). Several evolutionary mechanisms could explain these observations, including regulation of the balance between insertions and deletions during the repair of double-strand breaks in DNA (Schubert & Vu, 2016), and arguments suggesting that under certain conditions there might be selection against the accumulation of large plant genomes (Knight et al., 2005; Chen et al., 2014; Pellicer et al., 2014; Hidalgo et al., 2017a, 2017b). These arguments include the increased biochemical costs associated with building and maintaining a bigger genome (Hidalgo et al., 2017b) and a reduction in the competitiveness of taxa with large genomes in habitats with limited availability of nitrogen and phosphate (Guignard et al., 2016; Guigard et al., 2017). Genome size also affects the length of the cell cycle and thus the growth rate, which in turn could affect the competitiveness of plants requiring fast development of their body or organs (Francis et al., 2008; Gruner et al., 2010; Suda et al., 2014). Further arguments to suggest why there might be selection against larger genome sizes are based on molecular studies showing negative relationships between genome size and, for example, recombination rates (Tiley & Burleigh, 2015), mutation rates (Bromham et al., 2015; Gupta et al., 2016), and indeed, the rate of gene space evolution through the impact of repetitive DNA on genome dynamics (Dodsworth et al., 2015; Garrido-Ramos, 2015). Nevertheless, population level processes (e.g., genetic drift versus selection) might also influence genome size dynamics and evolution (Whitney et al., 2010), hence genome size is not only expected to show a non-random distribution across the phylogeny of ferns, but also spatial and ecological patterns that are biologically informative.

In this study, we aim to provide evidence for the following three predictions explaining the evolution of fern genome size that cannot only be explained by the repeated formation of polyploids followed by DNA conservation rather than genome downsizing (Leitch & Bennett, 2004). Instead, the observed data are better explained by constraints operating on the average size of fern chromosomes and/or the impact of ecological constraints.

Prediction 1. The genome size of ferns is not only shaped by changes in the chromosome number as a result of polyploidization. Instead, it is predicted that differences in the average amount of DNA per chromosome is an important factor shaping the range of monoploid genome sizes in ferns.

Prediction 2. The genome size of polyploid ferns is predictable as long as the genome size of their diploid parents has been reliably determined. The predictability of the genome size of polyploids is not restricted to newly formed polyploids but holds also for older polyploids that produce haploid species through regular meiosis.

Prediction 3. The monoploid genome size shows a phylogenetic pattern indicating evolutionary constraints, such as chromosome stability, or the influence of ecological factors.

To generate sufficient data to support or reject these predictions, we expanded the existing dataset of fern genome sizes generated by Clark et al. (2016) with additional data focused on European accessions of the genus *Asplenium* (spleenworts). This nearly globally distributed genus is especially suited to study these issues. With more than 700 species, it is not only the most species-rich genus of ferns but also the only genus to be equally represented in temperate

versus tropical climate regions and on all major continents. The base chromosome number of $x = 36$ is highly conserved as it has been reported for 95% of the species that have been counted. Speciation by polyploidy is very frequent, including polyploids up to $16x$ (Walker, 1966). Finally, an analysis of karyotype data for European species of *Asplenium* suggests a substantial variation in genome size independent of chromosome number and arising as a result of chromosome size variation. Such differences are clearly visible in the chromosome sets of allopolyploid hybrids involving *Asplenium scolopendrium L.* as one of the two parental species (Emmott, 1964; Schäfer & Rasbach, 2000). Although the study focuses on *Asplenium* as the model, we also analyzed existing fern genome size data (see Clark et al., 2016; Pellicer et al., 2018) to enable comparisons across other fern lineages, although it is recognized that the sampling density of all ferns is insufficient to provide the same resolution as the *Asplenium* data.

2 Material and Methods

Genome size measurements were obtained for 147 accessions belonging to 54 taxa of *Asplenium* by combining previously published data (Chang et al., 2013; Dyer et al., 2013; Henry et al., 2014; Clark et al., 2016; Li et al., 2016) with newly generated measurements for 62 accessions, including 12 previously unstudied specimens and adding new accessions to 29 taxa (Table S1; see also Data S1). The tetraploid *Asplenium quadrivalens (D.E.Mey.) Landolt* (=*A. trichomanes* subsp. *quadrivalens*), with 20 accessions, was the most densely sampled taxon followed by the diploid *Asplenium viridian Huds.* with 12 accessions, whereas 49% of the sampled taxa were represented by a single specimen (Data S1). The sampling comprised at least one representative of six out of the eight main clades of the crown group of *Asplenium* (Schneider et al., 2017), including the *Camptosorus* clade (32 spp.; Schneider et al., 2017), *Phyllitis* clade including the *Ceterach* subclade (7 spp.; Schneider et al., 2017), *Asplenium* clade (3 spp; Schneider et al., 2017), *Neottopteris* clade (3 spp; Schneider et al., 2017), *Pleurosorus* clade (6 spp; Schneider et al., 2017), and *Tarachia* clade (3 spp; Schneider et al., 2017).

In the context of polyploidy, the sampling included 22% diploid taxa, 7% triploid taxa, 49% tetraploid taxa, and 2% dodecaploid taxa. To establish a diploid–triploid–tetraploid genome size comparison, we estimated the genome sizes of six accessions of the tetraploid *Asplenium adulterine MildeAsplenium x poscharskyanum (Hofm.) Doerfl.*, and 12 accessions of the diploid *A. viride* and six accessions of *A. × poscharskyanum*, which represents the triploid cross between these two species (Data S1).

Another focus was on the genome size of *Asplenium scolopendrium L.* (five accessions), its European relative *Asplenium sagittatum (DC.) Bange* (one accession), the tetraploid relative *Asplenium komarovii Akasawa* (four accessions), and tetraploid *Asplenium hybridism (Milde) Bange* (one accession). The later taxon originating from hybridization between the diploid *Asplenium scolopendrium L.* and *Asplenium javorkeanum Vida*, the only European diploid of the *Ceterach* complex (Pinter et al., 2002). All data including voucher information are available in Data S1.

Genome sizes were measured using methods described previously (see Clark et al., 2016) using material collected from the Royal Botanic Garden Edinburgh, the private fern garden of A. Leonard, or during fieldwork at various locations in Europe and Asia Minor. Voucher specimens were obtained and deposited as necessary in public herbaria (CBFS, NHM). Species identifications were carried out by H. S. and L. E. For comparisons beyond *Asplenium*, we used the data published on ferns in Clark et al. (2016) and data available for gymnosperms and flowering plants in the Plant DNA C-values Database (release 6.0 available at https://cvalues.science.kew.org/; last checked January 2019) together with additional data from some recent publications not yet incorporated into the database. A comprehensive representation of all fern data available in early 2019 are available in Data S1. The seed plant DNA C-values used are all available in the Plant DNA C-values Database, whereas chromosome counts were obtained from the Chromosome Counts Database at http://ccdb.tau.ac.il (Rice et al., 2015). The chromosome dataset was further improved by an ongoing comprehensive review of all published chromosome counts of ferns (Schneider H, early 2018).

Statistical tests were carried out using R 3.2.5 (R Development Core Team, 2008), whereas the phylogenetic analyses were carried out using Mesquite 3.10 (Maddison & Maddison, 2018) combined with APE 5.3 (Paradis et al., 2004) and OUCH packages in R (Butler & King, 2004). Phylogenetic signal was tested by randomizing the character states observed in a matrix containing 500 characters. The phylogenetic tree used for the analysis was reconstructed using *rbcL* sequence data that were made available in previous phylogenetic analyses of Aspleniaceae (Schneider et al., 2017). The phylogenetic tree was reconstructed using RaxML (Stamatakis, 2014) and MrBayes (Ronquist & Huelsenbeck, 2003). The obtained topology was critically inspected by considering the topology of a large-scale phylogenetic analysis of spleenworts that included approximately half of the extant diversity (Schneider et al., 2017). The statistical test carried out included linear regression, generalized least squares, and phylogenetic independent contrast analyses (Felsenstein, 1985).

3 Results

3.1 Prediction 1: Monoploid genome size varies due to differences in the average amount of DNA per chromosome

In the genus *Asplenium* (Table S1), the holoploid 2C-values ranged 7.6-fold with the smallest holoploid genomes found in the diploids *Asplenium hispanicum (Coss.) Greuter & Burdet* ($2C = 6.77$ pg) and *Asplenium dalhousiae Hook.* ($2C = 6.79$ pg) and the largest one in the dodecaploid *Asplenium aetiopicum (Burm.f.) Becherer subsp. dodecaploideum A.F.Braithw.* ($2C = 51.35$ pg). At the monoploid ($1Cx$) level, the largest $1Cx$ genomes were found in the putative diploid *Asplenium normale D.Don* ($1Cx = 9.80$ pg) and the diploids *Asplenium marinum L.* ($1Cx = 9.66$) and *A. monanthes* ($1Cx = 9.74$ pg), whereas the smallest were encountered in the diploid *A. hispanicum* and the tetraploid *Asplenium septentrionale (L.) Hoffm.* – both with $1Cx = 3.39$ pg. The holoploid genome sizes (2C-values) of the 24 diploid species ($2n = 72$) ranged 2.90-fold from 6.77 to 19.6 pg, whereas the 25 tetraploid species

(2n = 144) ranged 2.69-fold from 13.74 to 37.00 pg (Table S2). Overall, diploid *Asplenium* samples had one of the largest ranges observed among diploid ferns, exceeded only by the genus *Equisetum* (Equisetales) where 2C-values ranged 2.88-fold (Table S3). As expected, given the conserved chromosome number, the holoploid genome size (2C) showed a linear correlation ($r^2 > 0.999$, $P < 0.0001$) with the average amount of DNA per chromosome (2C/2n) for each of the three ploidy levels that had more than four species with data (i.e., 2x, 3x, and 4x; Fig. 1). Thus, the difference in the holoploid genome size is shaped not only by polyploidy but also by the average amount of DNA per chromosome. The slope was seen to decrease with increasing ploidy level (i.e., 2x slope a = 0.014; 3x slope a = 0.009; 4x slope a = 0.007; Fig. 1). The same trend was also observed when different lineages across the phylogenetic diversity of ferns were analyzed (Fig. 2), although there were notable differences in the minimum start point and the range between minimum and maximum values observed. Furthermore, the majority of *Asplenium* taxa (>50%) analyzed showed an average chromosome size (2C/2n) of 0.1–0.15 pg (Fig. 3). Similar trends were observed in most other fern orders (Table S4). Indeed, species with an average DNA amount per chromosome of less than 0.05 pg were only found in *Azolla* and *Loxsoma R.Br. ex A.Cunn.*, whereas values above 0.5 pg were found nearly exclusively in Osmundales and Psilotales (Table S3). Both the plots of all ferns analyzed (308 taxa) and those belonging to the order Polypodiales (220 taxa) showed evidence for a skewed distribution towards average chromosomes size of approximately 0.1 pg (Fig. 3). This skewed distribution was also observed in *Asplenium* (68 species plus hybrid taxa).

### 3.2 Prediction 2: The genome size of polyploid ferns is predictable

In *Asplenium*, the predicted genome sizes for 17 polyploid species – based on the sum of genome sizes for the parental species – were higher than the estimated value for 14 of 17 taxa (Table S4). For the other polyploid ferns explored, 5 of 9 taxa had observed values below the estimated value (Table S4). Nevertheless, the proportional difference between observed and expected genome size was less than 10% for 15 of 17 polyploid *Asplenium* taxa and 8 of 9 other polyploid fern taxa. A 5% proportional difference between observed and expected genome size was found for 8 of 17 polyploid *Asplenium* taxa and 8 of 9 other polyploid fern taxa. Taxa of the reticulate species network arising from the hybridization between *Asplenium trichomanes Thunb.* (2x; 2C = 9.29 pg) and *A. viride* (2x; 2C = 9.14 +/− 0.29 pg) showed this pattern, for example, the allotetraploid *A. adulterinum* (4x; 2C = 17.96 +/− 0.50 pg) and the triploid *A. × poscharskyanum* (3x; 2C = 14.01 +/− 0.43 pg), which originated from backcrosses between *A. adulterinum* and *A. viride*. However, some polyploids showed a >10% deviation between observed and predicted genome sizes. These taxa include the hexaploid *Polypodium interjectum Shivas,* the dodecaploid *A. aethiopicum* subsp. *dodecaploideum*, and the tetraploid *Asplenium subglandulosum (Hook. & Grev.) Salvo, C.Prada & T.E.Diaz* (Table S4). The latter two belong to poorly understood species complexes. The apomictic *A. aethiopicum* subsp. *dodecaploideum* is part of the *A. aethiopicum* complex, which is a paleoploid lineage. The estimated 2C-value for the Australian *A. subglandulosum* was based on the diploid genome size estimated for its distant relative *A. hispanicum*, which occurs at the Iberian Peninsula and North Africa. The tetraploid *Asplenium adiantum-nigrum L.*, with 9%, also showed a notable discrepancy between observed and expected genome size. The observed genome size (2C = 17.67 pg +/− 0.15) is smaller than the expected genome size of 2C = 19.58 pg based on the assumption of an autopolyploid origin from *A. onopteris* (2C = 9.79 pg +/− 0.08). The alternative but less likely hypothesis of an allopolyploid origin from a hybrid between *Asplenium cuneifolium Viv.* (2C = 12.17 pg +/− 0.12) and *Asplenium onopteris L.* (2C = 9.79 pg +/− 0.08) would result in an even bigger discrepancy
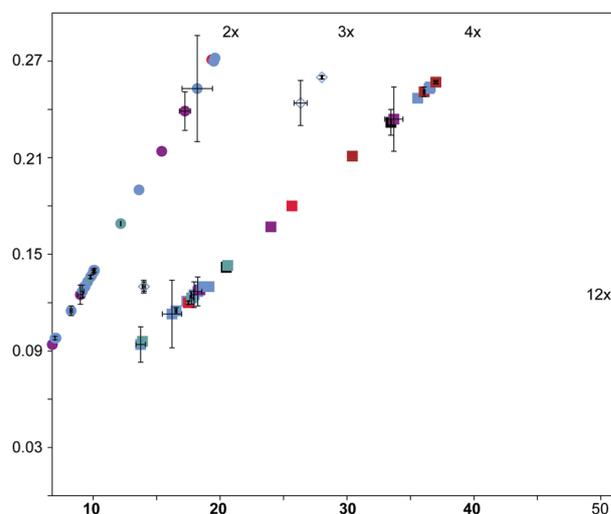
Fig. 1. Linear correlation between holoploid genome size (2C, x axis) and average DNA content per chromosome (2C/2n, y axis) in *Asplenium*. Filled circles, diploids (2x); open diamonds, triploids (3x); filled squares, tetraploids; filled triangles, dodecaploids (12x). Error bars show the variation recovered if more than one accession was available. Colors indicate phylogenetic relationships (clade names in Schneider et al., 2017): blue, *Camptosorus* clade; magenta, *Phyllitis* clade; red, *Asplenium* clade; brown, *Neottopteris* clade; green, *Pleurosorus* clade; black, *Tarachia* clade.
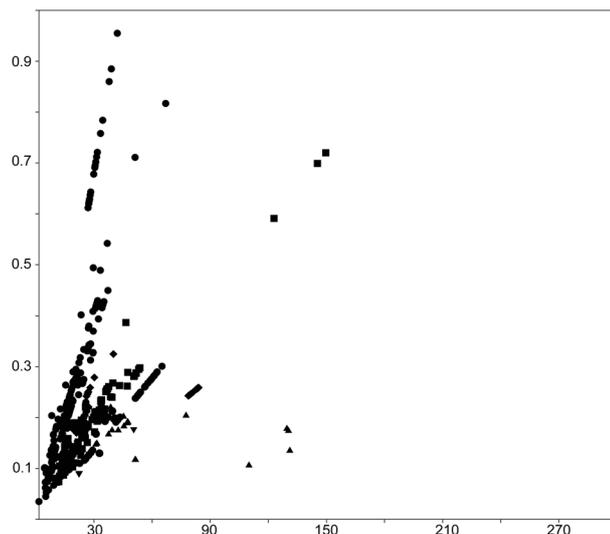
Fig. 2. Linear relationships between holoploid genome size (2C-value (*pg*), *x* axis) and average DNA amount per chromosome (2C/2n, *pg*, *y* axis) in ferns. Filled circles, diploids (2x); diamonds, triploids (3x); filled squares, tetraploids (4x); triangles, pentaploids (5x); inverted triangles, hexaploids (6x); filled triangles, ploidy levels >6x. For details see Fig. S1.

between the observed 2C = 17.76 *pg* and the predicted 2C = 21.96 *pg* (*Diff* = −19.0). The genome size estimates of *A. adiantum-nigrum* are based on four populations, of which one is a cultivar at the Royal Botanic Gardens Kew and the other three are newly studied populations from Armenia and Italy (see Table S1). Comparing these four populations, only limited variation was found with 2C-values ranging from 18.07 to 18.41 *pg* and indicating a relatively constant genome size in the tetraploid. This is notable because of the general trend of polyploid *Asplenium* species to show more variation in their genome sizes than their diploid relatives (with 0.27 as average standard deviation (SD) across 24 diploids, 0.50 as average SD

across 25 tetraploids, and 0.68 as average SD across four triploids). Constrasting genome size differences do not seem to pose a limitation in the formation of hybrids, as illustrated by *A. hybridum*, which is considered to have originated from hybridization between *A. javorkeanum* (2C = 8.99 *pg* +/− 0.40) and *A. scolopendrium* (2C = 17.23 *pg* +/− 0.89). However, hybrids formed between species with highly distinct genome sizes were rare, with more than 80% of the 17 *Asplenium* hybrids analyzed formed between parents having genome size differences of no more than 1.0 *pg* and less than 5% with a difference between 4.0 to 4.3 *pg* (Table S4).

3.3 Prediction 3: Monoploid genome size and average chromosome size shows a phylogenetic signal

In *Asplenium*, the overall monoploid genome size varied between 1Cx = 3.39 to 9.80 *pg* (Table S1) with considerable overlap in the ranges of the diploid (1Cx = 3.39 to 9.80 *pg*) and tetraploid (1Cx = 3.39 to 9.25 *pg*) taxa. Comparing the monoploid genome size of all 54 *Asplenium* species analyzed, 62.3% had 1Cx < 5.5 *pg*, 69.8% had 1Cx < 7.0 *pg*, and only 30.1% had a 1Cx > 7.0 *pg*. This pattern might be partially influenced by the dominance of European taxa (56.6% occurring in Europe plus Macaronesia) with 90.0% having a 1Cx < 7.0 *pg* in Europe (Fig. 4). Analysis of the range of average chromosome sizes (2C/2n) for the 54 *Asplenium* species showed that they varied 2.9-fold, ranging from 0.094 to 0.272 *pg* (Table S1, Fig. 3) with considerable overlap in the ranges between diploids (2C/2n = 0.094–0.272 *pg*) and tetraploid (2C/2n = 0.094–0.257 *pg*). Analyzing the data within a phylogenetic framework (Fig. 4) showed that there was considerable phylogenetic signal, indicating that closely related species have more similar 2C/2n values than expected by randomizing the observed data (mean observed 0.32 +/− 0.002; mean randomized data 0.096 +/− 0.012; *P* < 0.0001). Small 2C/2n values (<0.144 *pg*)


Fig. 3. Histogram showing the frequency distribution of the average DNA amount per chromosome (2C/2n) across all ferns (dark gray, front), Polypodiales (mid-gray, middle), and Aspleniaceae (light gray, back) plotted as the 2C/2n value (*pg* – *x* axis) versus proportion of the 2C/2n class per taxon (*y* axis).

were found mainly in three clades, namely the *A. trichomanes* clade (Tr in Fig. 4), the *Camptosorus* subclade (cCO in Fig. 4) and the *Pleurosorus* clade (PL in Fig. 4), whereas large $2C/2n$ values (>0.198 *pg*) were restricted to the *A. monanthes* clade (Mo in Fig. 4), *A. normale* clade (No in Fig. 4), *Euasplenium* clade (AS in Fig. 4), and the *Neotopteris* and *Taracchia* clades (NE and TA in Fig. 4). The *Ceterach–Phyllitis* clade (PH in Fig. 4) comprised a sister pair with distinct $2C/2n$ values including the *A. scolopendrium* complex (see Ph in Fig. 4) with large $2C/2n$ values (=0.239 *pg* +/− 0.012) and the *A. ceterach* complex (see Ce in Fig. 4) with small $2C/2n$ values (=0.128 *pg* +/− 0.009). Indications for gradual increases in the average chromosome sizes were apparent in the *A. monanthes* and *A. normale* clade (Fig. 4). However, no correlation was found between $2C/2n$ and the species occurrence in either temperate or tropical to subtropical climate zones ($P = 0.107$), although the majority of the 29 temperate *Asplenium* species typically showed smaller $1Cx$ values (mean $1Cx = 4.88$ *pg* +/− 4.88) compared with the 17 tropical *Asplenium* species (mean $1Cx = 7.37$ *pg* +/− 2.34),

with the exception of the *A. marinum* and the *A. scolopendrium* complex. Apart from these two exceptions, large $1Cx$ values were restricted to the tropical or subtropical clades. Some evidence for a correlation was found, however, in the *Camptosorus* clade (CA in Fig. 4) with a $P = 0.008$. The distribution of monoploid genome sizes ($1Cx$) and the $2C/2n$ values across all fern lineages for which data were available (308 species; Table S4) was also shown to exhibit phylogenetic structure (Table S4), especially across the phylogeny of Polypodiales with small values accumulating in the Dennstaedtiineae (DE in Fig. 5), Pteridiineae (PT in Fig. 5), and Aspleniineae (AS in Fig. 5) and medium to large genomes mostly found in the Polypodiineae (PO in Fig. 4) and the isolated genus *Didymochlaena* (Fig. 5). Nevertheless, each of the three species-rich lineages (Pteridiineae, Aspleniineae, and Polypodiineae) showed variation in the $2C/2n$ ratio (Table S3). Medium-sized $2C/2n$ ratios were found in the water-fern clade of the pteridoids including the mangrove fern *Acrostichum* (one species sampled, $2C/2n = 0.387$ *pg*) and the fresh water fern *Ceratopteris* (one species
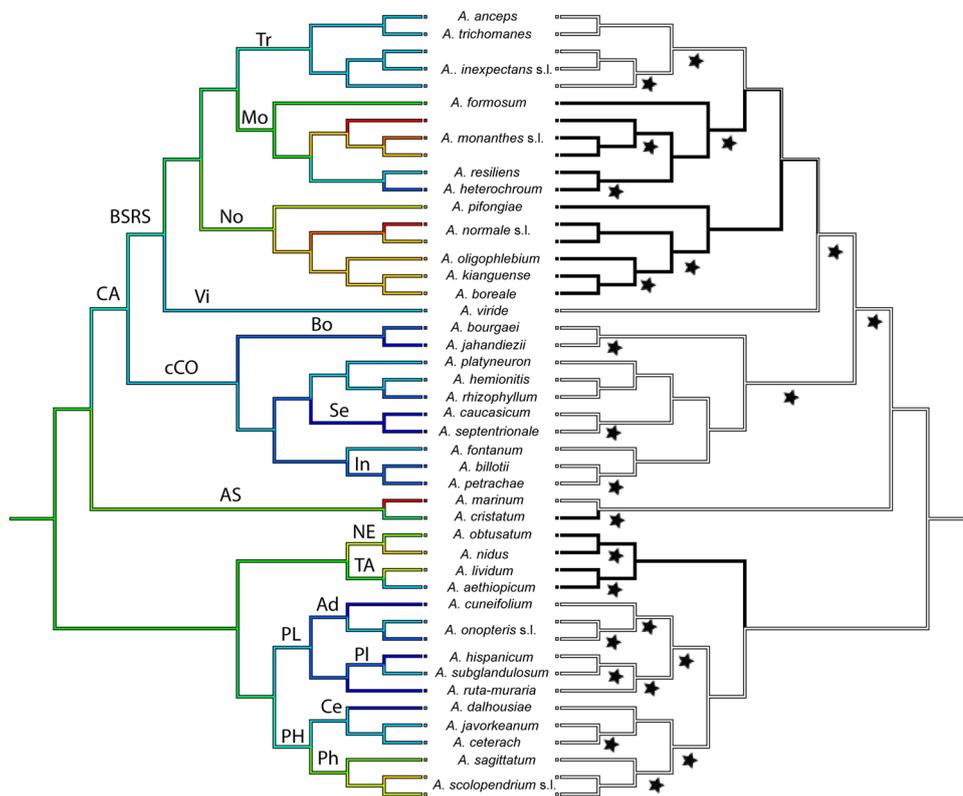
Fig. 4. Phylogenetic reconstruction of the average DNA amount per chromosome in *Asplenium* based on the Bayesian consensus phylogeny obtained using *rbcL* sequences. Left tree: Color of branches indicate the reconstructed $2C/2n$ value ranging from 0.09 *pg* (dark blue) to 0.29 *pg* (dark red). Right tree: preferred occurrence in climatic zones scored as either temperate (white) or tropical to subtropical (black). Stars indicate clades with posterior confidence values of $P \geq 0.95$; genome size is phylogenetically constrained ($P < 0.001$) compared to randomized characters. Ad, Asplenium adiantum comolex; AS, *Euasplenium* clade; Bo, Asplenium bourgei complex; BSRS, Black Stemmed Rock Spleenworts; CA, Camptosorus clade; cCO, *Camptosorus* subclade; Ce, *A. ceterach* complex; In, Asplenium incissum complex; Mo, *A. monanthes* clade; NE, *Neotopteris* clade; No, *A. normale* clade; Ph, Phyllitis complex; PH, *Ceterach–Phyllitis* clade; Pi, Pleurosorus complex; PL, *Pleurosorus* clade; Se, Asplenium septentronale complex; TA, *Taracchia* clade; Tr, *A. trichomanes* clade; Tr, Vi, Asplenium viride.
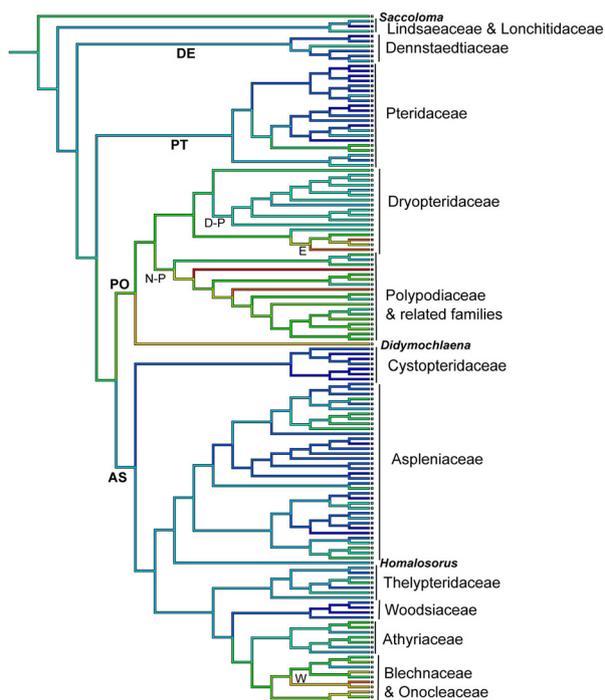
Fig. 5. Phylogenetic reconstruction of the monoploid genome size (1Cx-values, pg) in Polypodiales based on the Bayesian consensus phylogeny obtained from recent phylogenetic studies. Dark blues, small 1Cx-values; red colors, large 1Cx-values. Genome size is phylogenetic constrained (*P* < 0.001) compared to randomized characters. Classification of ferns follows PPG1 (The Pteridophyte Phylogeny Group, 2016) Families are shown on the left. Crucial nodes mentioned in the text are marked including four of the six suborders of Polypodiales, namely: AS, Aspleniineae; DE, Dennstaedtiinae; D-P, Dryopterioideae; E, *Elaphoglossum*; N=P, clade including Nephrolepidaceae to Polypodiaceae; PO, Polypodiineae; PT, Pteridineae; W, *Woodwardia*.

sampled, 2C/2n = 0.288). In the eupolypod I clade (PO in Fig. 5), large 2C/2n values were seen to arise in the tropical genera such as *Elaphoglossum* and relatives (E in Fig. 5; 2C/2n range from 0.289 to 0.817 among five species of *Elaphoglossum*) and the relatives of Polypodiaceae (N-P in Fig. 5; 2C/2n range from 0.102 to 0.450), whereas the smaller values were more frequently encountered in the clade comprising *Dryopteris* and *Polystichum* (D-P in Fig. 5; 2C/2n range from 0.098 to 0.334). Only temperate species were sampled for these two genera. In the Aspleniineae clade (AS in Fig. 4), small values dominated the clades containing frequently epilithic or epiphytic species (Aspleniaceae, Cystopteridaceae, and Wooodsiaceae; 2C/2n range from 0.073 to 0.308) but also the terrestrial Thelypteridaceae (2C/2n range from 0.138 to 0267). A trend towards larger values was observed in the Athyriaceae–Blechnaceae–Onocleaceae clade (2C/2n range from 0.062 to 0.542), with considerably larger values found in the genus *Woodwardia* (W in Fig. 5; 2C/2n range from 0.489 to 0.542).

4 Discussion

This study found results generally consistent with the three predictions based on the hypothesis that the genome size variation of ferns is only partly explained by the accumulation of polyploids that retain all the DNA from their diploid parents following polyploidization. Thus, although the expected additive pattern – that is, that the genome size of the polyploid is close or identical to the genome size predicted from the diploid parents – was supported by the analysis of some *Asplenium* polyploids (e.g., *A. adulterinum* and *A. septentrionale*), a trend towards genome downsizing was observed in other polyploid *Asplenium* species, such as *A. hybridum* and *A. aethiopicum* subsp. *dodecaploideum* (Table S4). Genome downsizing in polyploid ferns has not been recorded in previous studies focusing on diploidization patterns of polyploids (Haufler, 2014) or in recent reports investigating genome size stability following polyploidization, such as in the ophioglossoid genus *Botrychium Sw.* (Dauphin et al., 2016). Nevertheless, studies looking at the relationship between genome size and chromosome numbers (Clark et al., 2016) are also consistent with our findings. If evidence for substantial genome downsizing in ferns is reported in the future, as occurs in many angiosperms (Leitch & Bennett, 2004), it is important to highlight the possibility that they might be artefacts created by a lack of precision of the genome size measurements, incorrect identification of the parental species, and/or insufficient sampling density (Leitch & Bennett, 2004). Testing this hypothesis more extensively will therefore likely require not only expanded sampling and the application of best practice for genome size estimation, but also information on the estimated divergence time since the polyploids formed. For example, it has been assumed that the diploid genome size of the European–North African *Asplenium hispanicum* is conserved across all representatives of the polyploid *A. subglandulosum* complex, including in diploid cytotypes from South America and Australia–New Zealand (Schneider et al., 2017). Yet this is unlikely to be correct given the vast geographical distances involved. The second recorded exception in this work is *A. aethiopicum* subsp. *dodecaploideum*, which is an apomictic polyploid with a high chromosome number of 2n = 432. Given that previous studies have shown that apomictic *Asplenium* species can show distinct patterns of genome size evolution (Dyer et al., 2013) this could contribute to the differences noted here. The third example, *A. adiantum-nigrum*, might be the most promising target to study genome size variation and dynamics in polyploid ferns because of its large distribution range and variation of substrates on which it grows.

Phylogenetic conservatism of the average chromosome size (explored here 2C/2n values and monoploid (1Cx) genome sizes) was found to be another major factor shaping the distribution and evolution of fern genome sizes. Visual inspection of chromosome images provides support for the estimated differences in monoploid genome size and average chromosome sizes of the diploids *A. scolopendrium* (1Cx = 8.62 +/– 0.44 pg, 2C/2n = 0.239 +/– 0.012 pg), *A. javorkeanum* (1Cx = 4.50 +/– 0.20 pg, 2C/2n = 0.125 +/– 0.006), and *A. onopteris* (1Cx = 4.90 +/– 0.04 pg, 2C/2n = 0.136 +/– 0.001) reported here. For example, previous cytological studies have shown that two sets of 36 chromosomes with distinct sizes can easily be distinguished in meiotic preparations of hybrids between *A. scolopendrium* and these two diploid species (Emmott, 1964; Schäfer & Rasbach,

2000). In isolation without chromosome analysis, the larger genome size of *A. scolopendrium* might suggest it was a tetraploid, but it is clear from the images that its larger genome is due to an approximate doubling in the size of its chromosome. To confirm our interpretation of *A. scolopendrium*, we not only estimated the genome size of five different accessions originating from different localities across its range, including Bosnia and Herzegovina, Czech Republic, Italy, and Spain, but also obtained measurements of the sister species *A. sagittatum* (1Cx = 7.70 +/− 0.05 pg, 2C/2n = 0.214), the East Asian tetraploid *A. komarovii* (1Cx = 8.42 +/− 0.04 pg, 2C/2n = 0.234 +/− 0.008), and the allotetraploid *A. hybridum* (1Cx = 6.00 pg, 2C/2n = 0.167), which showed the expected intermediate 1Cx-value. The origin of the near doubling of the genome size in the *A. scolopendrium* complex (=*Phyllitis*) compared with the *A. ceterach* complex (=*Ceterach*) still needs to be further investigated. Two alternative hypotheses could be considered. One hypothesis might invoke an extreme form of Robertsonian chromosome fusion assuming a fusion of two chromosome sets in which each homologous chromosome pair fuses (Jones, 1998). The alternative hypothesis invokes better documented processes, at least in angiosperms, such as an increase in the amount of repetitive DNA (El Baidouri & Panaud, 2013; Bennetzen & Wang, 2014).

Our results are at least partly consistent with the hypothesis of phylogenetic conservation of chromosome complements in ferns (Manton, 1950; Haufler, 2014). For example, by 1950 Manton (1950) already noted that Osmundales and Psilotales were distinctive among ferns as they showed rather unusually large chromosomes compared with other ferns. Thus, although evidence for clade-specific changes in the 2C/2n ratio have clearly taken place (e.g., Dyer et al., 2013; Clark et al., 2016), our analyses not only within *Asplenium*, but more generally across all fern lineages show that the 2C/2n ratio is more often conserved among closely related ferns than expected under random evolution. Diploid taxa with relatively large genomes have therefore arisen either by duplicating the whole genome without chromosome number doubling or by other processes expanding the average DNA amount per chromosome. Such events are clade-specific, as illustrated by changes in genome size in sister lineages such as between the *A. ceterach* complex (=*Ceterach*) and the *A. scolopendrium* complex (=*Phyllitis*) noted above (Fig. 4). This hypothesis is also supported by the pattern of the *A. normale* complex with the newly described species *Asplenium pifongiae* (Li et al., 2016) having not only the smallest genome in the complex (2C-value = 15.84 pg) but also shown to be sister to the remaining complex (Fig. 4). This observation suggests a scenario of more gradual fluctuations in the average amount of DNA per chromosome across the phylogenetic tree with the range constrained by phylogenetic relationships. However, the chromosome numbers of taxa belonging to the *A. normale* complex require further investigation because some unpublished data indicate the possible occurrence of hexaploids and octoploids in this clade. A more gradual model is arguably also consistent with the data obtained for the *A. monanthes* complex (Dyer et al., 2013). Future studies should focus on determining the amounts and types of repetitive DNAs in these ferns to understand the mechanisms and dynamics underpinning these differences.

Phylogenetic constraints on the accumulation of medium to large genomes might therefore be partly explained by constraints acting on fluctuations in the average amount of DNA per chromosome. As a consequence, the monoploid genome size is not fully independent from chromosome number changes in most ferns, something that is especially apparent in lineages with highly conserved chromosome numbers, such as *Asplenium*. Beyond *Asplenium*, the largest chromosomes are found in Osmundales (mean 2C/2n = 0.71) and Psilotales (mean 2C/2n = 0.68), whereas the smallest ones are found in Gleicheniales (mean 2C/2n = 0.07) followed by the heterosporous Salviniales (mean 2C/2n = 0.11), with the latter including *Azolla,* the fern with the smallest 2C/2n value so far reported for any fern (mean 2C/2n = 0.04) for *Azolla microphylla Kaulf.* and mean 2C/2n = 0.01 for *Salvinia cucullata Bory.*). It is worth noting that the sister lineages Ophioglossales and Psilotales show highly distinct 2C/2n values with a mean 2C/2n value of 0.24 pg in the first lineage versus a mean value of 2C/2n of 0.68 in the latter (Table S3).

In addition to these differences in mean values for average chromosome sizes, differences are also found in the range of values for each lineage, suggesting substantial differences in the dynamics of chromosome size evolution among ferns. The lowest range is found in Gleicheniales (2C/2n range = 1.14-fold) followed by Psilotales (2C/2n range = 1.22-fold). The largest ranges are found not only in two highly specialized orders, namely the filmy ferns (Hymenophyllales: 2C/2n range = 4.73-fold) and the heterosporous water ferns (2C/2n range = 5.00-fold), but also in the most species-rich lineages, namely the tree ferns Cyatheales (2C/2n range = 3.80-fold) and Polypodiales (2C/2n range = 16.4-fold). Within Polypodiales, chromosome size variation is lowest in the species-poor Dennstaedtiineae (2C/2n range = 1.90-fold) and highest in the three species-rich lineages Pteridiineae (2C/2n range = 7.00-fold), Aspleniineae (2C/2n range = 9.00-fold), and Polypodiineae (2C/2n range = 8.20-fold).

Although there appears to be a good correlation between the range of 2C/2n values and species number per order and clade (log-log transformed, linear regression: slope *a* = 0.439, intercept *b* = −0.056, r^2 = 0.593, *P* = 0.0009), these statistics could be biased by taxon sampling density, which covers only approximately 3% of all extant fern diversity and approximately 7.6% of the diversity of *Asplenium*. Given the limited sampling, any conclusion is likely to be heavily affected by the small sampling size and the uneven distribution with some species-poor linages overrepresented (e.g., Equisetales represented by 13 of the 15 recognized species). Nevertheless, some evidence for a correlation between species richness of orders and range in monoploid genome sizes was not only found in ferns (log-log transformed, linear regression: *a* = 0.294, *b* = −0.132; r^2 = −0.434, *P* = 0.06) but also across the orders of flowering plants (log-log transformed, linear regression: *a* = 0.727, intercept *b* = 0.0007, r^2 = 0.680; *P* = 0.0001), all seed plants (log-log transformed, linear regression: slope *a* = 0.684, intercept *b* = 0.013. r^2 = 0.613, *P* = 0.0001), and all vascular plants (log-log transformed, linear regression: slope *a* = 0.693, intercept *b* = −0.068; r^2 = 0.624, *P* = 0.0001) (Schneider H, early 2019). As for ferns, these results are arguably influenced by the unevenness of available data for the different orders of plants, ranging from 0.1% in some

eudicot order (e.g., Celestrales) to 87.75% in horsetails (*Equisetum*), ignoring the 100% cover of some monotypic lineages. Although these correlations have to be interpreted carefully given the small sampling size, they do support the conclusion that ferns are perhaps not so different from flowering plants in terms of the distribution of monoploid genome size diversity. Such results indicate that the monoploid genome size of ferns evolved arguably with similar rates as the majority of angiosperm lineages, which is consistent with the recent attempt to compare rates of genome size evolution by Baniaga et al. (2016), but not that undertaken by Puttick et al. (2015). Indeed, we hesitated to carry out similar analyses because of the small proportion of genome size data available for most fern lineages and many flowering plants, and the known sensitivity of the methods used to estimate rates across phylogenetic trees to taxon sampling density (Cusimano & Renner, 2010; Cooper et al., 2016).

The observed pattern suggests a putative ecological constraint on the accumulation of medium to large monoploid genome size and average chromosome length in *Asplenium* caused by the strong seasonality of climatic regimes (Fig. 4; Table S5). However, this pattern was not significant in phylogeny-based comparative analyses of all ferns ($P > 0.01$), although it was somewhat supported in analyses restricted to the *Camptosorus* clade of *Asplenium* ($P < 0.01$). The underlying mechanisms might involve the selection of smaller genomes that can grow rapidly in habitats with a strong seasonality, such as rocky outcrops in the Mediterranean and temperate climate zones (e.g., *A. dalhousiae*, *A. hispanicum*, *Asplenium ruta-muraria L.*, and *A. septentrionale*), assuming the positive correlation between genome size and the cell cycle times reported in angiosperms (Greilhuber & Leitch, 2013) also applies to ferns. If so, then one would predict that such a selection pressure would be less pronounced in ferns occupying less seasonal habitats (e.g., rocky outcrops in Atlantic conditions as for *A. marinum*) or those ferns growing in more protected sides (e.g., *A. scolopendrium*), and leading to a larger range of genome sizes. The strongest evidence for this is arguably provided by the distribution of genome sizes reported in the black stemmed rock spleenworts (Fig. 4) with smaller monoploid genomes found in the mainly temperate black stemmed rock fern spleenworts (clade Tr and Vi in Fig. 4; *1Cx* range from 4.49 to 5.05 *pg*) and medium to large monoploid genomes found in those clades comprising subtropical to tropical species (clade Mo and No in Fig. 4; *1Cx* range from 4.14 to 9.80 *pg*). A comparable trend has been reported for the eudicot tree order Fagales in which tropical species typically had larger genomes than temperate species (Chen et al., 2014). Further evidence may be found in the trend to smaller genome sizes in the mainly temperate forest ferns within the Polypodiales, such as *Dryopteris Adans.* and *Polystichum Roth*, compared with the predominantly tropical species in the remaining Dryopteridaceae. Small *1Cx* and *2C/2n* values are also found in other mainly temperate clades, such as the *Cystopteris–Gymnocarpium* clade (Cystopteridaceae) and the *Adiantum–Pellaea* clade (Pteridaceae). However, larger values have been reported in the temperate representatives of the *Onoclea–Blechnum* clade, especially in the genus *Woodwardia* (Blechnaceae). Nevertheless, this requires further testing with a much expanded sampling. Given that the availability of nitrogen and phosphorous has also been shown to play a role in influencing species distributions

based on genome size in angiosperms (Kang et al., 2015; Guignard et al., 2016; Guigard et al., 2017), a negative correlation might be expected between genome size and epiphytism in ferns, as they are likely to be under nutrient limitation. So far, however, the limited available data suggest a trend towards accumulation of larger *2C/2n* ratios in the few sampled epiphytic *Asplenium* species (e.g., *Asplenium nidus L.*) and in the mainly epiphytic clades in the Polypodiineae (i.e., *Elaphoglossum* (Clade E; Fig. 5)) and the *Davallia–Polypodium* clade (Clade E; Fig. 5). Further sampling is required to test this hypothesis further to see whether the availability of nutrients, particularly N and P, play a role in influencing the evolution of genome size diversity in ferns.

Our results challenge the assumption that the small chromosome sizes are highly conserved in ferns, but are consistent with arguments put forward in the critical review on karyotypic analyses of fern chromosomes (Duncan & Smith, 1978). The results are also consistent with the well-documented observation that a few fern lineages possess larger chromosomes such as Osmundaceae and Psilotaceae (Manton, 1950; Clark et al., 2016). In addition, the results are supported by the observation of distinct sizes of chromosome sets in a few *Asplenium* hybrids (Emmott, 1964; Schäfer & Rasbach, 2000) but not the majority. Nevertheless, overall our results remain consistent with the hypothesis that the amount of DNA per chromosome is the key constraint that causes most ferns to have relative small chromosomes. Certainly, our approach of combining chromosome count data with DNA C-values to calculate the average chromosome size (*2C/2n*) overcomes some of the limitations of previous approaches (e.g., the use of light microscopy for karyotype analyses, which can lack sensitivity in estimating chromosome size differences; see Duncan & Smith, 1978). In the future there is clearly a need to follow up this study by both expanding the sample size and the karyological studies to include state-of-the art chromosome painting analyses to fully understand the biological context of the recorded differences.

5 Perspectives

This study has shown that constraints acting on the average size of chromosomes are one of the main factors that have limited the accumulation of medium to large genomes in many fern lineages. Indeed, it is possible that similar constraints are also operating in flowering plants. Whether fern genome sizes are also shaped by ecological constraints, as observed in many flowering plants, remains to be determined and will require further analyses based on much expanded taxon sampling and incorporation of data describing critical ecological characteristics, such as seasonality and availability of nutrients.

The results of this study will hopefully provide further stimulus to better understand why polyploidy is so frequent in ferns (see Wood et al., 2009; Clark et al., 2016), especially in the derived fern family Aspleniaceae (Schneider et al., 2017), and what the long term implications are at the genome level. It is possible that the limited changes in chromosome structure observed in ferns might contribute to enhancing the frequency of successful establishment of hybrids between diploids and recently formed polyploids, but could reduce the evolutionary potential of whole

genome duplications as a key innovation. Overall, it could be that angiosperms and ferns actually explore opposite evolutionary strategies in response to polyploidy. Thus, in contrast to ferns, the more dynamic angiosperm genomes and their ability to reorganize their genomes following polyploidy through a diversity of diploidization processes (Wendel, 2015) could contribute to their greater genome disparity and thus species diversity (see Cheng et al., 2018; Ren et al., 2018). In light of these observations, studying the fate of duplicated genomes in polyploid ferns seems an essential next step to understand how the genomic responses of angiosperm and fern polyploid genomes differ and what role these differences play in species diversification.

Acknowledgements

References

Bainard J, Henry T, Bainard L, Newmaster S. 2011. DNA content variation in monilophytes and lycophytes: Large genomes that are not endopolyploid. *Chromosome Research* 19: 763–775.

Baniaga AE, Arrigo N, Barker MS. 2016. The small nuclear genomes of *Selaginella* are associated with a low rate of genome size evolution. *Genome Biology and Evolution* 8: 1516–1525.

Barker MS. 2009. Evolutionary genomic analyses of ferns reveal that high chromosome numbers are a product of high retention and fewer rounds of polyploidy relative to angiosperms. *American Fern Journal* 99: 136–141.

Barker MS. 2013. Karyotype and genome evolution in pteridophytes. In: Greilhuber J, Dolezel J, Wendel J eds. *Plant genome diversity volume 2, physical structure, behaviour and evolution of plant genomes*. Vienna: Springer. 245–253.

Barker MS, Wolf PS. 2010. Unfurling fern biology in the genomics age. *Bioscience* 60: 177–185.

Bennetzen JL, Wang H. 2014. The contributions of transposable elements to the structure, function, and evolution of plant genomes. *Annual Review of Plant Biology* 65: 505–530.

Bromham L, Hua X, Lanfear R, Cowman PF. 2015. Exploring the relationships between mutation rates, life history, genome size, environment, and species richness in flowering plants. *American Naturalist* 185: 507–524.

Butler MA, King AA. 2004. Phylogenetic comparative analysis: A modeling approach for adaptive evolution. *American Naturalist* 164: 683–695.

Carta A, Peruzzi L. 2016. Testing the large genome constraint hypothesis: Plant traits, habitat and climate seasonality in Liliaceae. *New Phytologist* 210: 709–716.

Chang YF, Li J, Lu SG, Schneider H. 2013. Species diversity and reticulate evolution in the *Asplenium normale* complex (Aspleniaceae) in China and adjacent areas. *Taxon* 62: 673–687.

Chen SC, Cannon CH, Kua CS, Liu JJ, Galbraith DW. 2014. Genome size variation in the Fagaceae and its implications for trees. *Tree Genetics and Genomes* 10: 977–988.

Cheng F, Wu J, Cai X, Liang JL, Freeling M, Wang XW. 2018. Gene retention, fractionation and sub genome differences in polyploid plants. *Nature Plants* 4: 258–268.

Clark J, Hidalgo O, Pellicer J, Liu HM, Marquardt j, Robert Y, Christenhusz M, Zhang SZ, Gibby M, Leitch IJ, Schneider H. 2016. Genome evolution of ferns: Evidence for relative stasis of genome size across the fern phylogeny. *New Phytologist* 210: 1072–1082.

Cooper N, Thomas GH, Veditti C, Meade A, Freckleton RP. 2016. A cautionary note on the use of Ornstein Uhlenbeck models in macroevolutionary studies. *Biological Journal of the Linnean Society* 118: 64–77.

Cusimano N, Renner SS. 2010. Slowdowns in diversification rates from real phylogenies may not be real. *Systematic Biology* 59: 458–464.

Dauphin B, Grant J, Mraz P. 2016. Ploidy level and genome size variation in the homosporous ferns *Botrychium* s.l. (Ophioglossaceae). *Plant Systematics and Evolution* 302: 575–584.

Dodsworth S, Leitch A, Leitch IJ. 2015. Genome size diversity in angiosperms and its influence on gene space. *Current Opinion in Genetics & Development* 35: 73–78.

Duncan T, Smith AR. 1978. Primary basic chromosome numbers in ferns: Facts or fantasies? *Systematic Botany* 3: 105–114.

Dyer RJ, Pellicer J, Savolainen V, Leitch IJ, Schneider H. 2013. Genome size expansion and the relationship between nuclear DNA content and spore size in *Asplenium monanthes* fern complex (Aspleniceae). *BMC Plant Biology* 13: 219.

El Baidouri M, Panaud O. 2013. Comparative genomic paleontology across plant kingdom reveals dynamics of TE-driven genome evolution. *Genome Biology and Evolution* 5: 954––965.

Emmott JI. 1964. A cytogenetic investigation in a *Phyllitis–Asplenium* complex. *New Phytologist* 63: 306–308.

Felsenstein J. 1985. Phylogenies and the comparative method. *American Naturalist* 125: 1–15.

Francis D, Davis MS, Barlow PW. 2008. A strong nucleotypic effect on the cell cycle regardless of policy level. *Annals of Botany* 11: 747–757.

Garrido-Ramos MA. 2015. Satellite DNA in plants: More than just rubbish. *Cytogenetic and Genome Research* 146: 153–170.

Greihuber J, Dolezel J, Lysak MA, Bennett MD. 2005. The origin, evolution and proposed stabilization of the terms "genome size" and "C-value" to describe nuclear DNA contents. *Annals of Botany* 95: 255–260.

Greilhuber J, Leitch IJ. 2013. Genome size and the phenotype. In: Greilhuber J, Dolezel J, Wendel J eds. *Plant genome diversity volume 2, physical structure, behaviour and evolution of plant genomes*. Vienna: Springer. 323–344.

Gruner A, Hoverter N, Smith T, Knight CA. 2010. Genome size is a strong predictor of root meristem growth rate. *Journal of Botany* 2010: 390–414.

Guigard MS, Leitch AR, Acquisti C, Eizaguirre C, Elser JJ, Hessen DO, Jeyasingh PD, Neiman M, Riachardson AR, Soltis PS. 2017. Impacts of nitrogen and phosphorus: From genomes to natural ecosystems and agriculture. *Frontiers in Ecology and Evolution* 5: 70.

Guignard MS, Nichols RA, Knell RJ, MacDonald A, Romila CA, Trimmer M, Leitch IJ, Leitch AR. 2016. Genome size and policy influence angiosperm species' biomass under nitrogen and phosphorus limitations. *New Phytologist* 210: 1195–1206.

Gupta A, LaBar T, Miyagi M, Adami C. 2016. Evolution of genome size in asexual digital organisms. *Scientific Reports* 6: 25786.

Haufler CH. 2014. Ever since Klekowski: Testing a set of radical hypotheses revives the genetics of ferns and lycophytes. *American Journal of Botany* 101: 2036–2042.

Henry T, Bainard JD, Newmaster SG. 2014. Genome size evolution in Ontario ferns (Polypodiidae): Evolutionary correlations with cell size, spore size, and habitat type and an absence of genome downsizing. *Genome* 57: 1–12.

Hidalgo O, Pellicer J, Christenhusz M, Schneider H, Leitch AR, Leitch I. 2017b. Is there an upper limit to genome size? *Trends in Plant Science* 22: 567–566.

Hidalgo O, Pellier J, Christenhusz M, Schneider H, Leitch I. 2017a. Genomic gigantism in the whisk-fern family (Psilotaceae): *Tmesipteris obliqua* challenges record holder *Paris japonica*. *Botanical Journal of the Linnean Society* 183: 509–514.

Hudakova S, Kunzel G, Endo TR, Schubert I. 2002. Barley chromosome arms longer than half of the spindle axis interfere with nuclear divisions. *Cytogenetic and Genome Research* 98: 101–107.

Jones K. 1998. Robertsonian fusion and centric fission in karyotype evolution of higher plants. *Botanical Review* 64: 273–289.

Kang M, Wang J, Huang H. 2015. Nitrogen limitation as a driver of genome size evolution in a group of karst plants. *Scientific Reports* 5: 11636.

Knight CA, Molinari N, Petrov D. 2005. The large genome constrain hypothesis: Evolution, ecology, and phenotype. *Annals of Botany* 5: 177–190.

Landis JB, Soltis DE, Li Z, Marx HE, Barker MS, Tank DC, Soltis PS. 2018. Impact of whole-genome duplication events on diversification rates in angiosperms. *American Journal of Botany* 105: 1–16.

Leitch AR, Leitch IJ. 2012. Ecological and genetic factors linked to contrasting genome dynamics in seed plants. *New Phytologist* 194: 629–646.

Leitch IJ, Bennett MD. 2004. Genome downsizing in polyploid plants. *Biological Journal of the Linnean Society* 82: 651–663.

Leitch IJ, Leitch AR. 2013. Genome size diversity and evolution in land plants. In: Leitch IJ, Greilhuber J, Dolezel J, Wendel J eds. *Plant genome diversity*. Berlin: Springer-Press. 2: 307–322.

Li FW, Brouwer P, Carretero-Paulet L, Cheng SF, de Vries J, Delaux PM, Eily A, Koppers N, Kuo LY, Li Z, Simenc M, Small I, Wafula E, Angarita S, Barker MS, Bräutigam A, dePamphilis C, Gould S, Hosmani PS, Huang YM, Huettel N, Kato Y, Liu X, Maere S, McDowell R, Mueller LA, Nierop KGJ, Rensing SA, Robison T, Rothfels CJ, Sigel EM, Song Y, Timilsena PR, van de Peer Y, Wang H, Wilhelmsson PKI, Wolf PG, Xu X, Der JP, Schluepmann H, Wong GKS, Pryer KM. 2018. Fern genomes elucidate land plant evolution and cyanobacterial symbioses. *Nature Plants* 4: 460–472.

Li FW, Kuo LY, Chang YH, Hsu TC, Hung HC, Chiou WL, Rothfels CJ, Huang YM. 2016. *Asplenium pifongiae* (Aspleniaceae: Polypodiales), a new species from Taiwan. *Systematic Botany* 41: 24–31.

Li XR, Zhu CS, Lin ZW, Wu Y, Zhang DB, Bai GH, Song WX, Ma JX, Muehlbauer GJ, Scanlon MJ, Zhang M, Yu JM. 2011. Chromosome size in diploid eukaryotic species centers on the average length with a conserved boundary. *Molecular Biology and Evolution* 278: 1901–1911.

Maddison WP, Maddison DR. 2018. Mesquite: A modular system for evolutionary analysis. Ver. 3.10. Available from http://mesquiteproject.org [accessed 1 May 2018].

Manton I. 1950. *Problems of cytology and evolution in pteridophyta*. Cambridge: Cambridge University Press.

Mayrose I, Zhan SH, Rothfels CJ, Magnuson-Ford K, Barker MS, Rieseberg LH, Otto SP. 2011. Recently formed polyploid plants diversify at lower rates. *Science* 333: 1257.

Paradis E, Claude J, Strimmer K. 2004. APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics* 20: 289–290.

Pellicer J, Hidalgo O, Dodsworth S, Leitch IJ. 2018. Genome size diversity and its impact on the evolution of land plants. *Genes* 9: 88.

Pellicer J, Kelly LJ, Leitch IJ, Zomlefer WB, Fay MF. 2014. A universe of dwarfs and giants: genome size and chromosome evolution in the monocot family Melanthiaceae. *New Phytologist* 201: 1484–1497.

Pinter I, Bakker F, Barrett J, Cox C, Gibby M, Henderson S, Morgan-Richards M, Ramsey F, Russell SJ, Trewick SA, Schneider H, Vogel JC. 2002. Phylogenetic and biosystematics relationships in four highly disjunct polyploid complexes in the subgenera *Ceterach* and *Phyllitis* in *Asplenium*. *Organisms, Diversity & Evolution* 2: 299–311.

The Pteridophyte Phylogeny Group. 2016. A community-derived classification for extant lycophytes and ferns. *Journal of Systematics and Evolution* 54: 563–603.

Puttick MN, Clark J, Donoghue PCJ. 2015. Size is not everything: rates of genome size evolution, not C-value, correlate with speciation in angiosperms. *Proceedings of the Royal Society B: Biological Sciences* 282: 20152289.

R Development Core Team. 2008. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from http://www.R-project.org [1 May 2017].

Ren R, Wang HF, Guo CC, Zhang N, Zeng LP, Chen YM, Ma H, Qi J. 2018. Widespread whole genome duplications contribute to genome complexity and species diversity in angiosperms. *Molecular Plant* 11: 414–428.

Rice A, Glick L, Abadi S, Einhorn M, Kopelman NM, Salman-Minkov A, Mayzel J, Chay O, Mayrose I. 2015. The chromosome counts database (CCDB) – a community resource of plant chromosome numbers. *New Phytologist* 206: 19–26.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Scarpino SV, Levin DA, Meyers LA. 2014. Polyploid formation shapes flowering plant diversity. *American Naturalist* 184: 456–465.

Schäfer H, Rasbach H. 2000. *Asplenium x rouyi* Viane (*A. onopteris* L. x *A. scolopendrium* L.) in the Azores (Aspleniacae, Pteridophyta). *Willdenowia* 30: 219–227.

Schneider H, Liu HM, Chang YF, Ohlsen D, Perrie LR, Shepherd L, Kessler M, Karger DN, Hennequin S, Marquardt J, Russell S, Ansell S, Ngan Thi Lu, Kamau P, Loriga J, Regalado L, Heinrichs J, Ebihara A, Smith AR, Gibt M. 2017. Neo- and Paleopolyploidy contribute to the species diversity of *Asplenium* — the most species-rich genus of ferns. *Journal Systematics and Evolution* 55: 353–364.

Schneider H, Liu HM, Clark J, Hidalgo O, Pellicer J, Zhang SZ, Kelly LJ, Fay MF, Leitch IJ. 2015. Are the genomes of royal ferns really frozen in time? Evidence for coinciding genome stability and limited evolvability in the royal ferns. *New Phytologist* 207: 10–13.

Schubert I, Vu GTH. 2016. Genome stability and evolution: Attempting a holistic view. *Trends in Plant Science* 21: 749–757.

Soltis PS, Marchant DB, van de Peer Y, Soltis DE. 2015. Polyploidy and genome evolution in plants. *Current Opinion in Genetics & Development* 35: 119–125.

Stamatakis A. 2014. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312–1313.

Suda J, Meyerson LA, Leitch IJ, Pysek P. 2014. The hidden side of plant invasions: The role of genome size. *New Phytologist* 205: 994–1007.

Takuno S, Ran JH, Gaut BS. 2016. Evolutionary patterns of genic DNA methylation vary across land plants. *Nature Plants* 2: 5222.

Tiley GP, Burleigh JG. 2015. The relationship of recombination rate, genome structure, and patterns of molecular evolution across angiosperms. *BMC Evolutionary Biology* 15: 194.

van de Peer Y, Mizrachi E, Marchal K. 2017. The evolutionary significance of polyploidy. *Nature Reviews Genetics* 18: 411–424.

Vanneste K, Sterck L, Myburg AA, van de Peer Y, Mizrachi E. 2015. Horsetails are ancient polyploids: Evidence from *Equisetum giganteum*. *Plant Cell* 27: 1567–1578.

Walker TG. 1966. A cut-taxonomic survey of the pteridophytes of Jamaica. *Transaction of the Royal Society Edinburgh* 66: 169–237.

Wendel JF. 2015. The wondrous cycles of polyploidy in plants. *American Journal of Botany* 102: 1753–1756.

Whitney KD, Black EJ, Hamrick JL, Godt MJW, Barringer BC, Bennett MD, Eckert CG, Goodwillie C, Kalisz S, Leitch IJ. 2010. A role for nonadaptive processes in plant genome size evolution? *Evolution* 64: 2097–2109.

Wolf PG, Sessa EB, Marchant DB, Li FW, Rothfels CJ, Sigel EM, Gitzendanner MA, Visger CJ, Banks JA, Soltis DE, Soltis PS, Pryer KM, Der JP. 2015. An exploration into fern genome space. *Genome Biology and Evolution* 7: 2533–2544.

Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH. 2009. The frequency of polyploid speciation in vascular plants. *Proceedings of the National Academy of Sciences USA* 106: 13875–13879.

Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12525/suppinfo:

Fig. S1. Linear relationships between holoploid genome size (2C-value (*pg*), x-axis) and average DNA amount per chromosome (2C/2n, pg, y-axis) in ferns. Diploids (2x) are shown as filled circles, triploids (3x) as open diamonds, tetraploids as filled squares (4x), pentaploids (5x) as open circles, hexaploids (6x) as inverted triangles; ploidy levels > 6x as filled triangles. A: Equisetales (Blue), Marattiales (brown), Ophioglossales (green), Psilotales (black); B: Cyatheales (green), Gleicheniales (black), Osmundales (brown), Salvinailes (blue), Shcizaeales (purple); C: Aspleniineae (blue), Dennstaedtiineae (back), Lindsaeiineae (red), Polypodiineae (green), Pteridiineae (brown), Saccolomatiineae (purple).

Data S1. Providing the genome size data used in this study including classi-fication, generic and species names, genome size values, and voucher information. The classi-fication follows PPG I (2016). Column 1: Phylogenetic framework as given in Fig. 1 of PPG I (2016); columns 2: orders; columns 3: family; column 4: species; column 5: authority; column 6: comment; column 7: 2C DNA C-value in pg; column 8: standard deviation of the 2C value; col-umn 9: 1C DNA C-value in pg; column 10: 1Cx C-DNA value in pg; column 11: 2C/2n value; column 12: 2n chromosome count; column 13: x = policy level; column 14: BX base chromo-some number; column 15: voucher information; column 16: publication in which the value was reported for the first time; column 17: buffer used in Cytofluometric approach or notion as Feulgen if the later method was used; column 18: Standard used for Internal Ccallibration.

Table S1. Genomic size variation in Asplenium. Columns correspond to: Taxon = species name plus authority (cytotypes are recognized by their species name as long as the combination has been published); clade, subclade 1, species complex = species assignments based on their phylogenetic relationships (e.g., Chang et al., 2013; Dyer et al., 2014; Li et al., 2016; Schneider et al., 2017; 2C-value = mean holoploid genome size; SD – standard deviation (only calculated if more than one sample per species was available); 2n = Sporophytic chromosome number (bold = confirmed chromosome number; regular assumed chromosome number based on existing evidence); 1Cx-value = estimates monoploid genome size, SD – standard deviation of 1Cx (only calculated if more than one sample per species was available); 2C/2n = estimated average DNA content per chromosome, SD – standard deviation of 2C/2n (only calculated if more than one sample per species was available); NRAC = number of accessions analysed. To avoid confusion caused by inappropriate taxonomy, different ploidy levels with the same species name were recognized as distinct taxa. In some European complexes, we avoided the practice where which distinct taxa are merged into broader taxa to reflect the limited divergence in their morphology and the frequency of hybridiza-tion. Abbreviations in the clade column (CL) are as follows (see Schneider et al., 2017): AS = *Asplenium* clade, CA = *Camptosorus* clade, NE = *Neottopteris* clade, PH = *Phyllitis* clade, PL = *Pleurosorus* clade, TA = *Tarachia* clade; abbreviation in sub-clade column 1(SC1): BSRS = Black stemmed rock spleenworts, cCO = *Camptosorus* subclade, NA = not assigned; abbreviation of subclade column2 (SC2) with Mo = *A. monanthes* complex, No = *A. normale* complex, Tr = *A. trichomanes* complex, Vi = *Asplenium viride*, BSRS-H = hybrids between taxa belonging to different complexes belonging to BSRS; Bo = *A. bourgaei* complex, Ca = *A. rhizophyllum* complex, Fo = *A. fontanum*, He = *A. hemionitis* complex, In = *A. incisum* complex, Pl = *A. platyneuron*, Se = *A. septentrionale* complex; Ce = *A. ceterach* complex; CPH = Hybrids between members of *A. ceterach* complex and *A. scolpendrium* complex; Ph = *A. scolopendrium* complex; CRI = *A. cristatum* complex; Ma = *A. marinum*; Ne = *A. nidus* complex; Pr = *A. prolongatum* complex, Sp = South Pacific Complex; Ad = *A. adiantum-nigrum* complex; Pl = *A. subglandu-losum* complex; Ru = *A. ruta-muraria* complex; Ae = *A. aethopicum* complex. * One or more newly measured accessions were combined with previously published C-value data for this taxon. ** First time C-values were made available for this taxon.

Table S2. Genome size variation across different fern genera, families and lineages . To uncover differences caused by genome size variation independent from polyploidy and chromosome number changes, the OTUs were defined by taxonomic relationships, shared base chromosome number, and ploidy level. *Asplenium*, *Botrychium*, and Dryopteridaceae are known to have a highly conserved base chromosome number, species with other numbers were excluded. In Polypodiaceae, two basal chromosome numbers occur frequently x = 36 and x = 37, here only species with x = 37 were analysed. Columns: OTU = operational taxonomic unit, NRSP = number of species included in analysis, NRAC = number of accessions analysed, 2n = chromosome number of sporophytic generation, mean 2C = average holoploid genome size across all accessions analysed, min 2C = smallest 2C-value in data, max 2C = largest 2C-value in data, SD = standard deviation, x-fold (max/min) = range of genome size variation estimated by di-viding the maximum value by the minimum value. * Estimates for triploids assumes little differ-ences in the genome size among the two parents but this

may be not always the case. Thus, these estimates carry a substantial error.

Table S3. Monoploid genome size variation and average chromosome length variation in ferns reported for the eleven orders plus the four main lineages of Polypodiales. NrTa = number taxa (including hybrids); NRAC = Number of accessions analysed; *Mean 1Cx* = mean monoploid (1Cx-value) genome size; Min. 1Cx = minimum monoploid genome size; *Max. 1Cx* = maximum monoploid genome size; SD = standard deviation of monoploid genome size; *x-fold of 1Cx* = monoploid genome size range between smallest and largest values in data (*Max. 1Cx-value/Min. 1Cx-value*); *Mean 2C/2n* = mean average DNA amount per chromosome; *Min. 2C/2n* = minimum average DNA amount per chromosome; *Max. 2C/2n* = maximum average DNA amount per chromosome; *SD* = standard deviation of 2C/2x; *x-fold in 2C/2n* = range in average DNA amount per chromosome between smallest and largest values in data (*Max. 2C/2n / Min. 2C/2n*).

Table S4. Predicting genome sizes of hybrids and polyploid species originating from allopolyploidization or autopolyploidization events. Taxon = Name of taxon studied; *Obs. 2C* = observed holoploid genome size; *SD* = Standard deviation of observed genome size; *2n* = chromosome number of hybrid or polyploid; *Exp. 2C* = expected holoploid genome size of hy-brid or polyploid; *DIF* = difference between the observed and extrapolated genome size calcu-lated as $x = (obs.2C/exp.2C \times$ 100) - 100. Parent 1 = suspected parental genome donor 1; *2C* = genome size of parental taxon 1; *2n* = chromosome number parental taxon 1; Parent 2 = sus-pected parental genome donor 2; *2C* = genome size of parental taxon 2; *2n* = chromosome number of parental taxon 2. [1] The genome size of the triploid Asplenium x poscharskyanum was estimated based on the assumption that it contains two chromosome sets inherited from *A. viride* and one chromosome set of *A. trichomanes*. [2] The genome size of these triploids were calculated without knowing which of the two parents provided two and one chromosome set re-spectively. [3] The biosystematics of the *A. aethiopicum* complex is poorly understood, thus the interpretation of the parental taxa is ambiguous. [4] The biosystematics of the *A. normale* complex is still poorly understood see (Chang et al., 2013) and thus the parents of the tetraploid species need further investigation.

Table S5. Exploring evidence for a putative correlation between climate zone and monoploid genome size. Columns OTU = groups of ferns divided into their temperate and tropical species components; *mean 1Cx* = average monoploid genome size; *SD* = standard devia-tion; *Min. 1Cx* = minimum monoploid genome size; *Max. 1Cx* = maximum monoploid genome size; Range in *1Cx* = genome size range between smallest and largest mono-ploid genomes in data (= *Max. 1Cx-value/Min. 1Cx-value*).

Paper 8

Ekrt L., Holubová R., Trávníček P. & Suda J. (2010): Species boundaries and frequency of hybridization in the *Dryopteris carthusiana* (Dryopteridaceae) complex: A taxonomic puzzle resolved using genome size data. – American Journal of Botany 97: 1208–1219.

Species boundaries and frequency of hybridization in the *Dryopteris carthusiana* (Dryopteridaceae) complex: A taxonomic puzzle resolved using genome size data[1]

Libor Ekrt[2,5], Renata Holubová[3], Pavel Trávníček[3,4], and Jan Suda[3,4]

[2]Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic; [3]Department of Botany, Faculty of Science, Charles University in Prague, Benátská 2, CZ-128 01 Prague, Czech Republic; and [4]Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice 1, CZ-252 43 Průhonice, Czech Republic

- *Premise of the study*: Genome duplication and interspecific hybridization are important evolutionary processes that significantly influence phenotypic variation, ecological behavior, and reproductive biology of land plants. These processes played a major role in the evolution of the *Dryopteris carthusiana* complex. This taxonomically intricate group composed of one diploid (*D. expansa*) and two allotetraploid (*D. carthusiana* and *D. dilatata*) species in Central Europe. Overall phenotypic similarity, great plasticity, and the incidence of interspecific hybrids have led to a continuous dispute concerning species circumscription and delimitation.
- *Methods*: We used flow cytometry and multivariate morphometrics to assess the level of phenotypic variation and the frequency of hybridization in a representative set covering all recognized species and hybrids.
- *Key results*: Flow cytometric measurements revealed unique genome sizes in all species and hybrids, allowing their easy and reliable identification for subsequent morphometric analyses. Different species often formed mixed populations, providing the opportunity for interspecific hybridization. Different frequencies of particular hybrid combinations depended primarily on evolutionary relationships, reproductive biology, and co-occurrence of progenitors.
- *Conclusions*: Our study shows that genome size is a powerful marker for taxonomic decisions about the *D. carthusiana* complex and that genome size data may help to resolve taxonomic complexities in this important component of the temperate fern flora.

Key words: Central Europe; *Dryopteris carthusiana*; ferns; flow cytometry; interspecific hybridization; mixed populations; multivariate morphometrics; nuclear DNA content; polyploidy; Pteridophyta; taxonomy.

Hybridization has long been recognized as a prominent force in plant speciation, with up to 70% of extant plants being descendants of interspecific hybrids (Whitham et al., 1991). Hybridization can have several important evolutionary consequences, including increased genetic diversity, the origin of new ecotypes and taxa, and the reinforcement or breakdown of reproductive barriers (Rieseberg, 1997). However, interspecific crossing may not always be a source of genetic and functional novelty, because resulting hybrids can suffer from reduced fitness and thus merely represent evolutionary dead ends (Seehausen, 2004). Similarly, the detrimental effect of hybridization on the genetic integrity of rare species has been repeatedly documented (Ellstrand, 1992; Levin et al., 1996). In general, interspecific hybridization plays a central role in the evolutionary history of many plant species, although its impact varies by taxon and location (Ellstrand et al., 1996). From a taxonomic point, species prone to interspecific hybridization often pose serious problems because gene flow may blur species boundaries and affect levels of variability in natural populations. Considering the ploidy level of putative parents, homoploid and heteroploid hybridization can be distinguished (Grant, 1971; Rieseberg, 1997). While heteroploid hybridization is usually quite easy to detect because of the intermediate number of chromosomes (e.g., using conventional karyological techniques), recognition of homoploid crosses is a more challenging task (Rieseberg and Carney, 1998; Kron et al., 2007). Nevertheless, the last decade has seen significant advances in the understanding of patterns and dynamics of hybrid speciation, catalyzed mainly by the advent of novel molecular approaches (Hegarty and Hiscock, 2005; Uyeda and Kephart, 2006).

One of the taxonomically intricate plant groups that have been significantly affected by interspecific hybridization (often coupled with genome duplication) is the woodfern (*Dryopteris* Adans., Dryopteridaceae, Pteridophyta) (Manton, 1950). In temperate regions, *Dryopteris* belongs among the most hybrid-prone genera, with almost every combination of species pairs recorded (Wagner, 1971; Fraser-Jenkins, 1982; Dostál et al., 1984; Frey et al., 1995; Krause, 1998) as well as one intergeneric hybrid (Wagner et al., 1992). A paradigmatic example in the Central European flora is the *Dryopteris carthusiana* (formerly *D. spinulosa*) complex. This conspicuous component of

temperate woodlands consists of one diploid (2*n* = 2*x* = 82) species *D. expansa* (C. Presl) Fraser-Jenk. et Jermy and two tetraploids (2*n* = 4*x* = 164), *D. carthusiana* (Vill.) H. P. Fuchs and *D. dilatata* (Hoffm.) A. Gray. The latter (allo)tetraploid has been hypothesized to be derived from a cross between *D. expansa* var. *alpina* (Moore) Viane and *D. intermedia* subsp. *maderensis* (Alston) Fraser-Jenk. (Gibby and Walker, 1977; Viane, 1986; Krause, 1998), while *D. carthusiana* is thought to combine genomes of *D. intermedia* (Muhlenberg ex Willdenow) A. Gray subsp. *intermedia* from North America and an undiscovered diploid that has been variously identified as *D.* "*semicristata*" (Wagner, 1971; Werth and Lellinger, 1992) or *D.* "*stanley-walkeri*" (Fraser-Jenkins, 2001). The unknown diploid has been characterized using isozymes (Werth, 1989) and DNA (Hutton, 1992), and its morphology has been extrapolated by comparing its extant allotetraploid derivatives (Werth and Kuhn, 1989; Fraser-Jenkins, 2001). The species are classified in the subgenus *Dryopteris*, section *Lophodium* (Newman) C. Chr. ex H. Itô (Fraser-Jenkins, 1986), which includes ferns with usually thick, glossy, and bicolor stipe-base scales, narrow lobes of ultimate segments, terminating in long, hair-tipped aristate teeth, and unique spore morphology with minutely spinulose surface on the perispore. Formerly, *D. cristata* (L.) A. Gray was also a part of the *D. carthusiana* group (e.g., Walker, 1955, 1961; Widén et al., 1967). However, more recent treatments have placed *D. cristata* into the separate section *Pandae* Fraser-Jenk. (Fraser-Jenkins, 1986).

The characteristics of petiole scales and pinnule margins, shape and color of the frond, and the presence and density of glandular hairs are some of the diagnostic characters used for species recognition (Dostál et al., 1984; Fraser-Jenkins, 1993; Frey et al., 1995). However, overall phenotypic similarity, a high degree of phenotypic plasticity, and interspecific hybridization often make unambiguous species determination difficult. An important finding for the taxonomy of the group was the discovery that interspecific hybrids are sterile, producing only aborted, irregularly shaped spores (Manton, 1950; Wagner and Chen, 1965; Widén et al., 1967; Fraser-Jenkins, 1977; Jessen and Rasbach, 1987; Leonhards et al., 1990). All three possible hybrid combinations within the *D. carthusiana* group have been collected in the field in the past, though with markedly different abundances. Records exist for *D.* ×*ambroseae* Fraser-Jenk. et Jermy (= *D. dilatata* × *D. expansa*) and *D.* ×*deweveri* (Jansen) Jansen et Wachter (= *D. carthusiana* × *D. dilatata*) from western and northern Europe (Döpp and Gätzi, 1964; Widén et al., 1967; Benl and Eschelmüller, 1970; Piękoś, 1974; Fraser-Jenkins, 1977; Leonhards et al., 1990); more recently, these hybrids have also been repeatedly confirmed in Central and Eastern Europe (Holubová, 2006; Ivanova, 2006; Ekrt and Půbal, 2008). Conversely, *Dryopteris* ×*sarvelae* Fraser-Jenk. et Jermy (= *D. carthusiana* × *D. expansa*) is much rarer; occasional reports of its occurrence come only from Finland (Widén et al., 1967; Sorsa and Widén, 1968), Scotland (Corley and Gibby, 1981), and the island of Rügen in Germany (Jessen and Rasbach, 1987). This hybrid was synthesized experimentally (Walker, 1955) before it was documented from the wild.

In addition to the evaluation of morphological variation, the group has also been subjected to several cytological and chemotaxonomic analyses (Tryon and Britton, 1966; Widén et al., 1967, 1970; Sorsa and Widén, 1968; Widén and Sorsa, 1968; Britton, 1972; Gibby and Walker, 1977; Piękoś-Mirkowa, 1979, 1987, 1993; Benl and Eschelmüller, 1983; Gibby, 1983). Conventional chromosome counts provided robust evidence for

heteroploid hybridization (Manton, 1950), while studies of chromosome pairing during meiosis in both natural and artificial hybrids shed some light on species relationships (Manton and Walker, 1953; Walker, 1955, 1961). In recent years, another cytogenetic character, genome size, has become accessible (Leitch and Bennett, 2007). The knowledge that genome size is usually constant within the same taxonomic entity (Greilhuber, 2005) but may vary tremendously even among closely related taxa (Bennett and Leitch, 2005) provides a foundation for employing genome size as an important taxonomic marker. Indeed, this character has proven successful in resolving complex low-level taxonomies, delimiting species boundaries, and revealing cryptic taxa (Dimitrova et al., 1999; Mahelka et al., 2005; Kron et al., 2007; Suda et al., 2007). Despite its potential taxonomic value, genome size has largely been neglected in the biosystematics of ferns in general (but see Bureš et al., 2003; Ebihara et al., 2005; Ekrt and Štech, 2008; Ekrt et al., 2009), and in the *D. carthusiana* group in particular. The Plant DNA C-values database (Bennett and Leitch, 2005) contains only four estimates for the whole genus *Dryopteris*, including the value for *D. dilatata* (1C = 8.05 pg), as determined by Feulgen microdensitometry.

In this study, we used relative genome size data together with analysis of multivariate morphometric data to obtain new insights into phenotypic variation and the frequency of interspecific hybridization in the *D. carthusiana* group in Central Europe. Our investigation was inspired by the demonstration that genome size is usually stable within the same taxonomic entity (Greilhuber, 2005), while it often varies between different taxa (Bennett and Leitch, 2005). Consequently, genome size can be employed as a useful marker for resolving taxonomic complexities, circumscribing species, and unveiling cryptic diversity (Kron et al., 2007). An added benefit of genome size data is that they can also be used to differentiate between parental species and their hybrids, provided there are sufficient differences in the amount of nuclear DNA. This approach can be successfully applied even in groups with the same number of chromosomes, when traditional karyological treatments would be in vain (Loureiro et al., 2010).

Using flow cytometry and multivariate morphometrics, we addressed three main questions: (1) What is the level of genome size variation in the group? Can genome size be used as an informative marker for taxonomic decisions? (2) Which phenotypic traits are correlated with different genome size categories? What are the species- and hybrid-specific morphological characters? (3) What is the abundance and distribution of particular species and hybrids in the area studied?

MATERIALS AND METHODS

***Plant material*—**Plants were collected in the field between 2003 and 2007 from 85 localities (Fig. 1; see Appendix 1 for details), with 76 of them in the Czech Republic. Additional samples originated from Germany (five localities), Slovakia (three localities), and Austria (one locality). Special attention was paid to localities where more species grew in sympatry (to assess the frequency of interspecific hybridization) and localities where *D. expansa* was expected (i.e., a comparatively rare member of the complex). Plants were collected randomly in an area of approximately 20 × 20 m. In localities with multiple samples, we attempted to collect the whole morphological variation present at the locality. The number of samples per locality varied from one to 81, reflecting both the population structure (uniform vs. mixed populations) and locality size; at least five plants were collected at 34 localities. Collectively, 858 plants were available, involving all species and hybrid combinations (*D. carthusiana*: 237 samples; *D. dilatata*: 244 samples; *D. expansa*:
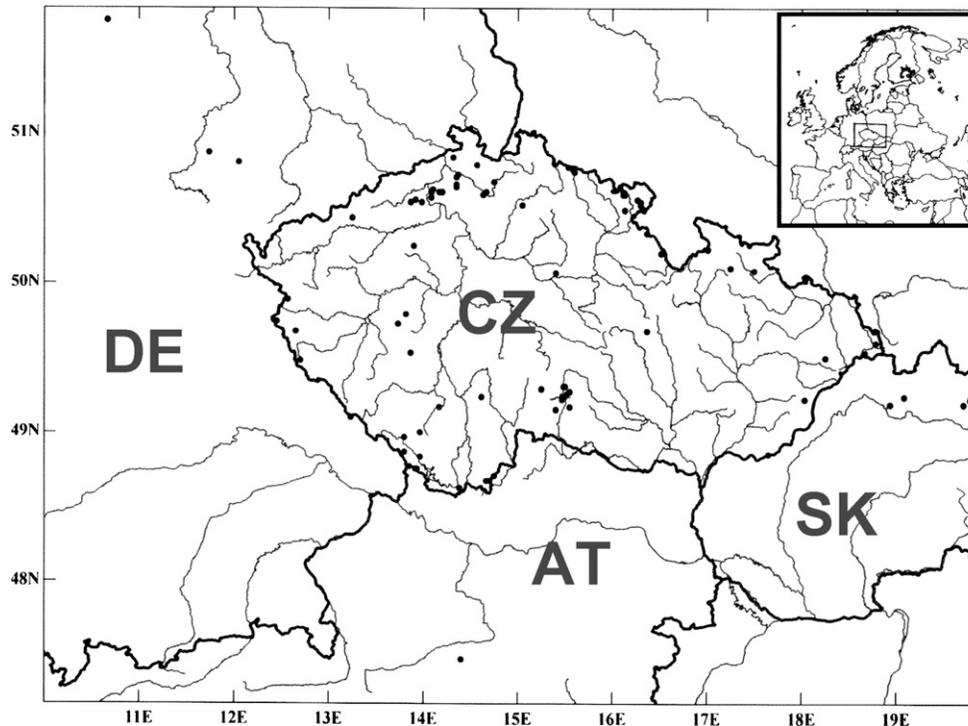
Fig. 1. Geographic distribution of 83 studied populations from the *Dryopteris carthusiana* group in Central Europe. AT = Austria, CZ = Czech Republic, DE = Germany, SK = Slovakia. Two additional localities from Rügen Island, Germany are not shown.

277 samples; *D. ×ambroseae*: 82 samples; *D. ×deweveri*: – 16 samples; *D. ×sarvelae*: 2 samples). Herbarium vouchers are kept in PR, PRC, HR and CB (see Appendix 1). Plants from Germany were deposited in the private herbarium of S. Jessen (Chemnitz).

Flow cytometry—Relative genome sizes were estimated by flow cytometry (FCM) using the two-step methodology originally developed by Otto (1990). The protocol generally followed Doležel et al. (2007). Briefly, approximately 4.5 cm^2 of fresh pinna tissue, devoid of sori, from the analyzed sample was chopped with leaf tissue of the internal standard, using a sharp razor blade, in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The suspension was filtered through a nylon mesh (42 μm) and incubated for 20 min at room temperature (20°C), with occasional shaking. Samples were stained with 1 ml of Otto II buffer (0.4 M Na$_2$HPO$_4$·12 H$_2$O) supplemented with AT-selective fluorochrome DAPI (at final concentration of 4 μg/mL) and β-mercaptoethanol (final concentration of 2 μL/mL). The staining lasted 1–2 min at room temperature, after which the relative fluorescence intensity of 3000–5000 particles was recorded on a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury-arc lamp for UV excitation. *Vicia faba* 'Inovec' (2C = 26.90 pg; Doležel et al., 1992) was selected as an appropriate primary internal reference standard (with genome size close to but not overlapping that of most samples). A secondary reference standard (*Pisum sativum* 'Ctirad'; 2C = 9.09 pg, Doležel et al., 1998) was used in analyses of *D. ×sarvelae* because genome size of this hybrid nearly overlapped with that of the primary standard. Resulting values were recalculated to the fluorescence intensity of *Vicia faba* (using a mean *Vicia* to *Pisum* ratio of 3.14, based on eight replications on different days). A karyologically confirmed plant of *D. expansa* ($n = 41^{II}$; counted by V. Jarolímová, Průhonice, Czech Republic) from the Czech Republic, Kostelní Myslová, Velký Hulišťský pond (loc. 40) was used to interpret FCM results.

Multivariate morphometrics—A subset of 609 plants, representing all species and hybrids except *D. ×sarvelae*, was subjected to morphometric analyses (*D. carthusiana*: 209; *D. dilatata*: 188; *D. expansa*: 163; *D. ×ambroseae*: 37; *D. ×deweveri*: 12; see Appendix 1). All the plants were classified to a particular species or hybrid on the basis of relative genome size as determined by flow cytometry. Only individuals with well-developed sori were considered. A total of

39 quantitative characters (21 primary characters and 18 ratios; Appendix S1, see Supplemental Data at http://www.amjbot.org/cgi/content/full/ajb.0900206/DC1) were measured and scored on dry herbarium vouchers. In addition, spore color was also recorded. All characters previously used for taxa recognition (e.g., Piękoś-Mirkowa, 1979; Chrtek, 1988; Ekrt, 2000; Kubát et al., 2002) were included, as well as additional potentially informative traits chosen on the basis of our own observations.

Morphometric data were analyzed with the SAS 8.1 statistical package (SAS Institute, Cary, NC, USA) using the procedures CANDISC, CORR, DISCRIM, PRINCOMP and UNIVARIATE (see Rosenbaumová et al., 2004 for details). In discriminant analyses, individual plants were selected as operational taxonomic units (OTUs), and genome size with spore fertility (v21) defined particular taxonomic groups (species and hybrids). Because the distribution within groups was not multivariate normal, the nonparametric *k*-nearest-neighbor method was employed (Klecka 1980). Discriminant power was determined by cross-validation. Various modifications of discriminant analyses (all species and hybrids together / parental species only / hybrids only / parental species with a corresponding hybrid) were performed.

Differences in genome size values (relative fluorescence intensities) were tested with a general linear model (procedure GLM) due to unbalanced data design (i.e., different sample size in different OTUs).

RESULTS

Genome size variation—Flow cytometric analyses of 858 *Dryopteris* plants yielded high-resolution histograms with distinct peaks of the sample and the internal reference standard, and with little background signal (Fig. 2). The average coefficients of variation of the G0/G1 peaks of the analyzed sample and internal reference standard were 2.67% (range 1.37–5.89) and 2.36% (range 1.18–4.44), respectively. Table 1 summarizes relative fluorescence intensities for all species and hybrids in the *D. carthusiana* group. The estimated values differed significantly (GLM procedure; $F_{5,852} = 15914$, $P < 0.0001$) and were
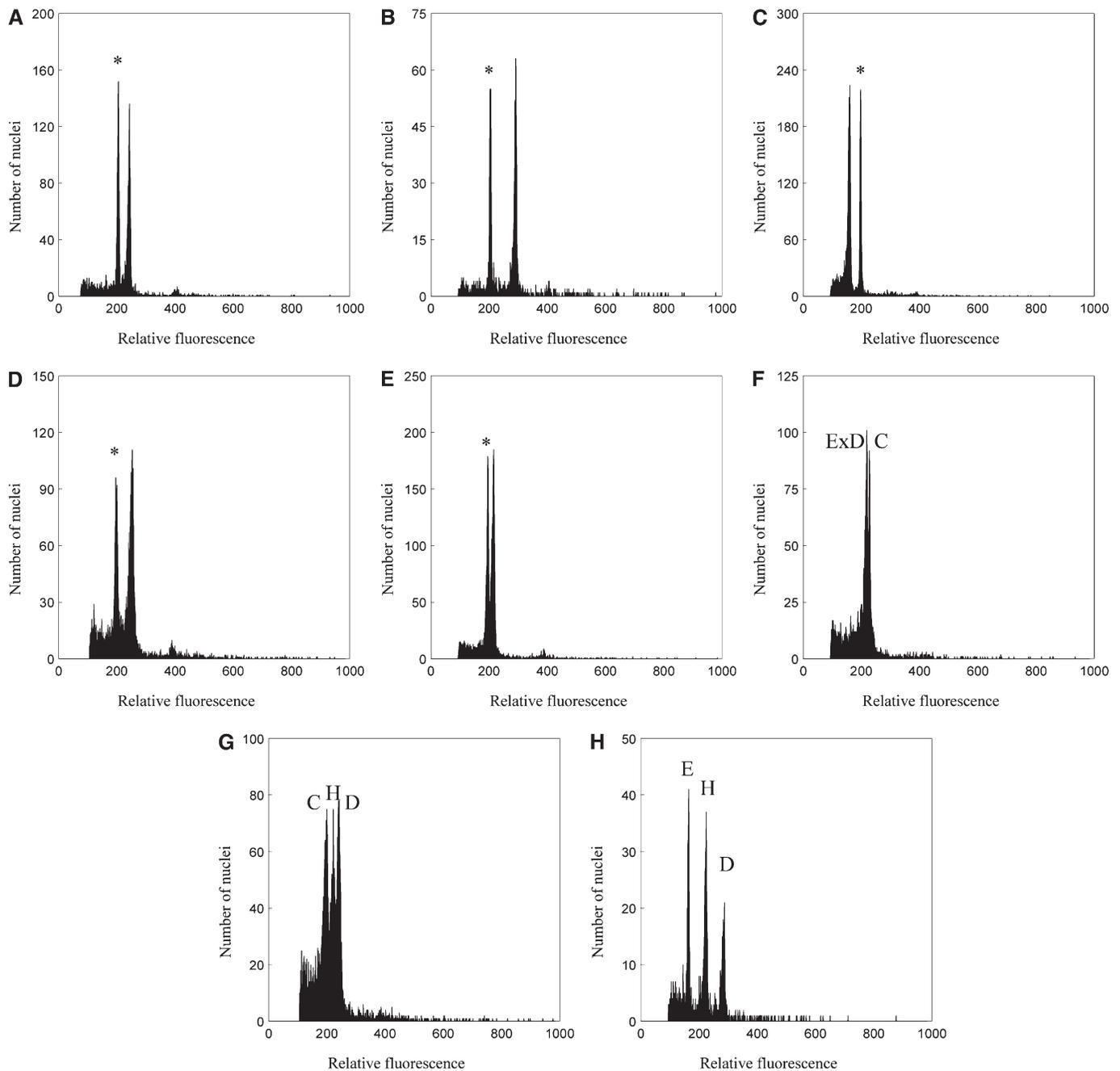
Fig. 2. Representative flow cytometric histograms for the estimation of relative genome sizes in species and hybrids from the *Dryopteris carthusiana* group. Analyses of (A) *D. carthusiana*, (B) *D. dilatata*, (C) *D. expansa*, (D) *D. ×deweveri*, and (E) *D. ×ambroseae* together with *Vicia faba* as an internal reference standard (marked by an asterisk). (F) Simultaneous analysis of species and hybrids with the most similar genome sizes: *D. ×ambroseae* and *D. carthusiana* (labeled as ExD and C, respectively). Simultaneous analyses of parental taxa and their putative hybrids: (G) *D. carthusiana*, *D. ×deweveri* and *D. dilatata* (labeled as C, H and D, respectively) and (H) *D. expansa*, *D. ×ambroseae* and *D. dilatata* (labeled as E, H and D, respectively). Nuclei from all samples were simultaneously isolated, stained with DAPI, and analyzed.

specific for species and hybrids. In fact, relative fluorescence intensities for particular species and hybrids were mostly non-overlapping; the only exception was *D. carthusiana* and *D. ×ambroseae*, with an average difference of about 4.7%. Nevertheless, the overlap among their variation intervals was not significant (Mann–Whitney U test; $Z = 9.64$, $U = 145$, df $= 1$, $P < 0.0001$). There was a good congruence between actual (= mea-sured by FCM) and theoretical (= calculated from the data of putative parents) relative genome size values of hybrid plants; the difference never exceeded 2.5%.

The knowledge of genome composition of individual species and hybrids (Table 1) allowed us to compare the relative size of parental genomes. Setting the genome of *D. expansa* (genome E) to a unit value, genomes of *D. intermedia* (genome I) and

Table 1.   Relative fluorescence intensities of 858 individuals corresponding to three parental species and their hybrids from the *Dryopteris carthusiana* group. Isolated nuclei were stained with DAPI, and *Vicia faba* 'Inovec' (2C = 26.90 pg) was set as the unit value.

Taxon	Genome composition [a]	DNA ploidy level	Relative fluorescence intensity (mean ± SD)	No. of samples
D. expansa	EE	2x	0.815 ± 0.020	277
D. carthusiana	IISS	4x	1.163 ± 0.014	237
D. dilatata	IIEE	4x	1.408 ± 0.019	244
D. ×sarvelae (= *D. carthusiana* × *D. expansa*)	ISE	3x	0.980 ± 0.015	2
D. ×ambroseae (= *D. dilatata* × *D. expansa*)	IEE	3x	1.111 ± 0.022	82
D. ×deweveri (= *D. carthusiana* × *D. dilatata*)	IIES	4x	1.254 ± 0.028	16

[a] Genome composition: E = *D. expansa*, I = *D. intermedia*, S = *D. "stanley-walkeri"/"semicristata"*

D. "stanley-walkeri"/"semicristata" (genome S) equal to 0.728 and 0.699, respectively.

Species abundance and hybrid frequency—All three species from the *D. carthusiana* complex together with all putative hybrid combinations were recorded from various locations within the areas studied. *Dryopteris carthusiana* and *D. dilatata* were the most common components of deciduous broad-leaved, mixed, and coniferous forests from lowlands to mountain regions (see Appendix 1). While *D. dilatata* has no special requirement for moisture, *D. carthusiana* occurs more frequently in moist habitats and swamps. Diploid *D. expansa* was also quite common (ca. 66% of localities), mainly in higher altitudes and/or at various humid stands such as bottoms of deep valleys or alder forests.

Hybrid plants were collected from 39 of the 85 localities (46%) and accounted for 11.7% of all samples (100 individuals). The frequency of particular hybrid combinations, however, differed markedly. The most common hybrid was *D. ×ambroseae* (= *D. dilatata* × *D. expansa*), only distantly followed by *D. ×deweveri* (= *D. carthusiana* × *D. dilatata*) and the rarest, *D. ×sarvelae* (= *D. carthusiana* × *D. expansa*). Disregarding localities with fewer than five sampled individuals, *D. ×ambroseae* was recorded in 72% of localities with the presence of both parents (see Fig. 3). The homoploid hybrid *D. ×deweveri* occurred at about 5% of localities inhabited by both parents (Fig. 3), while *D. ×sarvelae* was not found in 14 mixed *D. carthusiana–D. expansa* localities (this rare hybrid was only confirmed in two previously known sites; Jessen and Rasbach, 1987).

Sympatric occurrence of more species and/or hybrids was a very common phenomenon. Considering localities with multiple fern samples (≥5 sampled plants), only two of 34 localities contained a single species (see Appendix 1). The taxonomic composition in other localities was as follows: two species/hybrids, 12 localities; three species/hybrids, 9 localities; four species/hybrids, 10 localities; five species/hybrids, 1 locality. The most salient example was population no. 40 (Czech Republic, Českomoravská vrchovina, Kostelní Myslová), where all taxa, except for one hybrid (*D. ×sarvelae*), were recorded.

Morphometric analyses—Phenetic relationships among individual *Dryopteris* plants were visualized by principal component analysis (PCA). Particular species or hybrids usually showed high variation and formed loose, more or less overlapping clusters (results not shown).

Discriminant analyses were employed to select a set of characters that allowed the best separation of groups of objects (i.e., species and hybrids characterized by relative genome sizes) defined a priori and to determine the proportion of correctly classified plants. Discriminant analysis of all OTUs (i.e., three

parental species and two hybrids) revealed three distinct clusters (Fig. 4): (1) plants corresponding to *D. carthusiana*, (2) plants corresponding to *D. expansa* + *D. dilatata*, and (3) hybrid individuals: *D. ×ambroseae* + *D. ×deweveri*. Despite a high number of analyzed groups (five different OTUs), a very high rate of correctly classified objects was achieved (nearly 96%; Table 2). Because hybrid plants were unambiguously delimited based on aborted spores, separate analyses of parental species and hybrids were performed in the next step.

Discriminant analysis of three parental species corresponded well with the results of the whole data set. Once again, *D. carthusiana* formed an isolated cluster (all individuals correctly classified), while about 5% of individuals corresponding to *D. dilatata* and *D. expansa* were not assigned to the correct group. The characters most tightly correlated with the first canonical axis (presented according to a decreasing discrimination power) and thus separating *D. carthusiana* from the other two species were v33 (length of the dark central stripe/total length of the petiole scale), v18 (length of the dark central stripe on the scale), v16 (length of the petiole scale), v19 (glandularity of the rachis), v2 (width of the leaf lamina), v17 (scale width), and v6 (length of the lowermost pinna).

The second canonical axis discriminated between the phenotypically similar *D. dilatata* and *D. expansa*. The following
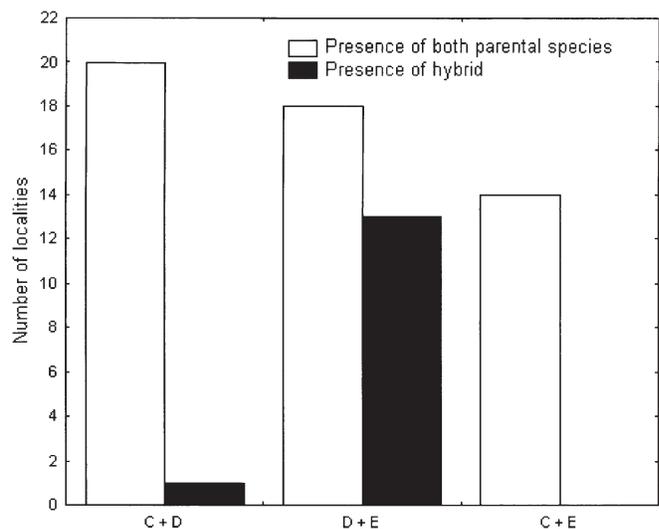

Fig. 3.   The occurrence of hybrid individuals in localities with sympatric growth of parental species. Only localities with at least five sampled plants were considered. C = *Dryopteris carthusiana*, D = *D. dilatata*, E = *D. expansa*.
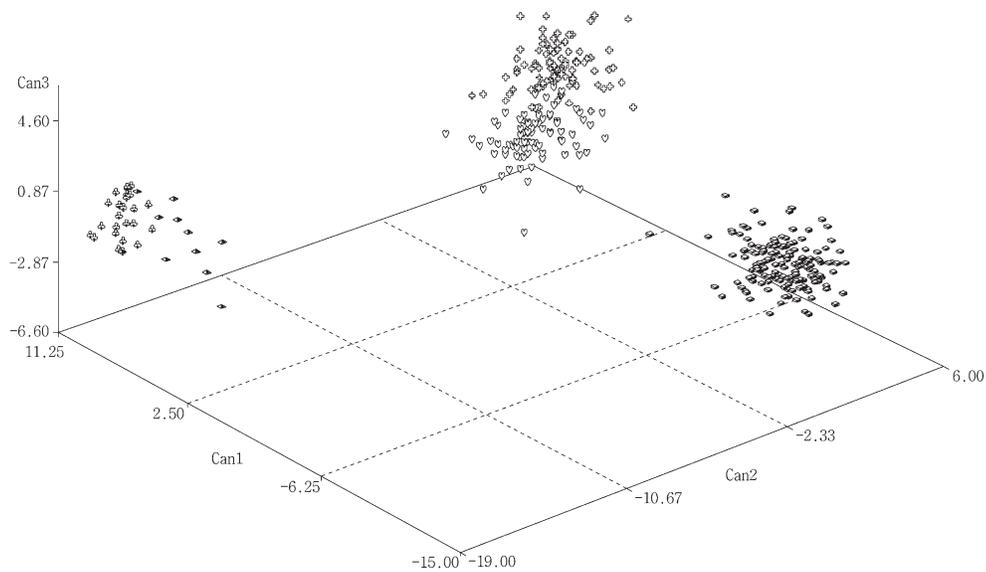
Fig. 4.   Canonical discriminant analysis of 609 individuals representing three parental species and two hybrids from the *Dryopteris carthusiana* group. Cube = *D. carthusiana*, heart = *D. dilatata*, cross = *D. expansa*, pyramid = *D. ×deweveri*, club = *D. ×ambroseae*.

characters contributed most to this division: v17 (scale width), v28 (length of the basal basiscopic pinnula of the lowermost pinna/length of the lowermost pinna), v32 (length/width of the petiole scale), v33 (length of the dark central stripe/total length of the petiole scale), v31 (length of the basal basiscopic pinnula of the lowermost pinna/length of the basal acroscopic pinnula of the lowermost pinna), v27 (length of the basal basiscopic pinnula of the lowermost pinna/lamina length), and v7 (length of the basal basiscopic pinnula of the lowermost pinna).

Hybrid plants (*D. ×ambroseae* and *D. ×deweveri*) can be successfully discriminated by scale characters (v18 [length of the dark central stripe], v33 [ratio between the length of the dark central stripe and the total length of the scale], and v16 [length of the scale]), glandularity of the rachis (v19), and length/width of the leaf lamina (v22).

Appendix S2 (see online Supplemental Data) summarizes descriptive statistics of 13 taxonomically most informative characters for the three parental species and two hybrids from the *D. carthusiana* group.

DISCUSSION

Genome size variation and its value for taxonomic decisions—Interspecific hybridization and polyploidization are processes of central importance in population and evolutionary

biology because of their impacts on the maintenance of species distinctions, enhancements of genetic diversity, and diversification (Hersch-Green and Cronn, 2009). The present work aimed to evaluate phenotypic variation and assess the frequency of interspecific hybridization in a taxonomically challenging polyploid fern group, the *Dryopteris carthusiana* complex. Flow cytometric analyses of a representative set of samples from Central Europe revealed large differences in relative genome size among the three species constituting this group. Average fluorescence intensities of two tetraploid species (*D. carthusiana* and *D. dilatata*) differed by 21%, while diploid *D. expansa* had a 43% smaller genome than *D. carthusiana* (the tetraploid with the smaller amount of nuclear DNA). Such differences in genome size not only allowed unambiguous determination of the species, but also reliable detection of interspecific hybrids of any parental combination. The closest fluorescence values were found between *D. carthusiana* and *D. ×ambroseae* (average difference 4.7%) and between *D. carthusiana* and *D. ×deweveri* (average difference 7.8%). Nevertheless, such plants can be easily discriminated by observing spore quality (well developed in *D. carthusiana* but aborted in hybrids). Collectively, the data obtained on genome size variation show that flow cytometry provides a powerful tool for reliable identification of species and hybrids in the *D. carthusiana* complex. In this fern group, neither conventional phenotype-based taxonomy nor conventional karyology can be easily used to recognize the

TABLE 2.   Results of classificatory discriminant analysis of 609 individuals corresponding to three parental species and two hybrids from the *Dryopteris carthusiana* group.

Actual group membership	Predicted group membership (number of observations / percentage classified into groups)				
	D	E	C	D × E	C × D
D	180 / 95.7	8 / 4.3	—	—	—
E	15 / 9.2	148 / 90.8	—	—	—
C	—	—	209 / 100.0	—	—
D × E	—	—	—	37 / 100.0	—
C × D	—	—	—	1 / 8.3	11 / 91.7

Notes: C = *D. carthusiana*, D = *D. dilatata*, E = *D. expansa*, D × E = *D. ×ambroseae*, C × D = *D. ×deweveri*

species and hybrids because of the considerable phenotypic plasticity and the difficulties inherent in counting high numbers of chromosomes, respectively.

Frequency of interspecific hybridization—Significant differences in relative genome size between different species allowed a reliable assessment of the frequency of interspecific hybridization. *Dryopteris* is considered one of the most hybrid-prone fern genera in temperate regions, as evidenced by 21 different hybrid combinations reported from Europe (Dostál et al., 1984) and 25 from North America (Montgomery and Wagner, 1993). However, the putative hybrids were almost exclusively identified based on morphological characters while more robust data (e.g., karyological and/or molecular) supporting their existence were often lacking. In our study, we detected hybrid individuals in nearly half of the localities (39 of 85) subjected to FCM investigation. This is certainly a much higher incidence of interspecific hybridization than would be expected based on previous works. For example, some studies of the *D. carthusiana* group performed in different European countries have not reported any crosses (e.g., Widén and Sorsa, 1968; Piękoś-Mirkowa, 1979; Seifert and Holderegger, 1995), while others found only a single hybrid individual (e.g., Döpp and Gätzi, 1964; Benl and Eschelmüller, 1970; Fraser-Jenkins, 1977; Leonhards et al., 1990). Triploid *Dryopteris ×ambroseae* (= *D. dilatata × D. expansa*) was the most common hybrid combination, detected in 32 localities (82 individuals). Despite the identical number of chromosomes in putative parental species, *D. ×deweveri* (= *D. carthusiana × D. dilatata*) occurred much less frequently, recorded from only nine localities (16 individuals altogether). Nevertheless, *D. ×deweveri* is likely distributed throughout Europe as indicated by reports of karyologically verified plants (Walker, 1955; Sorsa and Widén, 1968; Widén et al., 1970; Leonhards et al., 1990). The last hybrid combination *D. ×sarvelae* (= *D. carthusiana × D. expansa*) is extremely rare. Only two individuals from two previously known localities (Jessen and Rasbach, 1987) were encountered.

Marked differences in the frequency of particular hybrid combinations can be explained by the presence or absence of common genomic elements by the putative parental species. Based on evidence of genome homology between *D. expansa* and *D. dilatata* (*D. expansa* is hypothesized as one of the progenitors of the allotetraploid *D. dilatata*; Gibby and Walker, 1977; Viane 1986; Krause 1998; see also Table 1), extensive interspecific hybridization appears reasonable. An analogous situation is known in North America, where allotetraploid *D. carthusiana* hybridizes frequently with one of its diploid ancestors, *D. intermedia*, resulting in the common and widespread hybrid *D. ×triploidea* Wherry (Tryon and Britton, 1966; Xiang et al., 2000). *Dryopteris carthusiana* and *D. dilatata* also share the *D. intermedia* genome (Table 1), which may facilitate their interspecific hybridization. On the contrary, the lack of genome identity between *D. carthusiana* and *D. expansa* may reduce the likelihood of successful hybridization between them. A congruence between the frequency of interspecific hybridization and species relationship has been documented in several plant groups, both flowering (Mosseler, 1990) and spore-bearing (Yatabe et al., 2009).

Another, nonexclusive explanation of differences in the frequency of interspecific hybrids, takes into account breeding systems and associated sex expression in gametophytes of diploid vs. polyploid *Dryopteris* species. While the majority of diploids possess an outcrossing (or mixed) mating system,

polyploids are predominantly selfing (Soltis and Soltis, 1987, 1990; Soltis et al., 1988; Masayuma and Watano, 1990; Xiang et al., 2000; Flinn, 2006). Therefore, the probably selfing breeding system in tetraploid *D. carthusiana* and *D. dilatata* (Xiang et al., 2000; Flinn, 2006) likely reduces opportunities for interspecific hybridization, which is consistent with the lower number of crosses (*D. ×deweveri*) discovered in the current study.

Species and hybrid delimitation—A clear delimitation of species and hybrids in our study opened the way for a critical assessment of phenotypic variation and allowed us to select a set of species- and hybrid-specific morphological characters. Unlike previous studies, which either exclusively used subjective morphological criteria for species recognition (e.g., Nannfeldt, 1966; Widén et al., 1967; Benl and Eschelmüller, 1983) or karyologically examined only a few individuals (e.g., Widén et al., 1970; Nardi, 1976; Piękoś-Mirkowa, 1979; Leonhards et al., 1990), our approach involved unambiguous assignment of all samples to a particular species or hybrid based on the relative genome size. Therefore, we consider the present morphometric results and taxonomic conclusions to be highly robust and reliable. The results of discriminant analyses showed that *D. carthusiana* is phenotypically a well-defined species that possesses several unique characters and whose determination is usually clear. On the other hand, differentiation between *D. expansa* and *D. dilatata* is much more challenging, and some individuals will likely be misclassified even if a set of characters with the highest discrimination power is used. The taxonomically most informative characters in the *D. carthusiana* group include scale characteristics (length and width of the scale and the length of the dark central stripe), size of the leaf lamina together with the size and relative proportions of certain lamina parts (e.g., characteristics of the lowermost pinna, the basal basiscopic pinnula of the lowermost pinna), and the glandularity of the rachis. Despite their high degree of overall morphological similarity with putative parental species, hybrid ferns can be easily recognized by their aborted spores. Therefore, only fertile specimens allow reliable determination. All Central European members of the *D. carthusiana* groups (except the very rare hybrid *D. ×sarvelae*) can be determined according to the following key:

1a Spores abortive   2
1b Spores well-developed   3
2a Rachis sparsely glandular; petiole scales (8–)11–13(–16) mm long, with distinct, (6.5–)8.5–11(–13.5) mm long dark central stripe, usually extending to the upper half of the scale (occasionally up to the apex); lamina (0.9–)1.5–1.8(–1.9) mes longer than wide

D. ×ambroseae Fraser-Jenk. et Jermy
(= *D. dilatata × D. expansa*)

2b Rachis densely glandular; petiole scales (6–)8–10.5(–13.5) mm long, concolorous or with (2–)3–4(–6.5) mm long, dark, central stripe, typically terminating in the lower half of the scale; lamina (1.6–)1.7–2.1(–2.4) times longer than wide

D. ×deweveri (Jansen) Jansen et Wachter
(= *D. carthusiana × D. dilatata*)

3a Petiole scales (4–)5–6(–8.5) mm long, concolorous; rachis glabrous or very scarcely glandular (1–6 glands per rachis); lamina (10–)15–20(–35) cm wide, the lowermost pinna (4.5–)7–10(–17.5) cm long

D. carthusiana (Vill.) H. P. Fuchs

3b Petiole scales (6.5–)11–14(–17) mm long, with distinct dark central stripe; rachis densely glandular; lamina (14–)22–30(–41) cm wide, the lowermost pinna (7–)10.5–15(–21) cm long   4

4a Petiole scales (1.5–)3–4(–12) mm wide, (1.0–)3.2–4.2(–9.3) times longer than wide, with dark, central stripe often extending up to the apex, covering (65–)90–100% of the scale length; basal basiscopic pinnula of the lowermost pinna (2–)3.5–5.5(–9) cm long, its ratio to the lowermost pinna (0.15–)0.35–0.45(–0.70); spores dark brown

D. dilatata (Hoffm.) A. Gray

4b Petiole scales (2.5–)4.5–5.5(–7.5) mm wide, (1.9–)2.4–2.8(–4.7) times longer than wide, the dark central stripe usually not extending up to the apex, covering (40–)70–90(–100)% of the scale length; basal basiscopic pinnula of the lowermost pinna (2.5–)5.5–7.5(–10.5) cm long, its ratio to the lowermost pinna (0.25–)0.45–0.55(–0.65); spores yellowish to light brown

D. expansa (C. Presl) Fraser-Jenk. et Jermy

LITERATURE CITED

BENL, G., AND A. ESCHELMÜLLER. 1970. *Dryopteris dilatata × assimilis* in Bayern. *Berichte der Bayerischen Botanischen Gesellschaft* 42: 185–188.

BENL, G., AND A. ESCHELMÜLLER. 1983. Zum Vorkommen weniger bekanter *Dryopteris*-Sippen im bayerischen Alpen- und Voralpenraum. *Berichte der Bayerischen Botanischen Gesellschaft* 54: 77–102.

BENNETT, M. D., AND I. J. LEITCH. 2005. Nuclear DNA amounts in angiosperms: Progress, problems and prospects. *Annals of Botany* 95: 45–90.

BRITTON, D. M. 1972. Spore ornamentation in the *Dryopteris spinulosa* complex. *Canadian Journal of Botany* 50: 1617–1621.

BUREŠ, P., L. TICHÝ, Y. WANG, AND J. BARTOŠ. 2003. Occurrence of *Polypodium ×mantoniae* and new localities for *P. interjectum* in the Czech Republic confirmed using flow cytometry. *Preslia* 75: 293–310.

CHRTEK, J. 1988. *Dryopteris* Adanson, kapraď. *In* S. Hejný and B. Slavík [eds.], Květena ČSR 1, 262–272. Academia, Praha, Czech Republic.

CORLEY, H. V., AND M. GIBBY. 1981. *Dryopteris ×sarvelae* in Scotland: A new hybrid for the British Isles. *Fern Gazette* 12: 178.

DIMITROVA, D., I. EBERT, J. GREILHUBER, AND S. KOZHUHAROV. 1999. Karyotype constancy and genome size variation in Bulgarian *Crepis foetida* s. l. (Asteraceae). *Plant Systematics and Evolution* 217: 245–257.

DOLEŽEL, J., J. GREILHUBER, S. LUCRETTI, A. MEISTER, M. A. LYSÁK, L. NARDI, AND R. OBERMAYER. 1998. Plant genome size estimation by flow cytometry: Inter-laboratory comparison. *Annals of Botany* 82: 17–26.

DOLEŽEL, J., J. GREILHUBER, AND J. SUDA. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* 2: 2233–2244.

DOLEŽEL, J., S. SGORBATI, AND S. LUCRETTI. 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Physiologia Plantarum* 85: 625–631.

DÖPP, W., AND W. GÄTZI. 1964. Der Bastard zwischen tetraploider und diploider *Dryopteris dilatata*. *Bericht der Schweizerischen Botanischen Gesellschaft* 74: 45–53.

DOSTÁL, J., C. R. FRASER-JENKINS, AND T. REICHSTEIN. 1984. *Dryopteris*. *In* K. U. Kramer [ed.], Illustrierte Flora von Mitteleuropa. Band I, Teil 1. *Pteridophyta*, 3. Auflage, 136–169. Verlag Paul Parey, Berlin, Germany.

EBIHARA, A., H. ISHIKAWA, S. MATSUMOTO, S. LIN, K. IWATSUKI, M. TAKAMIYA, Y. WATANO, AND M. ITO. 2005. Nuclear DNA, chloroplast DNA, and ploidy analysis clarified biological complexity of the *Vandenboschia radicans* complex (Hymenophyllaceae) in Japan and adjacent areas. *American Journal of Botany* 92: 1535–1547.

EKRT, L. 2000. Komplex *Dryopteris carthusiana* agg. na Šumavě a v Předšumaví. Bc. thesis, České Budějovice, University of South Bohemia, Czech Republic.

EKRT, L., AND D. PŮBAL. 2008. Novinky v květeně cévnatých rostlin české Šumavy a přiléhajícího Předšumaví. I. *Silva Gabreta* 14: 19–38.

EKRT, L., AND M. ŠTECH. 2008. A morphometric study and revision of the *Asplenium trichomanes* group in the Czech Republic. *Preslia* 80: 325–347.

EKRT, L., P. TRÁVNÍČEK, V. JAROLÍMOVÁ, P. VÍT, AND T. URFUS. 2009. Genome size and morphology of the *Dryopteris affinis* group in Central Europe. *Preslia* 81: 261–280.

ELLSTRAND, N. C. 1992. Gene flow by pollen—Implications for plant conservation genetics. *Oikos* 63: 77–86.

ELLSTRAND, N. C., R. WHITKUS, AND L. H. RIESEBERG. 1996. Distribution of spontaneous hybrids. *Proceedings of the National Academy of Sciences, USA* 93: 5090–5093.

FLINN, K. M. 2006. Reproductive biology of three fern species may contribute to differential colonization success in post-agricultural forests. *American Journal of Botany* 93: 1289–1294.

FRASER-JENKINS, C. R. 1977. *Dryopteris ×brathaica* Fraser-Jenk. et Reichstein hybr. nov., the putative hybrid of *D. carthusiana × D. filix-mas*. *Fern Gazette* 11: 337.

FRASER-JENKINS, C. R. 1982. *Dryopteris* in Spain, Portugal and Macaronesia. *Boletim da Sociedade Broteriana Séries 2*, 55: 175–336.

FRASER-JENKINS, C. R. 1986. A classification of the genus *Dryopteris* (Pteridophyta: Dryopteridaceae). *Bulletin of the British Museum (Natural History). Botany* 14: 183–218.

FRASER-JENKINS, C. R. 1993. *Dryopteris*. *In* T. G. Tutin, N. A. Burges, A. O. Chater, et al., [eds.], Flora europaea, vol. 1, 2nd ed., 27–30. Cambridge University Press, Cambridge, UK.

FRASER-JENKINS, C. R. 2001. *Dryopteris stanley-walkeri* Fras.-Jenk., the missing diploid common ancestor of *D. carthusiana* and *D. cristata* (Pteridophyta: Dryopteridaceae). *In* P. C. Panda and S. S. Samant [eds.], Plant diversity of the Himalaya: Prof. Y. P. S. Pangtey commemoration volume, 119–152. Gyanodaya Prakashan, Nainital, India.

FREY, W., J. P. FRAHM, E. FISCHER, AND W. LOBIN. 1995. Kleine Kryptogamenflora Band IV, Die Moss- und Farnpflanzen Europas. Gustav Fischer Verlag, Stuttgart.

GIBBY, M. 1983. The *Dryopteris dilatata* complex in Macaronesia and the Iberian Peninsula. *Acta Botanica Malacitana* 8: 59–72.

GIBBY, M., AND S. WALKER. 1977. Further cytogenetic studies and a reappraisal of the diploid ancestry in the *Dryopteris carthusiana* complex. *The Fern Gazette* 11: 315–324.

GRANT, V. 1971. Plant speciation. Columbia University Press, New York, New York, USA.

GREILHUBER, J. 2005. Intraspecific variation in genome size in angiosperms: Identifying its existence. *Annals of Botany* 95: 91–98.

HEGARTY, M. J., AND S. J. HISCOCK. 2005. Hybrid speciation in plants: New insights from molecular studies. *New Phytologist* 165: 411–423.

HERSCH-GREEN, E. I., AND R. CRONN. 2009. Tangled trios?: Characterizing a hybrid zone in *Castilleja* (Orobanchaceae). *American Journal of Botany* 96: 1519–1531.

HOLMGREN, P. K., N. H. HOLMGREN, AND L. C. BARNETT. 1990. Index herbariorum, part I: The herbaria of the world, 8th ed. New York Botanical Garden, Bronx, New York, USA.

HOLUBOVÁ, R. 2006. Cytotaxonomická a morfologická studie okruhu *Dryopteris dilatata* v České republice s důrazem na České středohoří. M.S. thesis, Charles University, Prague, Czech Republic.

HUTTON, C. 1992. The common ancestor of the allotetraploid ferns *Dryopteris carthusiana* and *Dryopteris cristata*: A chloroplast DNA analysis. M.S. thesis, Mount Holyoke College, South Hadley, Massachussetts, USA.

IVANOVA, D. 2006. *Dryopteris ×ambroseae* (Dryopteridaceae: Pteridophyta) a hybrid new to Bulgaria. *Phytologica Balcanica* 12: 351–356.

JESSEN, S., AND H. RASBACH. 1987. *Dryopteris ×sarvelae*, erster Nachweis für Mitteleuropa. Abstammung, verwandte Arten und Hybriden (Pteridophyta). *Gleditschia* 15: 57–72.

KLECKA, W. R. 1980. Discriminant analysis. Sage University paper series: Quantitative applications in the social sciences series no. 07-019. Sage Publications, Beverly Hills, California, USA.

KRAUSE, S. 1998. *Dryopteris* Adans. *In* R. Wisskirchen and H. Haeupler [eds.], Standardliste der Farn- und Blütenpflanzen Deutschlands, 182–190. Ulmer, Stuttgart, Germany.

KRON, P., J. SUDA, AND B. C. HUSBAND. 2007. Applications of flow cytometry to evolutionary and population biology. *Annual Review of Ecology, Evolution, and Systematics* 38: 847–876.

KUBÁT, K., L. HROUDA, J. CHRTEK JR., Z. KAPLAN, J. KIRSCHNER, AND J. ŠTĚPÁNEK [eds.]. 2002. Klíč ke květeně České republiky. Academia, Praha, Czech Republic.

LEITCH, I. J., AND M. D. BENNETT. 2007. Genome size and its uses: The impact of flow cytometry. *In* J. Doležel, J. Greilhuber, and J. Suda, [eds.], Flow cytometry with plant cells, 153–176. Wiley-VCH, Weinheim, Germany.

LEONHARDS, W., H. RASBACH, W. JÄGER, AND H. W. BENNERT. 1990. Vorkommen und Cytologie von *Dryopteris ×deweveri* (=*Dryopteris carthusiana × dilatata*, Dryopteridaceae, Pteridophyta) in Nordrhein-Westfalen. *Tuexenia* 10: 17–24.

LEVIN, D. A., J. FRANCISCO ORTEGA, AND R. K. JANSEN. 1996. Hybridization and the extinction of rare plant species. *Conservation Biology* 10: 10–16.

LOUREIRO, J., P. TRÁVNÍČEK, J. RAUCHOVÁ, T. URFUS, P. VÍT, M. ŠTECH, S. CASTRO, AND J. SUDA. 2010. The use of flow cytometry in the biosystematics, ecology and population biology of homoploid plant. *Preslia* 82: 3–21.

MAHELKA, V., J. SUDA, V. JAROLÍMOVÁ, P. TRÁVNÍČEK, AND F. KRAHULEC. 2005. Genome size discriminates between closely related taxa *Elytrigia repens* and *E. intermedia* (Poaceae: Triticeae) and their hybrid. *Folia Geobotanica* 40: 367–384.

MANTON, I. 1950. Problems of cytology and evolution of the Pteridophyta. [Publisher unknown], Cambridge, UK.

MANTON, I., AND S. WALKER. 1953. Cytology of the *Dryopteris spinulosa* complex in eastern North America. *Nature* 171: 1116–1117.

MASUYAMA, S., AND Y. WATANO. 1990. Trend for inbreeding in polyploid pteridophytes. *Plant Species Biology* 5: 13–17.

MONTGOMERY, J. D., AND W. H. WAGNER JR. 1993. *Dryopteris. In* Flora of North America Editorial Committee [eds.], Flora of North America north of Mexico, 280–289. Oxford University Press, New York, New York, USA.

MOSSELER, A. 1990. Hybrid performance and species crossability relationships in willows (*Salix*). *Canadian Journal of Botany* 68: 2329–2338.

NANNFELDT, J. A. 1966. *Dryopteris dilatata* och *Dr. assimilis* i Sverige. *Botaniska Notiser* 119: 136–152.

NARDI, E. 1976. *Dryopteris assimilis* S. Walker in Italia. *Webbia* 30: 457–478.

OTTO, F. 1990. DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. *In* H. A. Crissman and Z. Darzynkiewicz [eds.], Methods in cell biology, vol. 33, 105–110. Academic Press, New York, New York, USA.

PIĘKOŚ, H. 1974. Mieszaniec *Dryopteris assimilis × dilatata* w Polsce. – The hybrid *Dryopteris assimilis × dilatata* in Poland. *Fragmenta Floristica et Geobotanica* 20: 333–338.

PIĘKOŚ-MIRKOWA, H. 1979. Paprocie z grupy *Dryopteris dilatata* w Polsce. *Monographiae Botanicae* 59: 1–75.

PIĘKOŚ-MIRKOWA, H. 1987. The *Dryopteris dilatata* complex in the Soviet Far East. *Botanica Helvetica* 97: 167–177.

PIĘKOŚ-MIRKOWA, H. 1993. Altitudinal distribution pattern of *Dryopteris dilatata* and *D. expansa* (Dryopteridaceae) in the Alps, the Carpathians and the Caucasus. *Floristica et Geobotanica* (supplementum 2): 307–312.

RIESEBERG, L. H. 1997. Hybrid origins of plant species. *Annual Review of Ecology, Evolution, and Systematics* 28: 359–389.

RIESEBERG, L. H., AND S. E. CARNEY. 1998. Plant hybridization. *New Phytologist* 140: 599–624.

ROSENBAUMOVÁ, R., I. PLAČKOVÁ, AND J. SUDA. 2004. Variation in *Lamium* subg. *Galeobdolon* (Lamiaceae): Insights from ploidy levels, morphology and isozymes. *Plant Systematics and Evolution* 244: 219–244.

SEEHAUSEN, O. 2004. Hybridization and adaptive radiation. *Trends in Ecology & Evolution* 19: 198–207.

SEIFERT, M., AND R. HOLDEREGGER. 1995. Morphologische Untersuchungen innerhalb der *Dryopteris carthusiana* Gruppe. *Farnblätter* 26/27: 58–77.

SOLTIS, P. S., AND D. E. SOLTIS. 1987. Population structure and estimates of gene flow in the homosporous fern *Polystichum munitum*. *Evolution* 41: 620–629.

SOLTIS, P. S., AND D. E. SOLTIS. 1990. Evolution of inbreeding and outcrossing in ferns and fern-allies. *Plant Species Biology* 5: 1–11.

SOLTIS, P. S., D. E. SOLTIS, AND K. E. HOLSINGER. 1988. Estimates of intragametophytic selfing and interpopulational gene flow in homosporous ferns. *American Journal of Botany* 75: 1765–1770.

SORSA, V., AND C. J. WIDÉN. 1968. The *Dryopteris spinulosa* complex in Finland, a cytological and chromatographic study of some hybrids. *Hereditas* 60: 273–293.

SUDA, J., A. KRAHULCOVÁ, P. TRÁVNÍČEK, R. ROSENBAUMOVÁ, T. PECKERT, AND F. KRAHULEC. 2007. Genome size variation and species relationships in *Hieracium* sub-genus *Pilosella* (Asteraceae) as inferred by flow cytometry. *Annals of Botany* 100: 1323–1335.

TRYON, R., AND M. BRITTON. 1966. A study of variation in the cytotypes of *Dryopteris spinulosa*. *Rhodora* 68: 59–92.

UYEDA, J. C., AND S. R. KEPHART. 2006. Detecting species boundaries and hybridization in *Camassia quamash* and *C. leichtlinii* (Agavaceae) using allozymes. *Systematic Botany* 31: 642–655.

VIANE, R. L. 1986. Taxonomical significance of the leaf indument in *Dryopteris* (Pteridophyta). I. Some North American, Macronesian and European taxa. *Plant Systematics and Evolution* 153: 77–105.

WAGNER, W. H. JR. 1971. Evolution of *Dryopteris* in relation to the Appalachians. *In* P. C. Holt [ed.], The distributional history of the biota of the southern Appalachians. Research Division Monograph 2, 147–192. Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

WAGNER, W. H. JR., AND L. K. CHEN. 1965. Abortion of spores and sporangia as a tool in the detection of *Dryopteris* hybrids. *American Fern Journal* 55: 9–29.

WAGNER, W. H. JR., F. S. WAGNER, A. A. REZNICEK, AND C. R. WERTH. 1992. *×Dryostichum singulare* (Dryopteridaceae), a new fern nothogenus from Ontario. *Canadian Journal of Botany* 70: 245–253.

WALKER, S. 1955. Cytogenetic studies in the *Dryopteris spinulosa* complex I. *Watsonia* 3: 193–209.

WALKER, S. 1961. Cytogenetic studies in the *Dryopteris spinulosa* complex II. *American Journal of Botany* 48: 607–614.

WERTH, C. R. 1989. Isozyme evidence on the origin of *Dryopteris cristata* and *D. carthusiana*. *American Journal of Botany* 76 (supplement): 208 [abstract].

WERTH, C. R., AND C. KUHN. 1989. A study of the morphological attributes of the hypothetical diploid fern *Dryopteris* "semicristata". *American Journal of Botany* 76 (supplement): 208 [abstract].

WERTH, C. R., AND D. B. LELLINGER. 1992. Genomically preserved plants and their nomenclature. *Taxon* 41: 513–521.

WIDÉN, C. J., J. SARVELA, AND T. AHTI. 1967. The *Dryopteris spinulosa* complex in Finland. *Acta Botanica Fennica* 77: 1–25.

WIDÉN, C. J., AND V. SORSA. 1968. On the intraspecific variability of *Dryopteris assimilis* S. Walker and *Dryopteris spinulosa* Watt, a chromatographic and cytological study. *Hereditas* 62: 1–13.

WIDÉN, C. J., V. SORSA, AND J. SARVELA. 1970. *Dryopteris dilatata* s. lat. in Europe and the Island of Madeira, a chromatographic and cytological study. *Acta Botanica Fennica* 91: 1–30.

WHITHAM, T. G., P. A. MORROW, AND B. M. POTTS. 1991. Conservation of hybrid plants. *Science* 254: 779–780.

XIANG, L., C. R. WERTH, S. N. EMERY, AND D. E. MCCAULEY. 2000. Population-specific gender-biased hybridization between *Dryopteris intermedia* and *D. carthusiana*: Evidence from chloroplast DNA. *American Journal of Botany* 87: 1175–1180.

YATABE, Y., W. SHINOHARA, S. MATSUMOTO, AND N. MURAKAMI. 2009. Patterns of hybrid formation among cryptic species of bird-nest fern, *Asplenium nidus* complex (Aspleniaceae), in West Malesia. *Botanical Journal of the Linnean Society* 160: 42–63.

APPENDIX 1. Locality details, including geographic coordinates (WGS-84 system), altitude (m a.s.l.), collector name(s), date of collection and herbarium collections where vouchers are stored (abbreviated according to Holmgren et al., 1990) for 85 populations of the *Dryopteris carthusiana* group. Total number of analyzed plants together with taxonomic composition and number of particular species and hybrids is also provided. Samples used for morphometric analyses are marked by an asterisk. Country abbreviations: AT = Austria, CZ = Czech Republic, DE = Germany, SK = Slovakia. Sample abbreviations: car = *D. carthusiana*, dil = *D. dilatata*, exp = *D. expansa*, ×amb = *D. ×ambroseae* (= *D. dilatata* × *D. expansa*), ×dew = *D. ×deweveri* (= *D. carthusiana* × *D. dilatata*), ×sar = *D. ×sarvelae* (= *D. carthusiana* × *D. expansa*).

No.	Locality details	Total number of samples	Species and hybrids detected (number of individuals)
1	CZ: Labské středohoří—Tlučenské údolí valley, 50°34′42.7″N, 14°05′13.8″E, 310 m, leg. R. Holubová, 17. VIII. 2004, PRC.	4	dil(4)*
2	CZ: Labské středohoří—Opárenské údolí valley, 50°32′19.8″N, 13°59′22.8″E, 275 m, leg. R. Holubová, 12. VI. 2005, PRC.	26	car(21)*, dil(5)*
3	CZ: Džbán—Bilíchov, 50°15′59.3″N, 13°54′50.7″E, 395 m, leg. R. Holubová, 25. VI. 2005, PRC.	48	car(15)*, dil(30)*, exp(3)*
4	CZ: Pardubické Polabí—Krakovany u Poděbrad, 50°04′14.3″N, 15°24′32.2″E, 220 m, leg. R. Holubová, 10.X. 2004, PRC.	20	car(14)*, dil(6)*
5	CZ: Krušnohorské podhůří vlastní—Místo, 50°26′17″N, 13°15′44″E, 405 m, leg. L. Ekrt, 2. IX. 2007, PR.	2	×dew(2)*
6	CZ: Český les—Pivoň, ruins of Starý Herštejn, 49°28′19″N, 12°42′48″E, 850 m, leg. L. Ekrt, 4. IX. 2007, PR.	5	dil(1), ×amb(1), ×dew(3)*
7	CZ: Český les—Broumov near Zadní Chodov, 49°53′33″N, 12°34′38″E, 595 m, leg. L. Ekrt, 3. IX. 2007, PR.	3	dil(1), exp(2)
8	CZ: Český les—Přimda, 49°40′54″N, 12°39′07″E, 595 m, leg. L. Ekrt, 3. IX. 2007, PR.	1	exp(1)
9	CZ: Český les—Ostrůvek near Lesná, 49°44′48″N, 12°27′08″E, 680 m, leg. L. Ekrt, 3. IX. 2007, PR.	2	exp(2)
10	CZ: Holoubkovské Podbrdsko—Strašice, 49°43′57″N, 13°44′01″E, 470 m, leg. L. Ekrt, 2. IX. 2007, PR.	2	dil(2)
11	CZ: Hořovická kotlina—Jivina near Hořovice, 49°47′19″N, 13°49′38″E, 600 m, leg. L. Ekrt, 2. IX. 2007, PR.	3	dil(3)
12	CZ: Prachatické Předšumaví—Kahov, Dehetník, 49°00′49″N, 13°58′22″E, 755 m, leg. L. Ekrt, 19. X. 2004, PR.	4	car(2)*+(1), dil(1)*
13	CZ: Kaplické mezihoří—Horní Dvořiště, 48°37′44″N, 14°23′02″E, 550 m, leg. L. Ekrt, 5. IX. 2007, PR.	1	dil(1)
14	CZ: Třeboňská pánev—Borkovická blata peatbog, 49°14′13″N, 14°37′56.2″E, 430 m, leg. R. Holubová, 27. IX. 2004, PRC.	28	car(26)*, dil(2)*
15	CZ: Třeboňská pánev—Mažická blata peatbog, 49°14′14″N, 14°37′00.8″E, 425 m, leg. R. Holubová, 28. IX. 2004, PRC.	23	car(16)*+(7)
16	CZ: Milešovské středohoří—Štěpánovská hora hill, 50°32′18.8″N, 13°52′18.7″E, 550 m, leg. K. Kubát & R. Holubová, 14. VIII. 2004, PRC.	12	dil(3)*, exp(9)*
17	CZ: Milešovské středohoří—Milešovka hill, 50°33′21.3″N, 13°55′45.5″E, 770 m, leg. R. Holubová, 26. VIII. 2004 & 11. VI. 2005, PRC.	43	car(7)*, dil(24)*+(6), exp(2)*, ×amb(4)*
18	CZ: Lovečkovské středohoří—Kalich hill, 50°36′30.2″N, 14°12′27.4″E, 435 m, leg. R. Holubová, 15. VIII. 2004, PRC.	3	dil(3)*
19	CZ: Lovečkovské středohoří—Panna hill, 50°36′31″N, 14°10′49.5″E, 475 m, leg. R. Holubová, 15. VIII. 2004, PRC.	9	car(1)*, dil(8)*
20	CZ: Lovečkovské středohoří—Čertova jizba hill, 50°36′59.4″N, 14°05′08.1″E, 305 m, leg. R. Holubová, 16. VIII. 2004, PRC.	9	exp(9)*
21	CZ: Lovečkovské středohoří—Bobří soutěska valley, 50°39′26.3″N, 14°21′29.3″E, 435 m, leg. R. Holubová, 4. IX. 2004 & 26. VI. 2005, PRC.	54	car(14)*, dil(19)*, exp(13)*, ×amb(8)*
22	CZ: Lovečkovské středohoří—Kamenec hill, 50°42′25.1″N, 14°21′20.4″E, 315 m, leg. R. Holubová, 6. IX. 2004 & 4. X. 2004, PRC.	81	car(49)*, dil(19)*, exp(13)*
23	CZ: Lovečkovské středohoří—Binov hill, 50°38′44.6″N, 14°21′52.5″E, 505 m, leg. R. Holubová, 7. IX. 2004, PRC.	11	car(1)*, dil(10)*
24	CZ: Lovečkovské středohoří—Průčelská rokle gorge, 50°37′03.8″N, 14°06′28.5″E, 450 m, leg. R. Holubová, 20. IX. 2004, PRC.	21	car(4)*, dil(16)*, exp(1)*
25	CZ: Lovečkovské středohoří—Starý Šachov, 50°43′12.7″N, 14°22′53.7″E, 230 m, leg. R. Holubová, 21. IX. 2004, PRC.	7	car(6)*, dil(1)*
26	CZ: Růžovská tabule—Děčín, Růžovský vrch hill, 50°50′17.7″N, 14°19′50.2″E, 470 m, leg. R. Holubová, 19. IX. 2004, PRC.	57	car(19)*, dil(11)*, exp(22)*, ×amb(5)*
27	CZ: Lužické hory—Klíč hill, 50°47′18.4″N, 14°34′14.4″E, 555 m, leg. R. Holubová, 5. IX. 2004, PRC.	24	car(3)*, dil(5)*, exp(13)*, ×amb(3)
28	CZ: Polomené hory—Polomené Hory, 50°36′11.3″N, 14°40′35″E, 365 m, leg. R. Holubová, 3. X. 2004, PRC.	1	car(1)*
29	CZ: Ralsko–bezdězská tabule—Ralsko u Mimoně, 50°40′31.7″N, 14°45′54.8″E, 600 m, leg. R. Holubová, 18. IX. 2004, PRC.	36	car(3)*, dil(18)*, exp(8)*, ×amb(7)*
30	CZ: Ralsko–bezdězská tabule—Staré Splavy, 50°35′57.3″N, 14°38′46.1″E, 260 m, leg. R. Holubová, 3. X. 2004, PRC.	1	car(1)*
31	CZ: Trosecká pahorkatina—Příhrazy, Krtola gorge, 50°31′48″N, 15°03′53″E, 340 m, leg. L. Ekrt, 25. VI. 2004, PR.	1	×amb(1)
32	CZ: Polická kotlina—Horní Adršpach, 50°38′23″N, 16°05′20″E, 620 m, leg. L. Ekrt, 19. X. 2004, PR.	2	×amb(2)*
33	CZ: Žaltman—Zbečník, Maternice, 50°29′31″N, 16°08′44″E, 435 m, leg. L. Ekrt, 9. IX. 2005, PR.	19	car(3), dil(10), exp(4), ×amb(2)
34	CZ: Broumovské stěny—Hlavňov, Koruna rock, 50°31′09″N, 16°18′45″E, 720 m, leg. L. Ekrt, 10. X. 2004, PR.	3	dil(1)*, exp(1)*, ×amb(1)
35	CZ: Broumovské stěny—Kovářova rokle gorge, 50°33′35″N, 16°16′07″E, 605 m, leg. L. Ekrt, 8. IX. 2005, PR.	17	exp(11), ×amb(6)
36	CZ: Broumovské stěny—Zaječí rokle gorge, 50°32′16″N, 16°17′57″E, 595 m, leg. L. Ekrt, 8. IX. 2005, PR.	17	car(3), dil(3), exp(6), ×amb(5)
37	CZ: Broumovské stěny—Božanov, 50°29′59″N, 16°20′43″E, 630 m, leg. A. Hájek, 27. VI. 2005, PR.	1	×dew(1)

APPENDIX 1. Continued.

No.	Locality details	Total number of samples	Species and hybrids detected (number of individuals)
38	CZ: Českomoravská vrchovina—Olšany u Telče, 49°10′46″N, 15°33′36″E, 600 m, leg. L. Ekrt, 28. VII. 2003, PR.	3	dil(1), exp(1), ×amb(1)*
39	CZ: Českomoravská vrchovina—Mysliboř, Obecní rybník pond, 49°13′23″N, 15°28′04″E, 550 m, leg. L. Ekrt, 30. X. 2004, PR.	2	car(1), dil(1)
40	CZ: Českomoravská vrchovina—Kostelní Myslová, Velký Hulišťský rybník pond, 49°09′09″N, 15°24′25″E, 520 m, leg. L. Ekrt, 8. VIII. 2005, PR.	22	car(4), dil(9), exp(1)*+(4), ×amb(2), ×dew(2)*
41	CZ: Českomoravská vrchovina—Třešť, Velký Špičák hill, 49°18′45″N, 15°30′40″E, 695 m, leg. L. Ekrt, 16. VII. 2005, PR.	18	car(4), dil(8), exp(6)
42	CZ: Českomoravská vrchovina—Stonařov, 49°16′57″N, 15°33′30″E, 630 m, leg. L. Ekrt, 19. IX. 2006, PR.	2	×dew(1)+(1)*
43	CZ: Českomoravská vrchovina—Polesí near Počátky, 49°17′16″N, 15°15′24″E, 630 m, leg. L. Ekrt, 11. VII. 2006, PR.	1	×dew(1)
44	CZ: Českomoravská vrchovina—Jezdovice, 49°18′28″N, 15°29′07″E, 545 m, leg. L. Ekrt, 15. IX. 2007, PR.	1	exp(1)
45	CZ: Českomoravská vrchovina—Třeštice, 49°14′40″N, 15°28′20″E, 605 m, leg. L. Ekrt, 9. IX. 2007, PR.	2	exp(2)
46	CZ: Českomoravská vrchovina—Panenská Rozsíčka, 49°14′43″N, 15°30′42″E, 585 m, leg. L. Ekrt, 8. IX. 2007, PR.	1	exp(1)
47	CZ: Českomoravská vrchovina—Bezděkov, 49°15′06″N, 15°31′16″E, 565 m, leg. L. Ekrt, 7. IX. 2007, PR.	1	exp(1)
48	CZ: Ceskomoravská vrchovina—Stašov, 49°40′32″N, 16°22′00″E, 650 m, leg. J. Košnar, 31. VIII. 2007, PR.	1	dil(1)
49	CZ: Jesenické podhůří—Krasov, 50°05′04″N, 17°30′02″E, 565 m, leg. L. Ekrt, 17. IX. 2007, PR.	2	exp(2)
50	CZ: Bílé Karpaty lesní—Vápenky, Velká Javořina hill, 48°51′58″N, 17°39′40″E, 620 m, leg. L. Ekrt, 18. IX. 2007, PR.	3	dil(1), exp(2)
51	CZ: Zlínské vrchy—Lidečko, 49°13′13″N, 18°02′48″E, 595 m, leg. L. Ekrt, 18. IX. 2007, PR.	1	exp(1)
52	CZ: Beskydské podhůří—Jablunkov, 49°35′30″N, 18°47′07″E, 430 m, leg. L. Ekrt, 17. IX. 2007, PR.	4	exp(2), ×amb(2)
53	CZ: Brdy—Leletice, 49°31′58″N, 13°52′06″E, 535 m, leg. L. Ekrt, 2. IX. 2007, PR.	5	exp(1), ×amb(2), ×dew(2)*
54	CZ: Královský hvozd—cirque of the Černé jezero lake, 49°10′29″N, 14°10′54″E, 1050 m, leg. L. Ekrt, 14. VII. 2005, PR.	12	dil(1), exp(9), ×amb(2)
55	CZ: Královský hvozd—Železná Ruda, Debrnické údolí valley, 49°06′57″N, 13°14′20″E, 770 m, leg. L. Ekrt & J. Hadinec, 29. VIII. 2007, PR.	1	exp(1)
56	CZ: Boubínsko–stožecká hornatina—České Žleby, Spáleniště hill, 48°52′30″N, 13°48′01″E, 870 m, leg. L. Ekrt, 19. X. 2004, PR.	13	car(5)*+(1), dil(1), exp(2)*, ×amb(4)*
57	CZ: Boubínsko–stožecká hornatina—Boubín hill, 48°58′31″N, 13°48′36″E, 1100 m, leg. L. Ekrt, 15. XI. 2003, PR.	2	exp(1), ×amb(1)
58	CZ: Trojmezenská hornatina—cirque of the Plešné jezero lake, 48°46′38.2″N, 13°51′51.5″E, 1120 m, leg. L. Ekrt & E. Hofhanzlová, 7. VIII. 2003 & 15. VII. 2005, PR.	13	dil(1), exp(6)+(2)*, ×amb(4)
59	CZ: 88e. Trojmezenská hornatina—Nová Pec, Smrčina hill, 48°45′27″N, 13°56′27″E, 880 m, leg. L. Ekrt, 25. IX. 2007, PR.	2	exp(1), ×amb(1)
60	CZ: Trojmezenská hornatina—Nová Pec, Bulík hill, 48°45′07″N, 13°55′32″E, 1150 m, leg. L. Ekrt, 25. IX. 2007, PR.	2	exp(2)
61	CZ: Želnavská hornatina—Pěkná, Černý les hill, 48°50′26″N, 13°58′38″E, 920 m, leg. L. Ekrt & E. Ekrtová, 19. X. 2004, PR.	5	car(1)*, dil(1)*, exp(2)*, ×amb(1)*
62	CZ: Novohradské hory—Šejby, Hojná voda, 48°42′23″N, 14°45′09″E, 850 m, leg. L. Ekrt & M. Lepší, 5. IX. 2007, PR.	2	exp(1), ×amb(1)
63	CZ: Novohradské hory—Pohorská Ves, Žofínský prales, 48°40′03.3″N, 14°42′04.5″E, 810 m, leg. M. Lepší, 10. VIII. 2005, CB.	20	car(3), dil(4), exp(10), ×amb(3)
64	CZ: Novohradské hory—Žofín, 48°40′30″N, 14°40′26″E, 765 m, leg. L. Ekrt & M. Lepší, 5. IX. 2007, PR.	1	exp(1)
65	CZ: Krkonoše lesní—Medvědí potok, 50°44′44.9″N 15°36′01.7″E, 820 m, leg. R. Holubová, 8. VII. 2005, PRC.	13	exp(12)*, ×amb(1)*
66	CZ: Krkonoše lesní—Medvědí boudy, 50°45′44.36″N, 15°35′33.1″E, 1025 m, leg. R. Holubová, 9. VII. 2005, PRC.	29	exp(27)*, ×amb(2)*
67	CZ: Krkonoše lesní—Labský důl valley, 50°46′09.4″N, 15°32′59.8″E, 1200 m, leg. R. Holubová, 10. VII. 2005, PRC.	23	exp(21)*, ×amb(2)*
68	CZ: Teplicko–adršpašské skály—Sibiř, 50°35′33″N, 16°07′32″E, 660 m, leg. L. Ekrt, 8. IX. 2005, PR.	19	exp(14), ×amb(5),
69	CZ: Teplicko–adršpašské skály—Homole cukru rock, 50°35′12.2″N, 16°7′10.4″E, 660 m, leg. A. Hájek, 2005, HR.	1	dil(1)
70	CZ: Teplicko–adršpašské skály—Divoká rokle gorge, 50°35′32.7″N, 16°08′25.1″E, leg. A. Hájek, 2005, HR.	1	exp(1)
71	CZ: Český hřeben—Bukačka, 50°20′20″N, 16°22′36″E, 1000 m, leg. L. Ekrt & J. Kučera, 17. IX. 2007, PR.	3	exp(2), ×amb(1)
72	CZ: Český hřeben—Panské Pole, Černý důl valley, 50°12′06″N, 16°31′05″E, 860 m, leg. L. Ekrt & J. Kučera, 17. IX. 2007, PR.	2	exp(2)
73	CZ: Hrubý Jeseník—Vidly, 50°06′13″N, 17°15′17″E, 845 m, leg. L. Ekrt, 17. IX. 2007, PR.	2	exp(2)
74	CZ: Radhošťské Beskydy—Pustevny, 49°29′34″N, 18°15′57″E, 1090 m, leg. L. Ekrt, 18. IX. 2007, PR.	5	exp(2), ×amb(1), ×dew(2)*
75	CZ: Radhošťské Beskydy—Dolní Lomná, Mionší reserve, 49°31′52″N, 18°40′08″E, 625 m, leg. L. Ekrt, 18. IX. 2007, PR.	2	exp(2)
76	CZ: Slezské Beskydy—Nýdek, 49°40′09″N, 18°46′24″E, 485 m, leg. L. Ekrt, 17. IX. 2007, PR.	3	exp(2), ×amb(1)
77	SK: Krivánska Malá Fatra—Krasňany, 49°11′34″N, 18°56′01″E, 605 m, leg. L. Ekrt, 30. IX. 2004, PR.	2	dil(1)*, exp(1)*
78	SK: Malá Fatra—Terchová, 49°14′39″N, 19°05′24″E, 945 m, leg. L. Ekrt, 30. IX. 2004, PR.	1	exp(1)*

APPENDIX 1. Continued.

No.	Locality details	Total number of samples	Species and hybrids detected (number of individuals)
79	SK: Západní Tatry—Liptovský Mikuláš, Parichvost valley, 49°11′17″N, 19°43′51″E, 1250 m, leg. L. Ekrt & E. Hofhanzlová, 24. VI. 2003, PR.	1	exp(1)
80	AT: Rottenmanner Tauren—Rottenmann, 47°28′21″N, 14°24′56″E, 1600 m, leg. L. Ekrt & E. Hofhanzlová, 24. VIII. 2003, PR.	3	exp(2), ×amb(1)
81	DE: Harz—Bärenklippe near Schirke, 51°45′N, 10°40′E, 860 m, leg. S. Jessen, 8. VI. 1981, cult. in Arktisch–Alpine Garten, Chemnitz, SJ359.	1	exp(1)
82	DE: Thüringen—Stadtroda, Borntal valley near Ruttersdorf, 50°52′N, 11°44′E, leg. S. Jessen & J. Riethausen, 1992, cult. in Arktisch–Alpine Garten, Chemnitz, SJ2821.	1	car(1)
83	DE: Thüringen—Fuchsgraben near Wolfsgefärth near Gera, 50°48′N, 12°03′E, leg. S. Jessen, 17. VII. 1982, cult. in Arktisch–Alpine Garten, Chemnitz, SJ147/1.	1	×dew(1)
84	DE: Rügen—Kranichbruch near Neu Mukran, 54°28′N, 13°34′E, 3 m, leg. S. Jessen, 28. VI. 1983, cult. in Arktisch–Alpine Garten, Chemnitz, SJ337.	1	×sar(1)
85	DE: Rügen—Stubbnitz, Herthasee, 54°32'N, 13°38′E, 115 m, leg. S. Jessen, 12. VI. 1984, cult. in Arktisch–Alpine Garten, Chemnitz, SJ374.	1	×sar(1)

Paper 9

Kaplan Z., Danihelka J., Štěpánková J., Ekrt L., Chrtek J. Jr., Zázvorka J., Grulich V., Řepka R., Prančl J., Ducháček M., Kúr P., Šumberová K. & Brůna J. (2016): Distributions of vascular plants in the Czech Republic. Part 2. – Preslia 88: 229–322.

Distributions of vascular plants in the Czech Republic. Part 2

Rozšíření cévnatých rostlin v České republice. Část 2

Zdeněk K a p l a n[1], Jiří D a n i h e l k a[1, 2], Jitka Š t ě p á n k o v á[1], Libor E k r t[3],
Jindřich C h r t e k Jr.[1], Jiří Z á z v o r k a[1], Vít G r u l i c h[2], Radomír Ř e p k a[4],
Jan P r a n č l[1, 5], Michal D u c h á č e k[6], Pavel K ú r[3], Kateřina Š u m b e r o v á[1]
& Josef B r ů n a[1]

[1]*Institute of Botany, The Czech Academy of Sciences, CZ-252 43 Průhonice, Czech Republic, e-mail: kaplan@ibot.cas.cz, stepankova@ibot.cas.cz, chrtek@ibot.cas.cz, zazvorka @ibot.cas.cz, hroudova@ibot.cas.cz, prancl@ibot.cas.cz, katerina.sumberova@ibot.cas.cz, bruna@ibot.cas.cz;* [2]*Department of Botany and Zoology, Masaryk University, Kotlářská 2, CZ-611 37 Brno, Czech Republic, e-mail: danihel@sci.muni.cz, grulich@sci.muni.cz;*
[3] *Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, CZ-370 05 České Budějovice, Czech Republic, email: libor.ekrt@gmail.com, pavel.kur@prf.jcu.cz;* [4]*Department of Forest Botany, Dendrology and Geobiocenology, Faculty of Forestry and Wood Technology, Mendel University, Zemědělská 3, CZ-613 00 Brno, Czech Republic, e-mail: repka@mendelu.cz;* [5]*Department of Botany, Faculty of Science, Charles University in Prague, Benátská 2, CZ-128 01 Prague, Czech Republic;* [6]*Department of Botany, National Museum, Cirkusová 1740, CZ-193 00 Praha 9-Horní Počernice, Czech Republic, e-mail: duchace@seznam.cz*

The second part of the publication series on the distributions of vascular plants in the Czech Republic includes grid maps of 87 taxa of the genera *Antennaria*, *Aposeris*, *Astragalus*, *Avenula*, *Bidens*, *Carex*, *Cenchrus*, *Centunculus*, *Convallaria*, *Crocus*, *Cryptogramma*, *Cyperus*, *Dryopteris*, *Gladiolus*, *Gratiola*, *Helictochloa*, *Hierochloë*, *Lindernia*, *Maianthemum*, *Myriophyllum*, *Notholaena*, *Nymphoides*, *Radiola*, *Schoenoplectus*, *Sisyrinchium*, *Spergularia*, *Tillaea*, *Veratrum* and *Veronica*. The maps were produced by taxonomic experts based on all available herbarium, literature and field records. The plants studied include 56 taxa registered in the Red List of vascular plants of the Czech Republic, some of which showed remarkable declines. *Astragalus arenarius*, *Hierochloë odorata* and *H. repens*, as representatives of vegetation of inland sand dunes, are critically threatened due to conversion of their habitats to arable land, local sand mining, afforestation, changes in landscape management and eutrophication followed by succession. Each of them survives at a few localities and their populations are poor. Competitively weak wetland annuals, confined to open habitats such as exposed fishpond littorals and river beds, abandoned sand-pits and wet arable fields, have considerably declined and disappeared from large areas as a result of agriculture and fish-farming intensification, in particular fertilization and restriction of summer drainage of fishponds, and other changes in land-use. These include *Centunculus minimus*, *Cyperus flavescens*, *C. michelianus*, *Lindernia procumbens*, *Radiola linoides* and *Tillaea aquatica*. Observed recently at a few sites only, they are all classified as critically threatened. A map is for the first time provided also for *Spergularia kurkae*, a newly recognized species and a central-European endemic. *Astragalus asper*, *Schoenoplectus supinus* and *Veronica pumila* are now extirpated from the country's flora. In contrast, *Spergularia marina*, until recently confined to natural saline habitats and very rare, has been spreading along roads that are treated by de-icing salts. Examination of an old herbarium voucher showed that the only record of *Astragalus alopecuroides* in the Czech flora actually refers to the species whose correct name is *A. alopecurus*.

Further introduced casuals mapped in this paper include *Bidens pilosus*, *Cenchrus echinatus*, *Gratiola neglecta* and *Lindernia dubia*, each introduced to only a few sites. *Bidens connatus* was recorded at two dozen sites and appears to have spread as a consequence of the great floods in 2002. Typical examples of naturalized neophytes are *Veronica filiformis* and *V. peregrina*, both currently known from many parts of the country. Invasive aliens are represented by *Bidens frondosus*, which began to spread in the 1930s and now is frequent throughout the country. Spatial and temporal dynamics of individual species are shown in maps and documented by records included in the Pladias database and available in Electronic appendices. The maps are accompanied by comments, which include additional information on distribution, habitats, taxonomy and biology of the species.

K e y w o r d s : alien species, central Europe, chorology, Czech Republic, distribution atlas, distribution patterns, endangered species, endemic, flora, grid maps, herbaria, phytogeography, plant record, vascular plants

Introduction

A recent review on the Czech flora (Kaplan 2012) emphasized that no comprehensive piece of work with distribution maps in this country is available in spite of a long history of botanical research. The project of mapping plant distributions in the Czech Republic was launched two years ago with the aim to establish a modern plant record database and to prepare the first sets of distribution maps as a basis for a future complete atlas of the distribution of vascular plants in the Czech Republic. The first results of our effort were published a half year ago (Kaplan et al. 2015) within the PLADIAS project (www.pladias.org). The paper contained 75 grid distribution maps produced by taxonomic experts and based on critically evaluated and sorted records.

From September 2015 to February 2016 the plant record database has increased by ca 154,000 new records. Of these about 45,000 records resulted from critical examination of herbarium specimens by taxonomic experts. Maps of further 87 taxa, both native and alien, were finished at the beginning of February 2016 and these are published in this paper.

Current revisions of national plant diversity have brought several species new to the flora of the Czech Republic, which include both newly recognized native endemics (Kolář et al. 2015, Lepší et al. 2015) as well as recently introduced alien species (Kocián 2014, Hadinec & Lustyk 2015). Two changes in identification and nomenclature involve genera dealt with in this paper, which require an update of the checklist of vascular plants of the Czech Republic (Danihelka et al. 2012). Examination of an old herbarium voucher and the nomenclatural history of the respective plant group showed that the only record on the casual occurrence of *Astragalus alopecuroides* in the Czech flora actually refers to the species whose correct name is *A. alopecurus*. The name *Crocus albiflorus* has to be replaced by *C. vernus*, which was shown to be the correct name for the species largely known as *C. albiflorus* (Peruzzi et al. 2013).

Materials and methods

Taxonomic scope

The following groups of vascular plants are mapped: native taxa, naturalized aliens and most casuals, and selected hybrids. Distribution maps are produced for species and sub-

species, in exceptional cases also for varieties or infrageneric taxa (e.g. sections). Plants of species groups that are difficult to assign to species may be mapped as species aggregates. Field crops and plants deliberately cultivated in gardens and parks are not included in the mapping project. Nomenclature, taxonomic concepts and delimitation of species aggregates mostly follow Danihelka et al. (2012), with differences indicated where necessary. For taxa not included in that checklist, a taxonomic reference is given. Publication of maps does not follow any alphabetical or systematic order but those maps that have resulted from recent revisions are preferably printed.

Data sources

All relevant floristic data sources are used. Major national herbaria and some local and foreign collections were consulted, incl. BRA, BRNL, BRNM, BRNU, CB, CBFS, CESK, CHOM, FMM, GM, HOMP, HR, KHMS, LIM, LIT, MJ, MMI, MP, MZ, NJM, OH, OL, OLM, OMJ, OP, OSM, OSTR, OVMB, PL, PR, PRA, PRC, ROZ, SAV, SLO, SOB, SOKO, SUM, VM, VYM, W, WU and ZMT (acronyms follow Thiers 2016), as the main source of taxonomically revised records. Most records for maps of common and easy-to-identify taxa come from the recently developed Pladias database (hosted at the Institute of Botany, Průhonice; previously tentatively named CzechDistrib database), which has integrated all available records on the distribution of vascular plants in the Czech Republic. Among the most important incorporated databases are the Database of the Distribution of Vascular Plants in the Czech Republic (FLDOK), the Czech National Phytosociological Database (CNPD), plant records from the Floristic Summer Schools and other activities of the Czech Botanical Society, the Species Occurrence Database of the Nature Conservation Agency of the Czech Republic (NDOP) and the Database of Forest Typology of the Forest Management Institute of the Czech Republic (DLT). Unpublished field records previously entered into the Pladias database by the authors of maps or regional contributors were also considered.

Procedure of mapping

All records used for mapping are entered into the Pladias database and geographically sorted according to the traditionally used CEBA (Central European Basic Area) grid template (Niklfeld 1999) divided into quadrants of 5 × 3 arc minutes (corresponding to approximately 5.5 × 5.9 km). The territory of the Czech Republic is covered by 2551 quadrants, of which 2181 are completely within the border of the country. Individual records as well as the whole distribution pattern of each taxon are checked and evaluated by the author of a particular map in a web-based mapping interface of the Pladias database. Because maps of taxonomically critical groups are often highly inaccurate in distribution atlases (Gregor 2009), maps of such taxa are based solely or mainly on herbarium records revised by taxonomic experts; these cases are indicated in the text accompanying the particular map. Maps of all other taxa are based on records from databases, literature and herbaria, which were scrutinized by the authors of the respective maps. Records used for producing maps are listed in Electronic Appendices 1–87. In selected maps, native versus introduced occurrences are distinguished and corresponding records in the database classified accordingly. Draft distribution maps and the background records are released in a web-based review process for scrutiny to field botanists, regional collabora-

tors and members of the Czech Botanical Society. Their comments and additional records are collected in the database and returned to the responsible specialists for consideration before producing final distribution maps.

Final maps and comments

The treatment of each taxon consists of a grid distribution map and of an accompanying text; authors of maps are indicated in the figure captions, and they also took major part in preparing the first drafts of the respective texts. Maps are displayed using spherical Mercator projection (EPSG:3857) where meridians and parallels are shown perpendicular, and the mapping CEBA grids are thus nicely displayed. The background relief was derived from the SRTM data (http://www2.jpl.nasa.gov/srtm/, the version provided by http://srtm.csi.cgiar.org), and the river network was adapted from data provided by CENIA (www.cenia.cz). When appropriate, different symbols are used in the maps in order to distinguish one of the following attributes of the plant distribution records: (1) recent versus old records, (2) native occurrences versus introductions, or (3) records based on revised herbarium specimens versus all other records. These classifications of records are used only for those taxa where such distinction provides important information and, in addition, the amount and quality of records are sufficient. The mapping symbols used to indicate the different attributes of the records in the particular grid cell are shown in Table 1. Symbols specific to individual maps are explained in their captions. In the caption to each map, counts of occupied quadrants are indicated according to the symbols used in the map; uncertain occurrences are not included in the counts. The accompanying text includes the accepted scientific name, a brief outline of the total distribution, information on habitats occupied by the species and a description of its distribution in the Czech Republic. Where appropriate, comments on the taxonomy, biology and details of the spatial and temporal dynamics of the distribution are given.

Table 1. – The mapping symbols used in the distribution maps to indicate the different attributes of the occurrence in a particular grid cell.

Attribute distinguished	Symbol	Attribute state
None	●	all records
Time	●	recent occurrence (at least one record since 2000)
	◐	old occurrence (all records before 2000, or demonstrably being extirpated from all localities after 2000, or all records undated)
Origin	●	native (at least one record)
	✕	alien
Source data	●	a revised herbarium specimen (at least one record)
	▲	all other
All	?	only record(s) uncertain regarding identification and/or locality

Distribution maps and comments

Antennaria dioica (Fig. 1)

Antennaria dioica is a boreo-temperate species occurring throughout the temperate zone of Eurasia as far as Japan; in North America it is confined to the western Aleutian Islands. It is found in most of Europe except the Iberian Peninsula, southern Italy and the Balkan Peninsula, where it is scattered; it is absent from the Mediterranean islands (Meusel & Jäger 1992, Bayer 2000, Greuter 2006). In Europe *A. dioica* is a species characteristic of slightly buffered soils with pH 4.5–6.0 (van den Berg et al. 2005). In the Czech Republic it grows on nutrient-poor soils in dry pastures, heathlands, on well-drained mountain slopes and rock edges, in pine forests and other types of rather acidophilous forests with open canopy. It is distributed throughout the country from the lowlands to the mountains, where it is more frequent. In contrast, it is markedly less frequent in dry deforested parts of north-western, central and eastern Bohemia. In Moravia it is absent from the dry and warm lowlands with prevailing arable land and usually with base- and nutrient-rich soils. It has recently disappeared from many sites, particularly in lowlands, as a result of the abandonment of pastures, increasing canopy closure in forests and atmospheric deposition of sulphate and inorganic nitrogen (van den Berg et al. 2005, Chytrý 2007a). *Antennaria dioica* is classified as endangered (Grulich 2012).

Aposeris foetida (Fig. 2)

Aposeris foetida has a small distribution range situated in central and south-eastern Europe. It occurs in most of the calcareous parts of the Alps, and is scattered throughout the Carpathians and the mountains of the north-western Balkan Peninsula (Meusel & Jäger 1992). In the Czech Republic *A. foetida* usually grows in beech and oak-hornbeam forests on flysch sediments. It has been only recorded from several localities in the Bílé Karpaty Mts around the Brumov-Bylnice village where it was discovered as late as in 1922 by S. Staněk. It used to be also rarely grown as an ornamental in château parks, sometimes surviving there for a long time. It is classified as critically threatened (Grulich 2012).

Astragalus alopecurus (Fig. 3)

Astragalus alopecurus, described from the Southern Ural Mts, has a large fragmented distribution range, mainly in the mountains of the temperate zone of western and central Siberia, Kazakhstan and north-western China. It is also found in the Caucasus Mts and Anatolia, as well as in the Rodopi Mts. in Bulgaria and the Western Alps in France and Italy (Vydrina 1994, Podlech & Zarre 2013). In the Czech Republic it was collected at a single site, namely in the Prokopské údolí valley west of the village of Hlubočepy in the south-western outskirts of the city of Prague by L. J. Čelakovský in June 1872. He correctly identified the specimen as a "Russian-Siberian" plant and published his find as *A. alopecuroides* (Čelakovský 1875: 675), which was the name then in current use (see the synonymy in Podlech & Zarre 2013). More than a century later the Linnaean name *A. alopecuroides* was typified by D. Podlech (in Turland & Jarvis 1997: 463) with a specimen collected in Spain that is attributable to a different species native to south-western Europe and northern Africa. The typification remained unnoticed by Czech botanists,

and the single record from Bohemia was further referred to as *A. alopecuroides* (e.g. Pyšek et al. 2012b). We examined the voucher specimen (preserved at PR) using a modern monograph (Podlech & Zarre 2013) and confirmed Čelakovský's identification, with the correct name being now *A. alopecurus*. The specimen's label describes the habitat as "in a slope below rocks behind the mill" (we assume he referred to Dalejský mlýn). The species was either deliberately planted or sown (it was rarely cultivated due to its peculiar habit) or accidentally introduced with cereals. Čelakovský harvested the single specimen for his herbarium and, as he noted (Čelakovský 1875: 675), the plant was no longer present at the site in the following year.

Astragalus arenarius (Fig. 4)

The more or less continuous distribution range of *Astragalus arenarius* includes north-eastern Germany east of the Elbe river, Poland, Belorussia, northern Ukraine and northern-central part of the European Russia. It occurs also near the Baltic coast of Latvia and Lithuania, in southern Sweden and Finland; rather isolated occurrences are found in the Czech Republic and the German province of Bavaria (Meusel et al. 1965, Podlech & Zarre 2013). The populations in eastern, eastern-central and northern Bohemia are situated on the south-western border of its distribution range. In this area *A. arenarius* is considered a relict from late glacial or early postglacial ages and its origin is associated with the Sarmatian migration route (Kaplan 2012). Most of the Czech populations are found in open vegetation of inland sand dunes dominated by the perennial tussock-forming grass *Corynephorus canescens*, developed on acidic sand that is free of calcium carbonate (Chytrý & Sádlo 2007). At one of its sites the species temporarily colonized an edge of the gravel embankment of the adjacent railway soon after its rebuilding. *Astragalus arenarius* used to be found at 14–17 sites in the surroundings of the town of Doksy in northern Bohemia, in the Labe river basin in central and eastern Bohemia, and in the Divoká Orlice and Tichá Orlice rivers basin in eastern Bohemia. Most of these populations disappeared in late 19th century or during the first half the 20th century as a result of direct habitat destruction, including conversion to arable land, local sand mining, afforestation, abandonment of pastures and eutrophication followed by succession. Currently it survives at only 4 sites. They host poor populations, which have declined during the last decades and have consisted of one individual to several dozen individuals during recent years. It is therefore currently classified as critically threatened (Grulich 2012).

Astragalus asper (Fig. 5)

Astragalus asper is distributed in eastern Austria, south-western Slovakia, Hungary, northern Croatia, northern Serbia, Romania, Bulgaria, Ukraine and the southern part of European Russia, in the east reaching the lower Volga river (Meusel et al. 1965, Podlech & Zarre 2013). It used to be found also at two sites in the south-eastern part of the Czech Republic near the towns of Klobouky u Brna and Čejč about 35 km SE of Brno. It was discovered there by A. Makowsky in 1859 (Podlech & Zarre 2013: 1862) and last collected by J. Otruba in 1930. It may be assumed that all native populations, representing the north-westernmost outpost of the species' range, were destroyed before World War II. Based on rather general information on herbarium labels, it may have been found there in semi-ruderal dry grasslands and meadows. In 1993 *A. asper* was collected by Č. Deyl
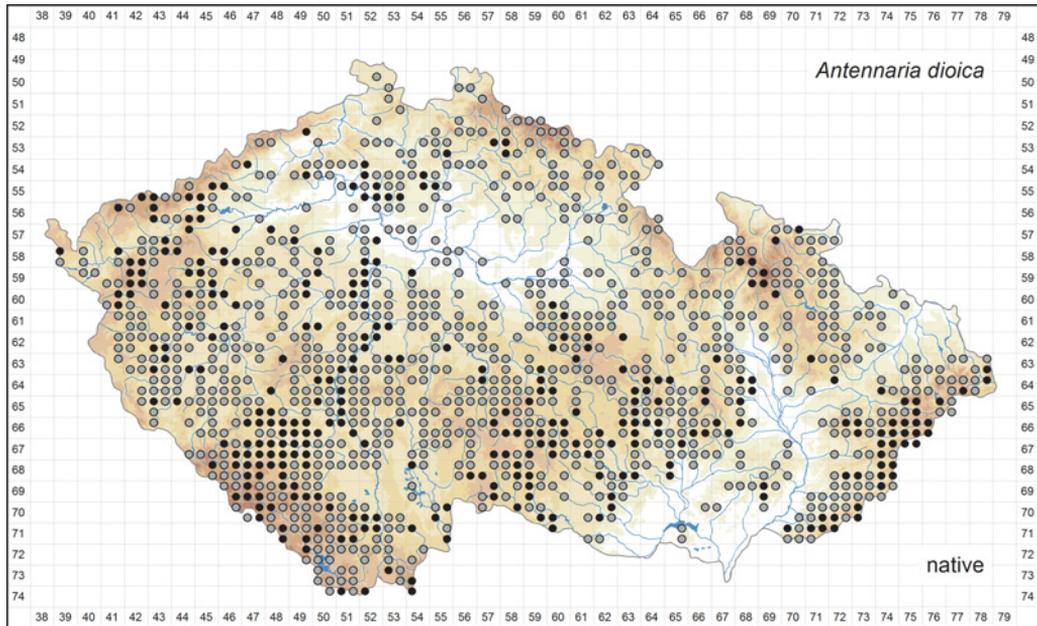
Fig. 1. – Distribution of *Antennaria dioica* in the Czech Republic: ● at least one record in 2000–2016 (319 quadrants), ◉ pre 2000 records only (842 quadrants). Prepared by Jitka Štěpánková.
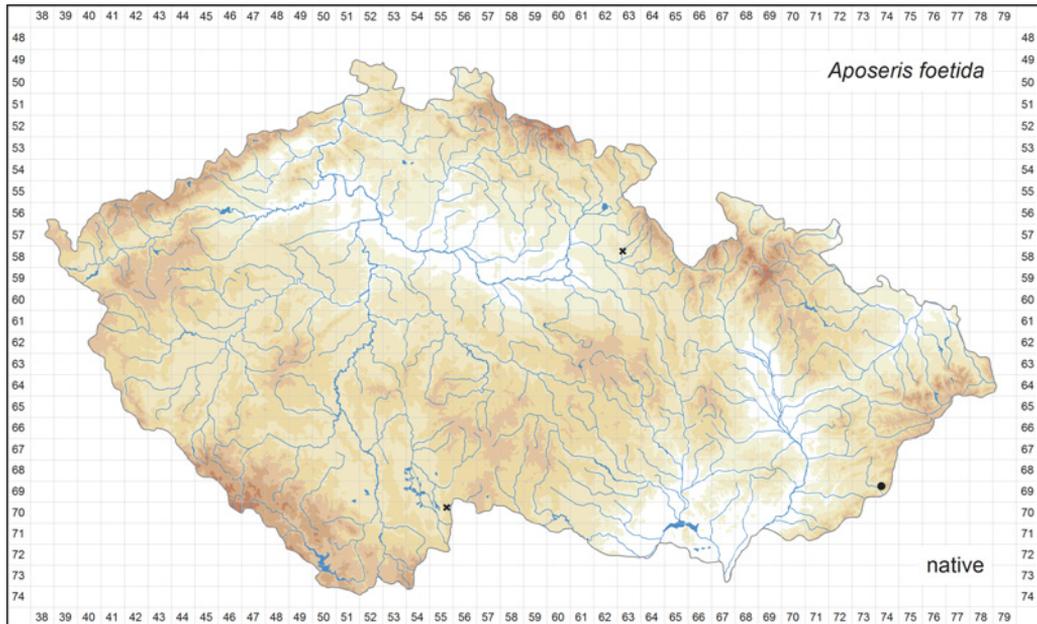

Fig. 2. – Distribution of *Aposeris foetida* in the Czech Republic: ● native (1 quadrant), ✕ alien (2 quadrants). Prepared by Jitka Štěpánková.
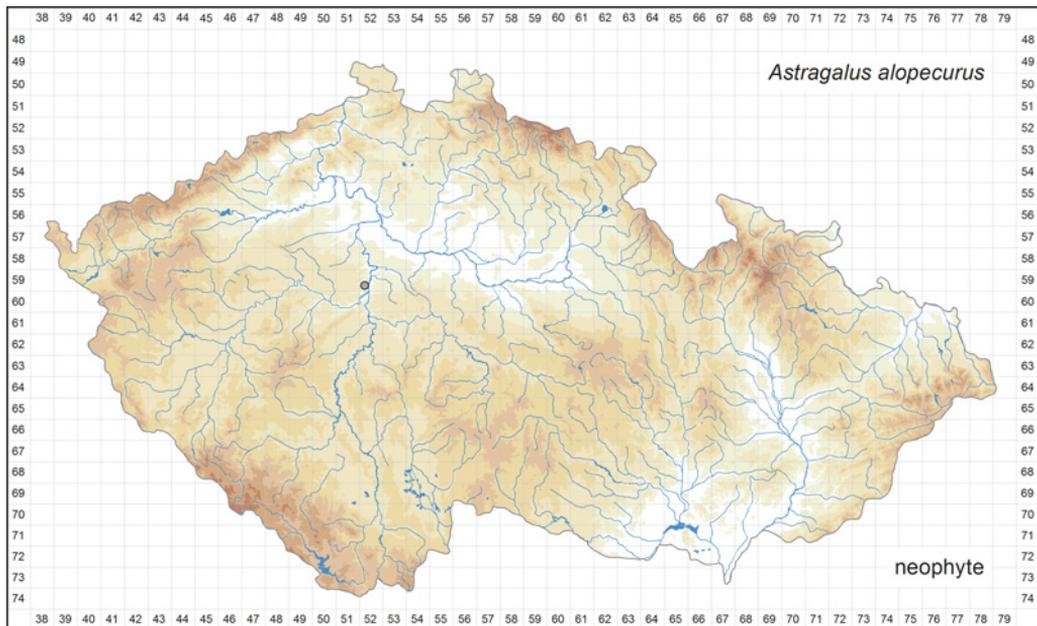
Fig. 3. – Distribution of *Astragalus alopecurus* in the Czech Republic (1 occupied quadrant). Prepared by Zdeněk Kaplan & Jiří Danihelka.
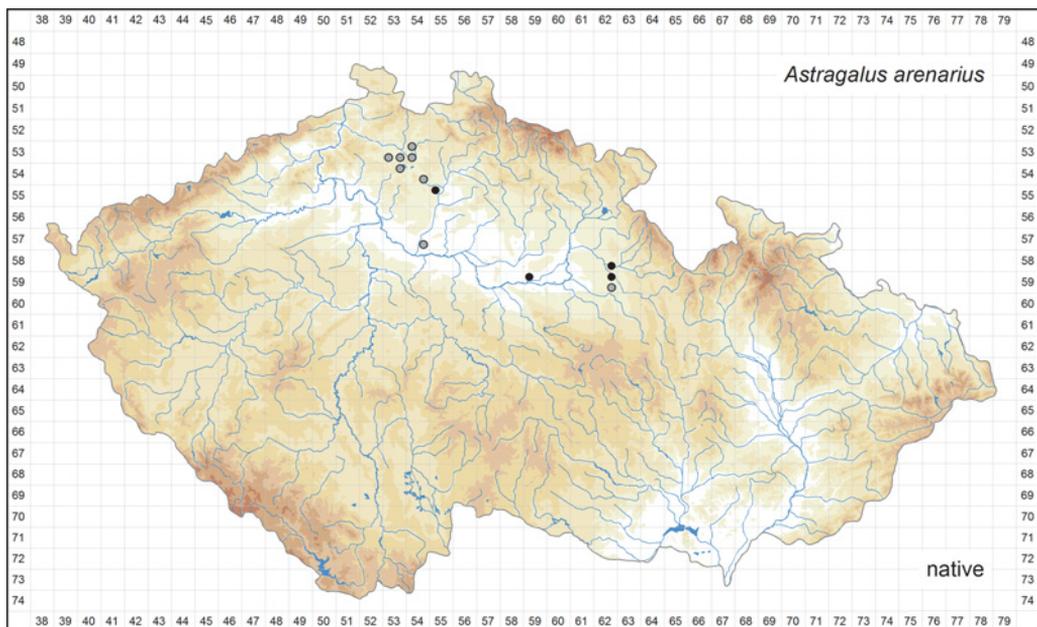

Fig. 4. – Distribution of *Astragalus arenarius* in the Czech Republic: ● at least one record in 2000–2016 (4 quadrants), ◉ pre 2000 records only (8 quadrants). Prepared by Zdeněk Kaplan & Jiří Danihelka.
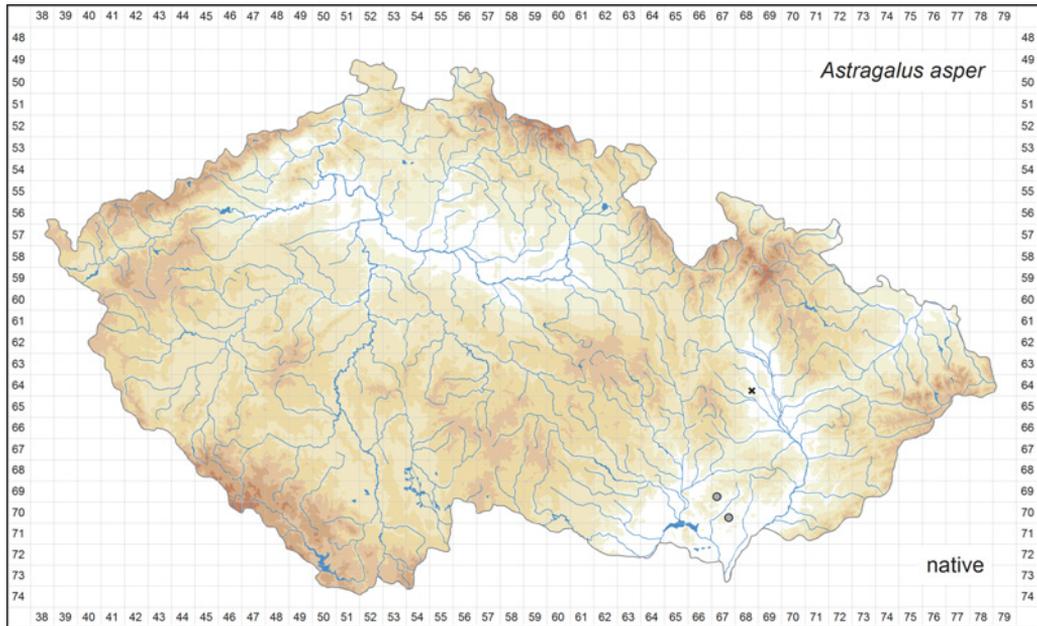
Fig. 5. – Distribution of *Astragalus asper* in the Czech Republic: ◉ pre 2000 native records (2 quadrants), ✕ pre 2000 alien records (1 quadrant). Prepared by Zdeněk Kaplan & Jiří Danihelka.
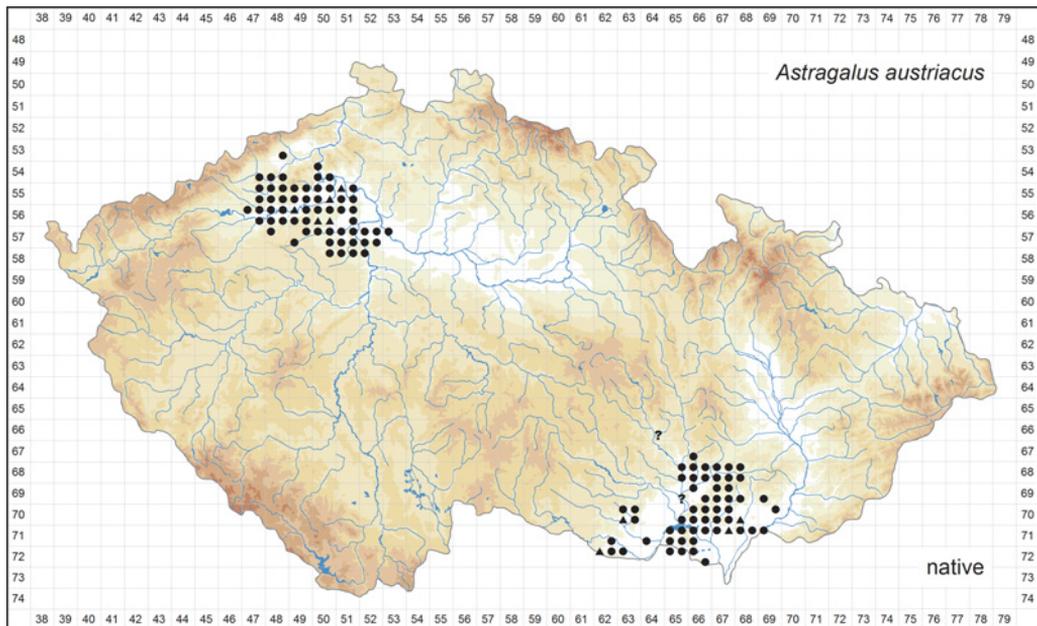

Fig. 6. – Distribution of *Astragalus austriacus* in the Czech Republic: ● occurrence documented by herbarium specimens (110 quadrants), ▲ occurrence based on other records (9 quadrants). Prepared by Zdeněk Kaplan & Jiří Danihelka.
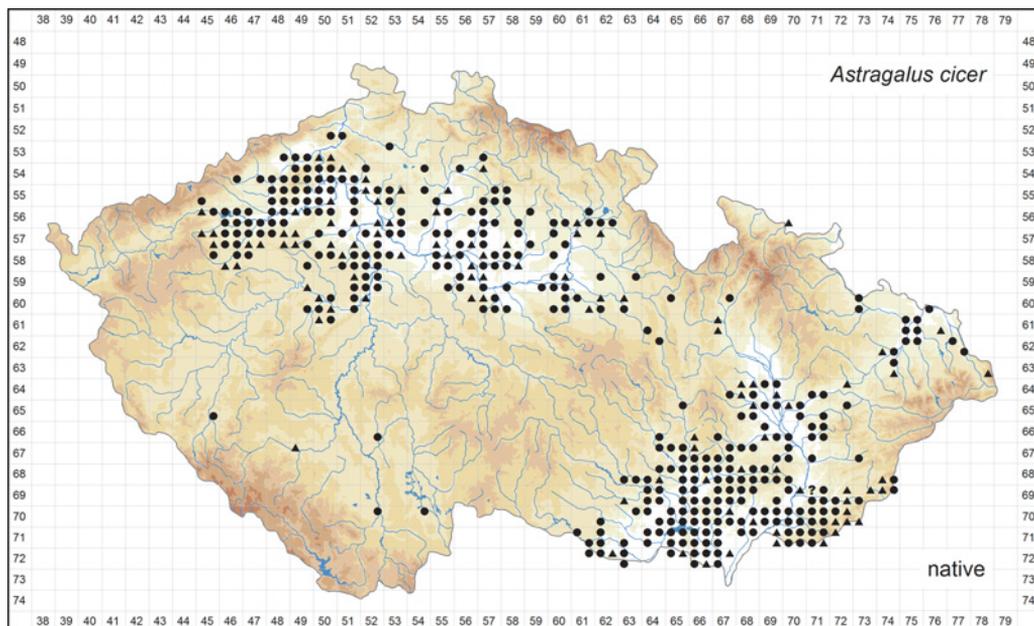
Fig. 7. – Distribution of *Astragalus cicer* in the Czech Republic: ● occurrence documented by herbarium specimens (335 quadrants), ▲ occurrence based on other records (100 quadrants). Prepared by Zdeněk Kaplan & Jiří Danihelka.
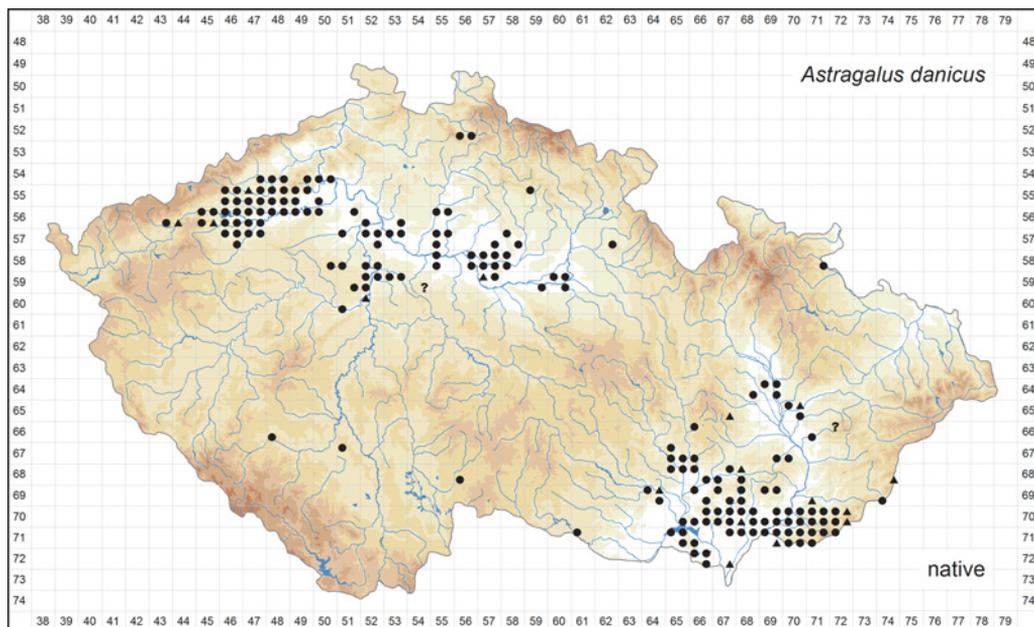

Fig. 8. – Distribution of *Astragalus danicus* in the Czech Republic: ● occurrence documented by herbarium specimens (171 quadrants), ▲ occurrence based on other records (16 quadrants). Prepared by Zdeněk Kaplan & Jiří Danihelka.

on a grassy slope above the railway near the village of Kaple in central Moravia north of the town of Prostějov. It was probably accidentally introduced to that site, and the population no longer exists. The species is currently classified as extinct (Grulich 2012).

Astragalus austriacus (Fig. 6)

Astragalus austriacus has a large distribution range including north-western Spain, south-western France, north-western Italy, large parts of central and south-eastern Europe (apart from Albania and Greece), Ukraine and the south-eastern part of European Russia in Europe, as well as the southern part of western and central Siberia and Kazakhstan in Asia (Meusel et al. 1965, Vydrina 1994, Podlech & Zarre 2013). In the Czech Republic it occurs in various types of dry grasslands; it is particularly well represented in the communities of continental steppes dominated by narrow-leaved tussock-forming grasses of the genera *Festuca* and *Stipa* (Chytrý 2007b), developed usually on base-rich and often calcareous bedrocks, including loess. It is found in the country's warmest and driest parts, i.e. in the hilly area north and north-west of Prague and in the České středohoří Mts in Bohemia, and south-west, south and south-east of Brno in Moravia. The localities in Bohemia are situated at the northern edge of the species' distribution range. Though *A. austriacus* may have somewhat declined during the last decades, it is not immediately endangered and it is therefore classified only as vulnerable (Grulich 2012).

Astragalus cicer (Fig. 7)

Astragalus cicer is native to Europe, mainly to the European Mediterranean area and south-eastern Europe, as well as Anatolia and Transcaucasia (Podlech & Zarre 2013). It has been introduced to and naturalized in the British Isles (Stace 2010), northern Europe and other parts of the World, such as North America (USDA, NRCS 2016). In the Czech Republic it is found in dry meadows and grasslands, pastures, forest fringes, road verges, railway stations, along railways and in similar semi-ruderal sites in settlements. It is fairly common in the northern half of Bohemia, southern and central Moravia south of Olomouc and scattered in the northernmost Moravia and adjacent Czech Silesia. It is difficult to distinguish between "native" and recent secondary occurrences as *A. cicer* usually grows in secondary habitats but it is quite certain that, e.g., scattered records from southern Bohemia, eastern Bohemia south-east of Pardubice and northern Moravia represent rather recent introductions (cf. Procházka 1977: 33). The species' distribution pattern clearly shows its affinity to warm and moderately warm parts of the country.

Astragalus danicus (Fig. 8)

Astragalus danicus is a species with a remarkably large distribution range, extending from Spain and the British Isles in the west over central and eastern Europe and Siberia as far as the Russian Far East; in North America it is replaced by its sibling species *A. agrestis*. The westernmost part of the distribution range is discontinuous and includes outposts in southern Scandinavia and the Caucasus Mts, but the species is absent from the Balkan Peninsula. In Asia, *A. danicus* occurs also in Kazakhstan, Kyrgyzstan, Mongolia and north-eastern China (Meusel et al. 1965, Vydrina 1994, Podlech & Zarre 2013). In the

Czech Republic *A. danicus* is found in semi-dry grasslands dominated by *Brachypodium pinnatum* or *Bromus erectus*, less frequently in continental thermophilous oak forests and various types of dry meadows, usually on deep heavy soils (but also on sand), developed on loess, marl, marlstone but also above basalt, usually well supplied with carbonates. It is almost continuously distributed in north-western Bohemia and scattered in central Bohemia. In the eastern part of the country, it is scattered in central Moravia and almost continuously distributed from the city of Brno towards the southeast, being particularly frequent in the south-western part of the Bílé Karpaty Mts. Mainly in the past *A. danicus* was accidentally introduced to other parts of the country outside its range, being able to establish viable and rather persistent populations, frequently in road verges and other secondary habitats. Numerous records found in literature, in particular those from southern and south-western Moravia, may be erroneous, based on misidentifications of *A. onobrychis*. The species is declining and currently classified as vulnerable (Grulich 2012).

Astragalus exscapus (Fig. 9)

Astragalus exscapus is a species with a discontinuous distribution range including central Germany, the Czech Republic, eastern Austria, the western Alps in Switzerland and Italy, Hungary, Romania, the Balkan Peninsula, Moldavia, southern Ukraine, southern European Russia and northern Anatolia. All central-European populations are assigned the type subspecies, while *A. exscapus* subsp. *transsilvanicus* is endemic to Romania and *A. e.* subsp. *pubiflorus* is found in south-eastern and eastern Europe (Meusel et al. 1965, Vydrina 1994, Podlech & Zarre 2013). In the Czech Republic *A. exscapus* is considered to be a postglacial relict now restricted to isolated refugia (Kaplan 2012). It is confined to vegetation of continental steppes dominated by narrow-leaved species of *Festuca* and *Stipa*; less frequently it is found in open scrub communities, both developed usually over calcareous or base-rich bedrock, such as loess, calcareous sand, sandstone or basalt on south-facing slopes. The vegetation is frequently disturbed by rabbits or landslides. Most of the sites may be considered naturally tree-less, but the species is able of colonizing secondary habitats, such as railway embankments (Dřevojan 2012). In the Czech Republic *A. exscapus* occurs in the western part of the České středohoří Mts, the adjacent hilly area south of the town of Chomutov and north of Prague in Bohemia as well as south and south-east of Brno in Moravia. The species is declining, mainly because of abandonment of former pastures, previous planting and later spread of *Robinia pseudoacacia* and vegetation succession; it is classified as endangered (Grulich 2012).

Astragalus glycyphyllos (Fig. 10)

Astragalus glycyphyllos is distributed over most of Europe, in the northern part of Anatolia, the Caucasus Mts, south-western Siberia and eastern Kazakhstan (Meusel et al. 1965, Vydrina 1994, Podlech & Zarre 2013). It has been recorded as alien in Quebec in Canada and the eastern USA (USDA, NRCS 2016). In the Czech Republic it occurs in thermophilous oak forests, oak-hornbeam forests, beech forests and their canopy openings, forest fringes, scree slopes, abandoned meadows, orchards and stone quarries, along roads and railways. It is common throughout most of the country, being absent only from its mountainous parts, such as the Krkonoše Mts and Hrubý Jeseník Mts, areas covered by plantations of coniferous trees, with arable fields as dominant land use type or
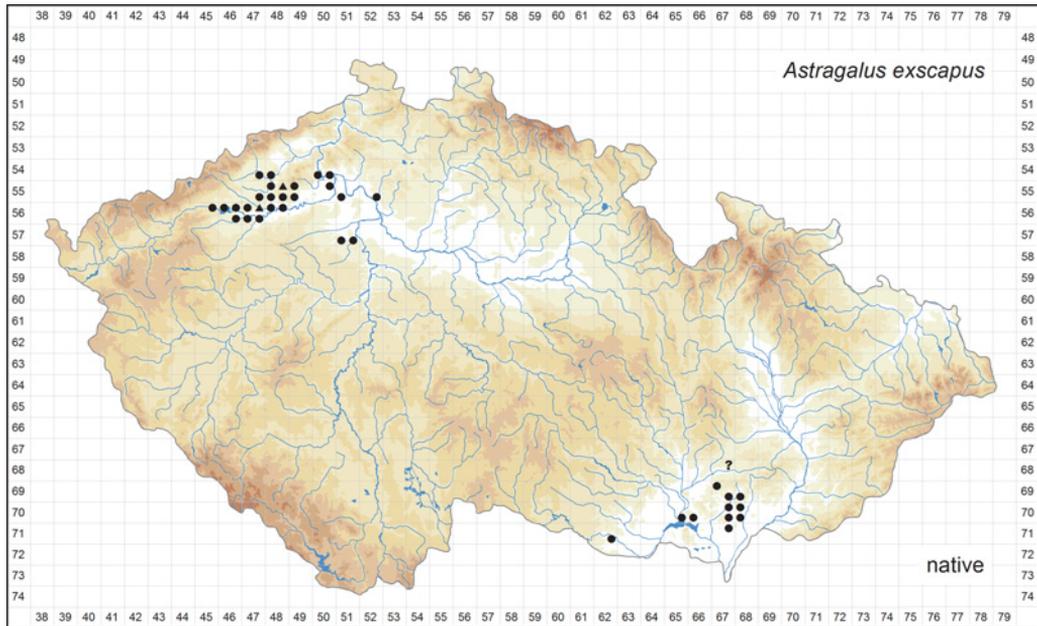
Fig. 9. – Distribution of *Astragalus exscapus* in the Czech Republic: ● occurrence documented by herbarium specimens (35 quadrants), ▲ occurrence based on other records (2 quadrants). Prepared by Zdeněk Kaplan & Jiří Danihelka.
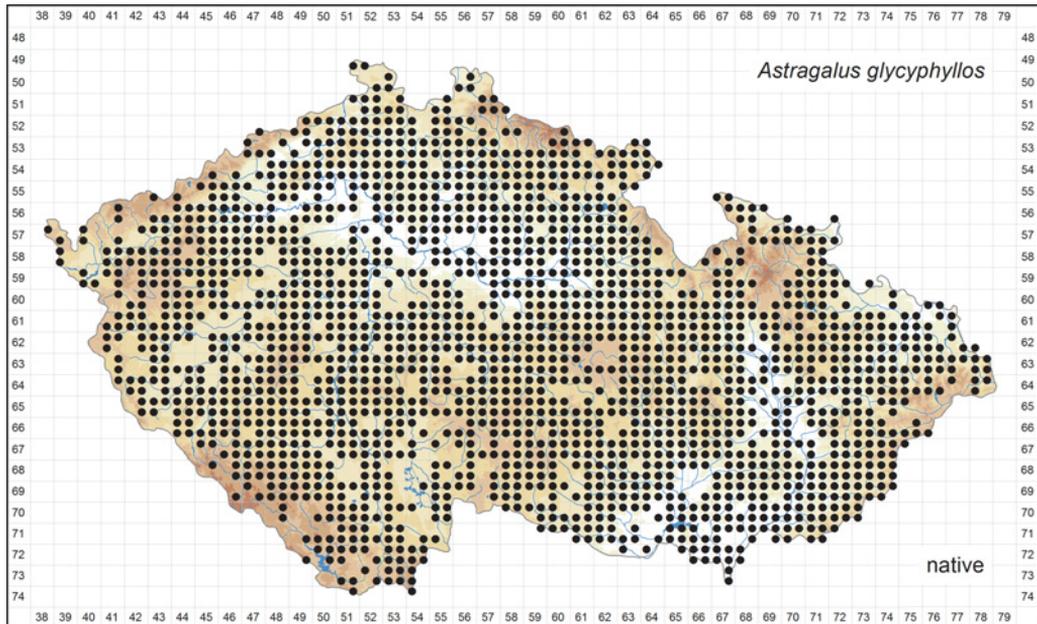

Fig. 10. – Distribution of *Astragalus glycyphyllos* in the Czech Republic (1876 occupied quadrants). Prepared by Zdeněk Kaplan & Jiří Danihelka.
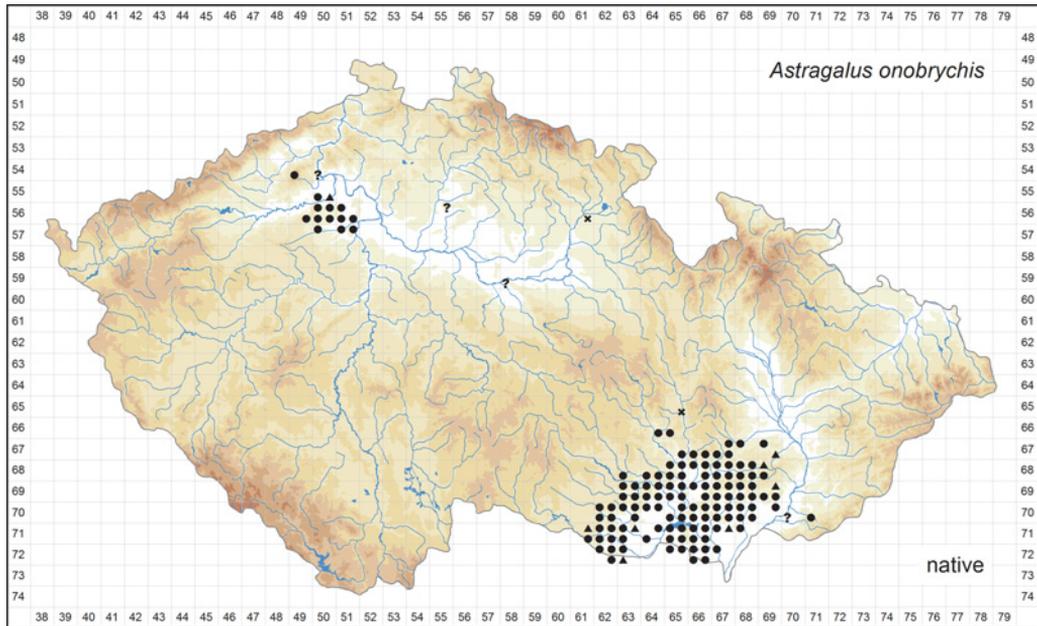
Fig. 11. – Distribution of *Astragalus onobrychis* in the Czech Republic: ● occurrence documented by herbarium specimens (123 quadrants), ▲ occurrence based on other records (8 quadrants), × alien (2 quadrants). Prepared by Zdeněk Kaplan & Jiří Danihelka.
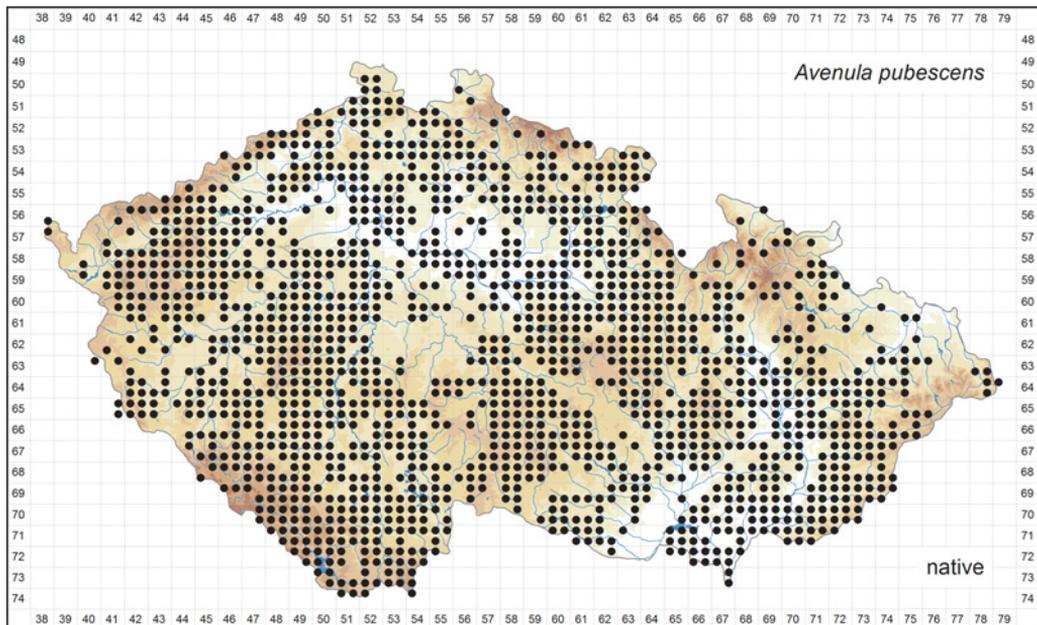

Fig. 12. – Distribution of *Avenula pubescens* in the Czech Republic (1567 occupied quadrants). Prepared by Jiří Zázvorka.

with very acidic soils, such as the Třeboňská pánev basin. Still, the gaps in the map may combine true absences with under-recording at least in warm and moderately warm parts of the country.

Astragalus onobrychis (Fig. 11)

Astragalus onobrychis is the most widespread member of *A.* sect. *Onobrychoidei*, a taxonomically difficult group of about 75 species in the Old World, with a distribution range reaching from France in the west over central, south-eastern and eastern Europe as far as western Siberia, Mongolia and Kazakhstan. It occurs also in Anatolia, the Caucasus Mts and Transcaucasia (Vydrina 1994, Podlech & Zarre 2013). As casual alien it has been introduced to Germany. In the Czech Republic *A. onobrychis* is usually found in vegetation of continental steppes dominated by narrow-leaved tussock-forming grasses of the genera *Festuca* and *Stipa* and in subcontinental semi-dry grasslands dominated by *Brachypodium pinnatum* or *Bromus erectus* (Chytrý 2007b, Novák & Chytrý 2007). The species occurs in a small area north-west of Prague with an outpost, documented by a single herbarium specimen, in the western part of the České středohoří Mts in Bohemia; in Moravia, its continuous local distribution range may be delimited by the lines connecting the city of Brno with the towns of Znojmo in the south-west and Veselí nad Moravou in the south-east, again with some outposts towards the north and east. It was accidentally introduced, probably with hay, to the garrison town of Josefov in eastern Bohemia (first recorded in 1933 and still present in 2009); in addition the northernmost occurrence Moravia north of the town of Blansko, recorded in 2014, may be due to accidental introduction. Both the Czech and Moravian localities are situated on the northern edge of the species' distribution range. The map is well supported by herbarium specimens so that most wrong records based on misidentifications of *A. danicus* have been eliminated. *Astragalus onobrychis* may have been slightly declined because of abandonment of former pastures and eutrophication, both enhancing succession by scrub vegetation. It is therefore classified as vulnerable (Grulich 2012).

Avenula pubescens (Fig. 12)

Avenula pubescens is a Euro-Siberian species with a large distribution range consisting of two separate parts. The western part extends throughout most of Europe, in Norway reaching as far as 70°N, eastwards to Ural Mts and southwards to Italy, and isolated patches are located in western Asia. The central-Asian part of its distribution range covers vast territories including the Ob and Irtysh basins, the Altai and Tian Shan Mts, central Siberia, Transbaikalia and northern Mongolia (Conert 1998). It is found in various types of grasslands, such as meadows, pastures and steppes, forest clearings, road and railway embankments, usually on dry or humid, more or less neutral soils rich in nutrients. In the Czech Republic it is scattered through the country, being rare in or absent from the highest elevation of the mountains. At lower altitudes it is less frequent in or absent from westernmost and south-eastern Bohemia as well as from western, southwestern and north-eastern Moravia and adjacent Silesia. It is most frequently found at altitudes of 150–800 m, in the Krušné hory Mts reaching 1000 m and in the Šumava Mts 1150 m.

Bidens cernuus (Fig. 13)

Bidens cernuus occurs in the temperate zone of the Northern Hemisphere, from Europe eastwards through south-western Asia as far as eastern Siberia, and scattered even in China and Japan. It is widespread in the warmer part of the temperate zone of North America, towards the north reaching 60°N (Hultén & Fries 1986, Chen & Hind 2011). In Europe it is widely distributed from southern Scandinavia and the Baltic countries southwards as far as northern Spain, central Italy and southern Greece (Meusel & Jäger 1992, Greuter 2006). *Bidens cernuus* grows on nutrient-rich soils on the banks of slow-flowing rivers, canals and streams, on the shores of fishponds, oxbows and around temporary pools in meadows. It also occurs on the bottoms of summer-drained fishponds and other water reservoirs. In the Czech Republic it is abundant predominantly in the fishpond landscapes of southern Bohemia, in the Českomoravská vrchovina highlands and the Železné hory hills. It tends to be rare in or absent from the dry part of north-western Bohemia, the Krkonoše Mts, Jizerské hory Mts and Krušné hory Mts; in the Šumava Mts it occurs only along the Vltava river and in adjacent areas. In Moravia it is markedly less frequent than in Bohemia, with most localities situated in the floodplains of lowland rivers around the cities of Ostrava and Olomouc, and the towns of Břeclav and Pohořelice. It occurs at altitudes about 150–750 m, reaching its altitudinal maximum in the Žďárské vrchy Mts.

Bidens connatus (Fig. 14)

Bidens connatus is native to eastern North America, including southern Canada and the USA. It was introduced to Europe probably in 1865 (Meusel & Jäger 1992). It is now scattered in central Europe, eastwards reaching as far as Ukraine, southwards to France and Italy, and westwards to the British Isles (Greuter 2006). In the Czech Republic it inhabits banks of lowland rivers, oxbows and canals, and humid waste places. *Bidens connatus* was first collected in 1934 in Prague along the Vltava river and in Litoměřice along the Labe river. In the eastern part of the country it was first recorded in 1964 in the Silesian town of Karviná. In the last decade it has spread to many new sites, especially along the Labe and Vltava rivers, and in the vicinity of Karviná, apparently as consequence of the great floods in 2002. It is currently classified as casual neophyte (Pyšek et al. 2012b).

Bidens frondosus (Fig. 15)

Bidens frondosus is native to North America, from southern Canada southwards to the southern USA. It has been introduced to Europe (Meusel & Jäger 1992), and isolated records are known from Morocco, Lebanon, Tajikistan, China, South Korea, Japan, French Guyana, and New Zealand (Mouterde 1986, Pyke et al. 2008, Han et al. 2009, Nobis & Nowak 2011, Chen & Hind 2011). In Europe, it was first recorded as escaped in 1777 in Poland (Lhotská 1966). Today it is naturalized almost in all European countries except Scandinavia and the Baltic countries, where it is classified as casual (Greuter 2006). In the Czech Republic it grows on nutrient-rich moist soils, invading banks of rivers, streams and canals, shores of oxbows, water reservoirs and fishponds, and also has been recorded in wet waste places, road ditches, along railways, in wet depressions in arable fields and meadows. The earliest record for the Czech flora from the town of Žďár
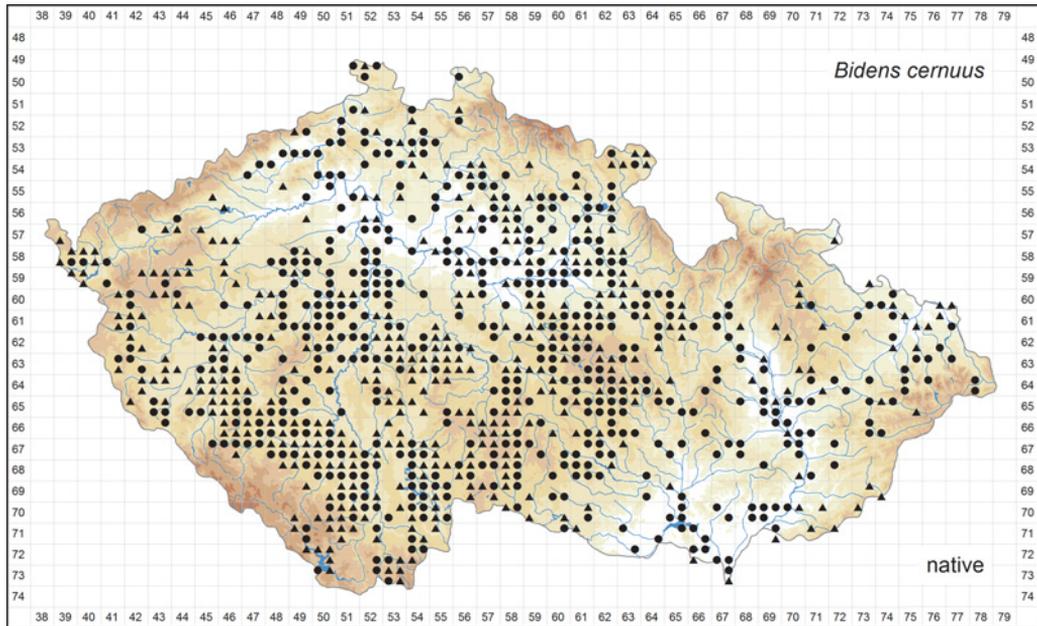
Fig. 13. – Distribution of *Bidens cernuus* in the Czech Republic: ● occurrence documented by herbarium specimens (522 quadrants), ▲ occurrence based on other records (388 quadrants). Prepared by Jitka Štěpánková.
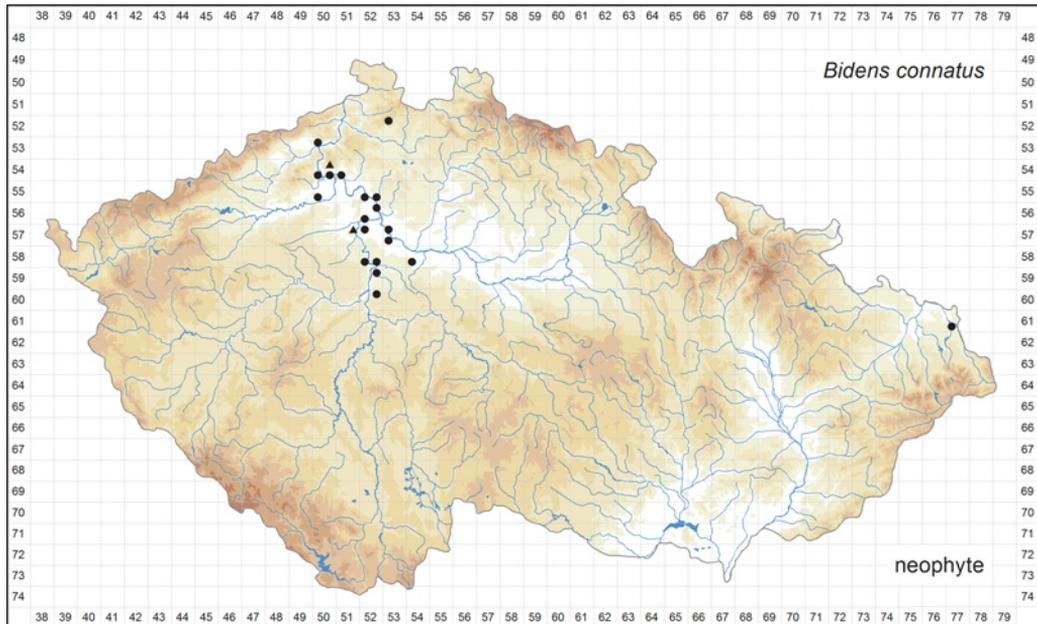

Fig. 14. – Distribution of *Bidens connatus* in the Czech Republic: ● occurrence documented by herbarium specimens (19 quadrants), ▲ occurrence based on other records (2 quadrants). Prepared by Jitka Štěpánková.
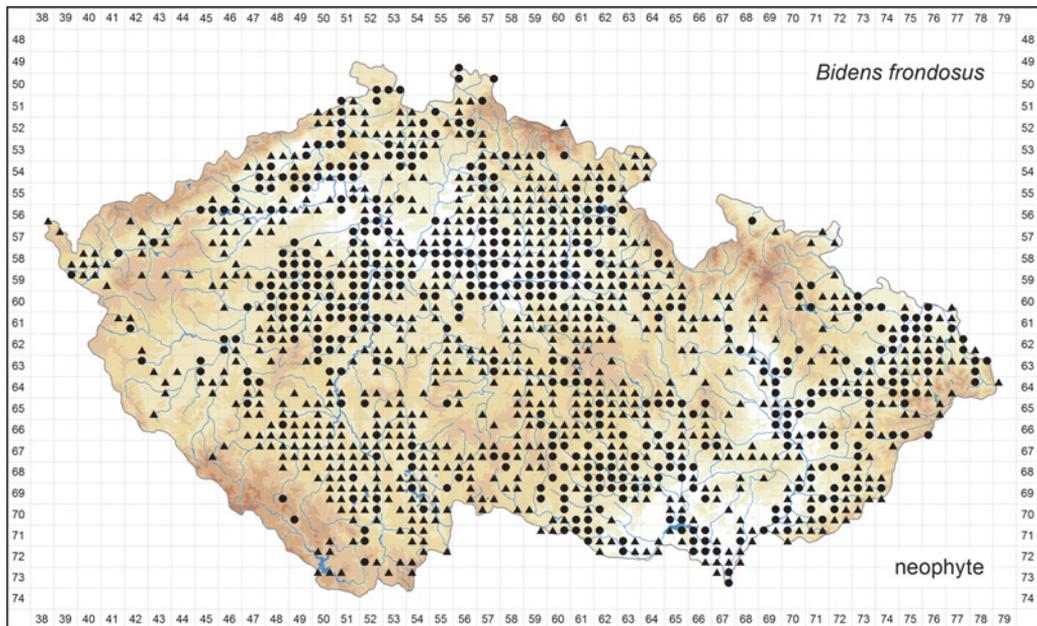
Fig. 15. – Distribution of *Bidens frondosus* in the Czech Republic: ● occurrence documented by herbarium specimens (466 quadrants), ▲ occurrence based on other records (788 quadrants). Prepared by Jitka Štěpánková.
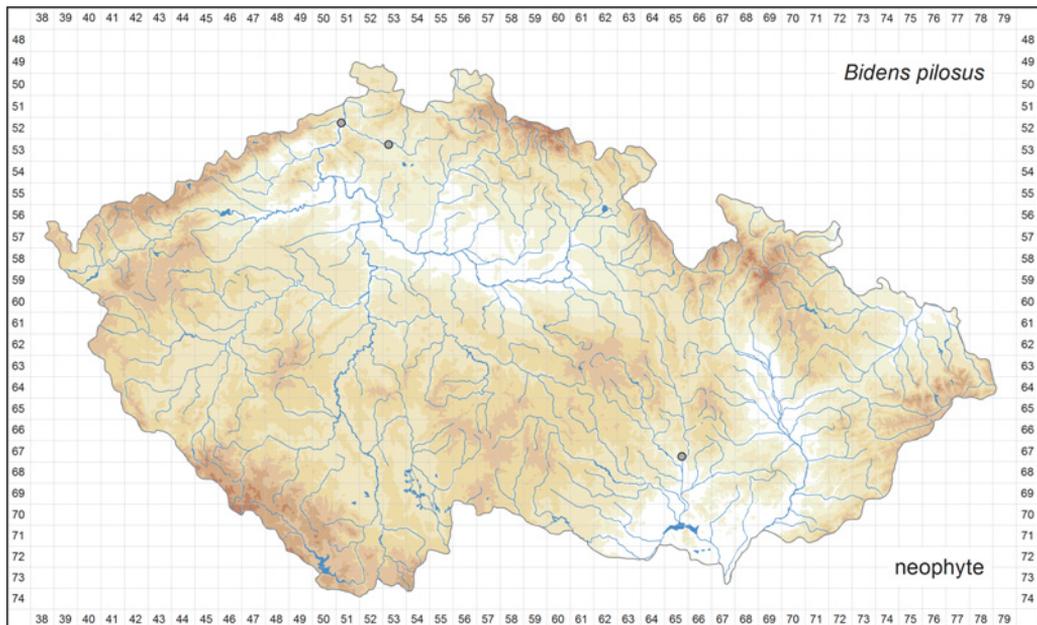

Fig. 16. – Distribution of *Bidens pilosus* in the Czech Republic (3 occupied quadrants). Prepared by Jitka Štěpánková.

nad Sázavou in Moravia dates back to 1907. The year 1894 given in Pyšek et al. (2012a, b) for the first record ever of *B. frondosus* in the Czech Republic is erroneous, relating in fact to the first record in Germany (Hegi 1925, Hejný 1948). The growing number of herbarium specimens and literature records suggests that it began to spread intensively as late as in the 1930s along the Labe and Vltava rivers. The distribution maps published by Hejný (1948) and Lhotská (1968) show records of *B. frondosus* almost exclusively from the alluvia of lowland rivers. At present it is distributed almost throughout the country. In Bohemia it is most abundant in lowlands, while being less frequent in middle altitudes, particularly in the west, and rare in or absent from the Krkonoše Mts, Jizerské hory Mts, Krušné hory Mts and Šumava Mts. It occurs frequently in most of Moravia, being rare in or absent only from the Hrubý Jeseník Mts and reaching its altitudinal maximum at 880 m in the Javorníky Mts. It is classified as an invasive neophyte (Pyšek et al. 2012b).

Bidens pilosus (Fig. 16)

Bidens pilosus is native to southern North America and South America, but now it is a noxious weed introduced to most of the tropical zone of the World, reaching occasionally the temperate zones of both hemispheres (Hadač & Hadačová 1969). In Europe *B. pilosus* occurs sparsely in its central and southern parts, northwards reaching Germany and Poland and southwards the Mediterranean area including northern Africa (Greuter 2006). It grows on mesotrophic to eutrophic neutral to basic, moist or dry soils (Hadač & Hadačová 1969). It is capable of invading various types of disturbed habitats such as railway embankments, road edges and dumping ground. The earliest herbarium record for the Czech Republic is from the town of Česká Lípa, dating back to 1913. It was further recorded in 1934 in Brno and in 1981–1983 in the railway station of Děčín-Loubí. It is classified as a casual neophyte (Pyšek et al. 2012b).

Bidens radiatus (Fig. 17)

The distribution range of *Bidens radiatus* is confined to Europe and the temperate zone of Asia (Hultén & Fries 1986, Meusel & Jäger 1992). In Europe it occurs from the Netherlands northwards to southern Scandinavia and the Baltic countries, eastwards to Ukraine and southwards to France and Italy (Greuter 2006). It grows on nutrient-rich soils on the shores of fishponds and oxbows, banks of slow-flowing rivers, canals and streams, and on the bottoms of summer-drained fishponds and other water reservoirs. In the Czech Republic it is abundant in the fishpond landscapes of southern Bohemia and in the Českomoravská vrchovina highlands. Recently, as possible results of climatic changes and the great floods in 2002, it has been spreading in central and eastern Bohemia, where it used to be rare before. In Moravia it is generally rare, occurring more frequently only in its south-western part. It is found at altitudes about 160–700 m, reaching its maximum elevation in the Žďárské vrchy Mts.

Bidens tripartitus (Fig. 18)

Bidens tripartitus is native to Europe, northern Africa and Asia, except its north and north-western part. It has been introduced to many other parts of the World, now being frequent in North America, Australia and New Zealand. In Europe it is found over the

whole of the continent, excluding its northernmost and southernmost parts. In Asia it is widespread through central and south-eastern Siberia to China, Sakhalin, the Kuril Islands, the Korean Peninsula and Japan (Hultén & Fries 1986, Meusel & Jäger 1992, Greuter 2006). It grows on nutrient-rich soils on the banks of slow-flowing rivers, canals and streams, on the shores and exposed bottoms of fishponds and oxbows and in wet depressions in grasslands and arable fields, less frequently in road ditches and humid ruderal sites in human settlements. It is distributed throughout the Czech Republic from the lowlands to middle altitudes, reaching its altitudinal maximum at 900 m in the Novohradské hory Mts. It is fairly common in central, southern and eastern Bohemia, less frequent in dry areas of western Bohemia and north-central Bohemia and rare in or absent from the highest mountains. It is abundant in Moravia, becoming less frequent in its north-western part.

Carex appropinquata (Fig. 19)

Carex appropinquata has been recorded across most of Europe, from the British Isles and southern parts of the Scandinavian Peninsula in the north-west as far as Greece in the south-east. It is also found in the Caucasus Mts, Anatolia, western and central part of southern Siberia, in the east reaching as far as Lake Baikal (Hultén & Fries 1986, Egorova 1999). It occurs over base-rich substrates (limestone, marl and serpentine) on gley or organic, slightly acidic to slightly basic soils with a high groundwater level. It is most frequent, sometimes even forming monodominant stands, in rich fens, fen and wet meadows, less frequent in alder carrs. In the Czech Republic *C. appropinquata* occurs scattered in the warm and moderately warm parts of the country, being rather rare in the mountains. In Moravia it has been known from its south-eastern and central parts, becoming rarer towards the north and missing from the Moravian part of the Carpathians. Its altitudinal range is 165–750 m. *Carex appropinquata* has been declining both in number of sites and in population size. It is particularly threatened by habitat destruction, most frequently drainage, and less so by abandonment of meadows and subsequent encroachment. It has therefore been classified as endangered (Grulich 2012). The literature records may be contaminated by misidentifications of the similar *C. paniculata*.

Carex chordorrhiza (Fig. 20)

Carex chordorrhiza is a boreal circumpolar species occurring only rarely in central and southern Europe. It has been known from central Spain, the Pyrenees, the Alps, Germany, the Czech Republic, Poland and Slovakia. It is widespread throughout the boreal zone of eastern Europe and Asia as far as Sakhalin and the Kamchatka Peninsula. In North America it grows across the north from Alaska to Newfoundland, southwards as far as the Great Lakes region (Meusel et al. 1965, Page & Rieley 1985, Hultén & Fries 1986). The Czech Republic is situated at the southern limit of the species' distribution range. *Carex chordorrhiza* is confined to transitional peat bogs. It has been found at only 15 localities in the moderately warm and cold areas. Most of its localities are situated in Bohemia; in Moravia it is found only in the Jihlavské vrchy hills and Žďárské vrchy hills. The altitudinal range of this species in the Czech Republic is 275–1210 m. The records of *C. chordorrhiza* from the Jizerské hory Mts are either doubtful or refer to the Polish part of the mountains (Plocek 1986). *Carex chordorrhiza* occurs in habitats that are prone to
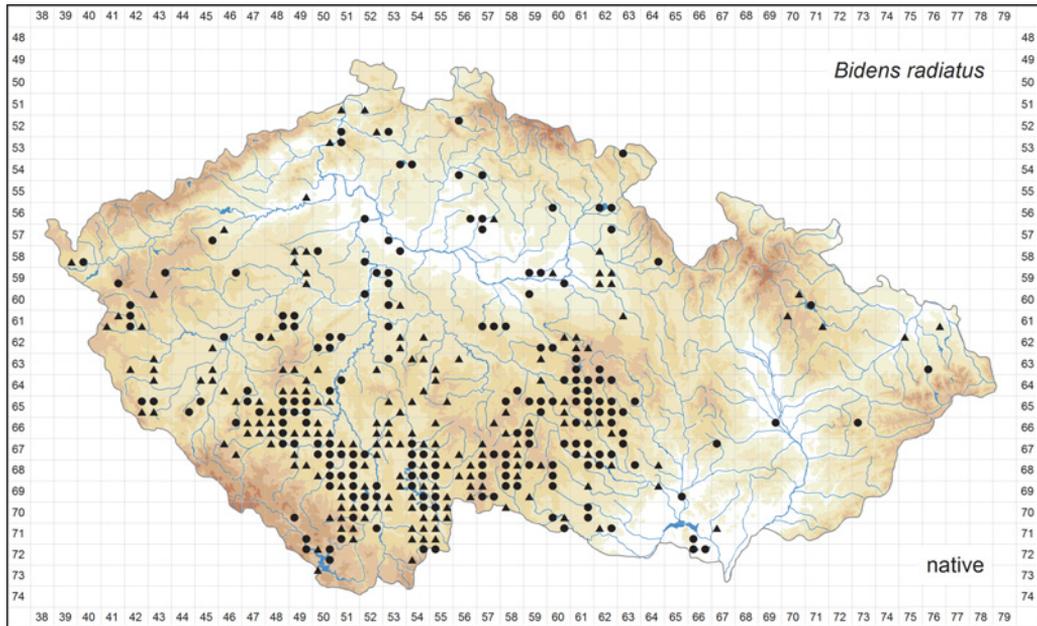
Fig. 17. – Distribution of *Bidens radiatus* in the Czech Republic: ● occurrence documented by herbarium specimens (188 quadrants), ▲ occurrence based on other records (178 quadrants). Prepared by Jitka Štěpánková.
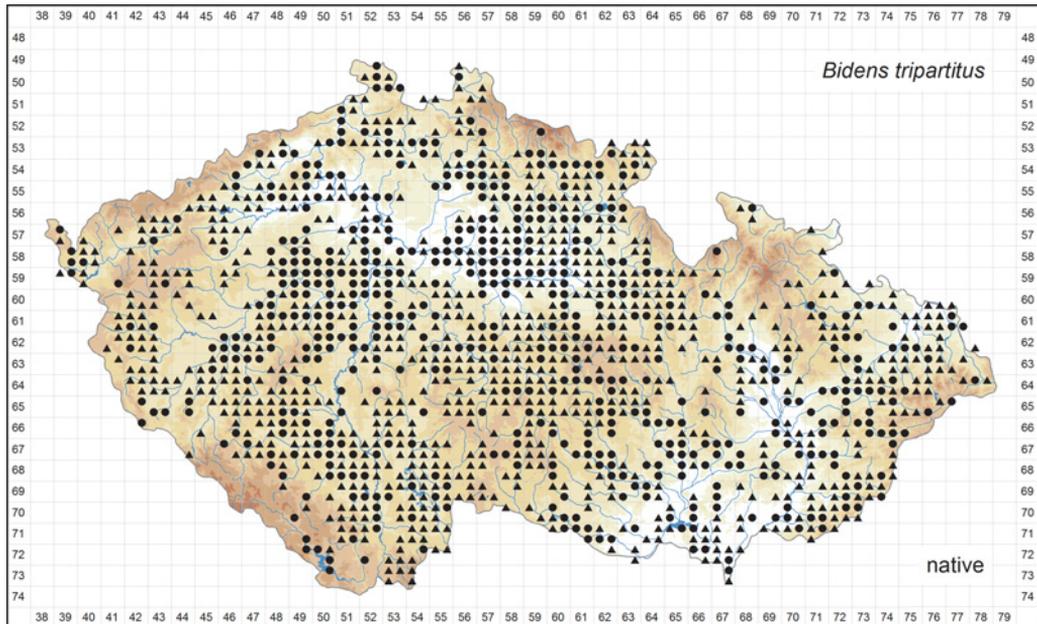

Fig. 18. – Distribution of *Bidens tripartitus* in the Czech Republic: ● occurrence documented by herbarium specimens (546 quadrants), ▲ occurrence based on other records (804 quadrants). Prepared by Jitka Štěpánková.
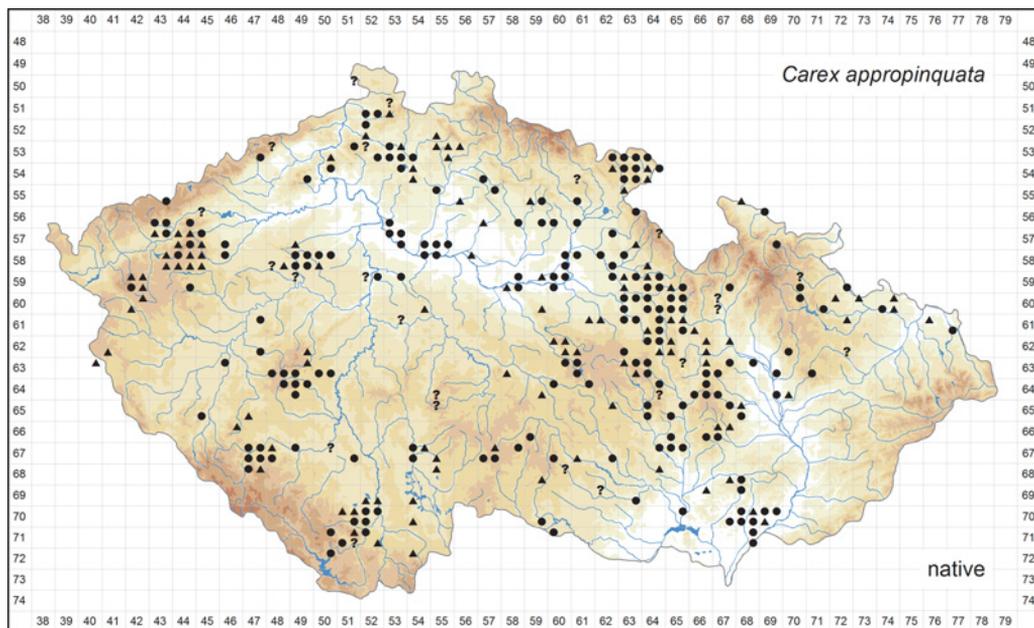
Fig. 19. – Distribution of *Carex appropinquata* in the Czech Republic: ● occurrence documented by herbarium specimens (190 quadrants), ▲ occurrence based on other records (110 quadrants). Prepared by Vít Grulich & Radomír Řepka.
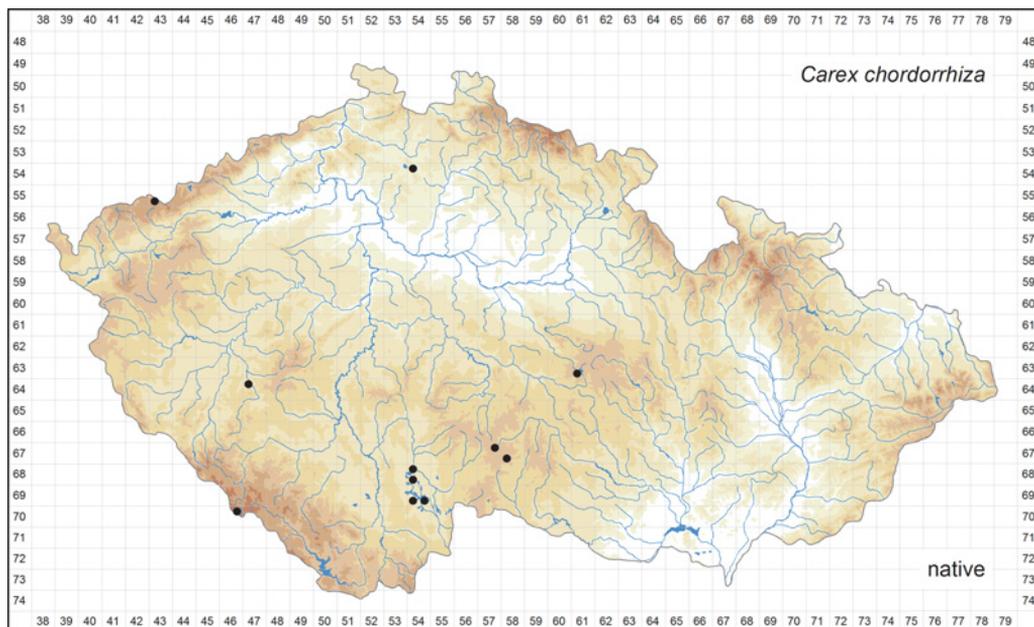
Fig. 20. – Distribution of *Carex chordorrhiza* in the Czech Republic (11 occupied quadrants). Prepared by Vít Grulich & Radomír Řepka.
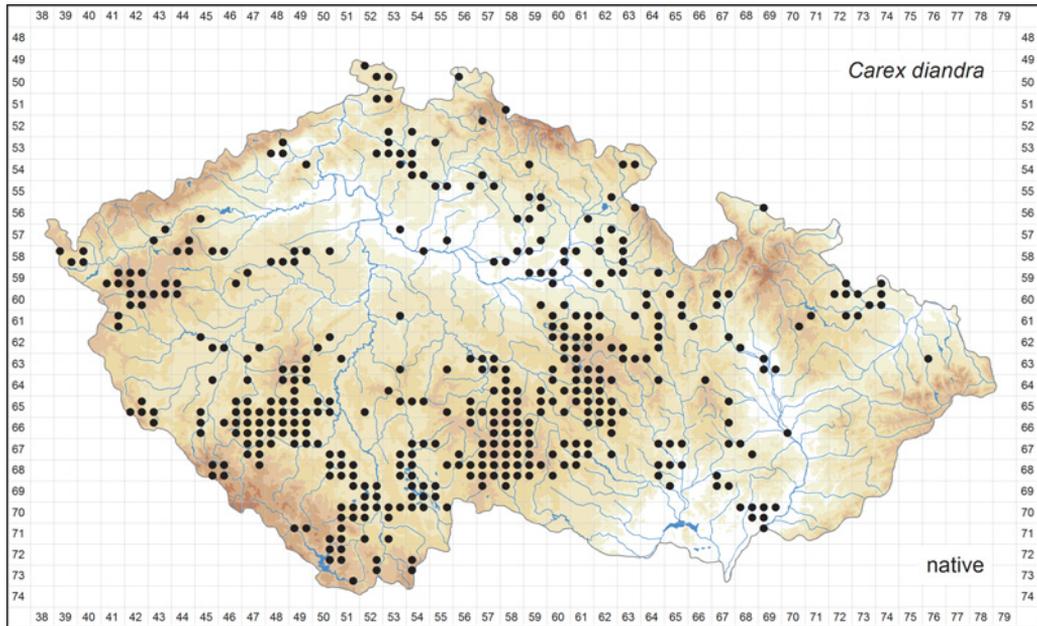
Fig. 21. – Distribution of *Carex diandra* in the Czech Republic (417 occupied quadrants). Prepared by Vít Grulich & Radomír Řepka.
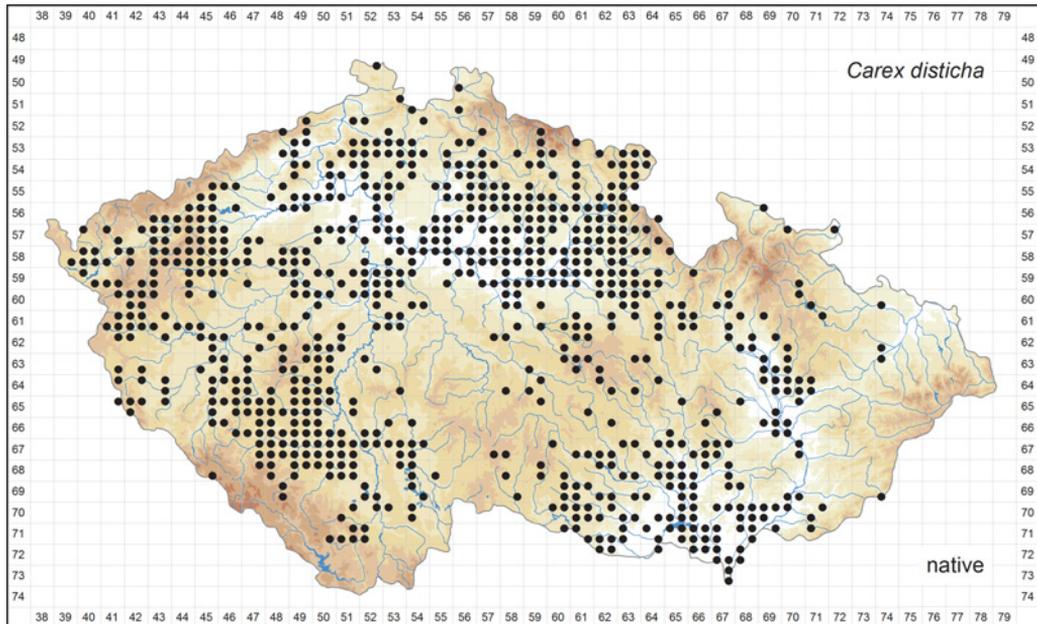
Fig. 22. – Distribution of *Carex disticha* in the Czech Republic (793 occupied quadrants). Prepared by Vít Grulich & Radomír Řepka.
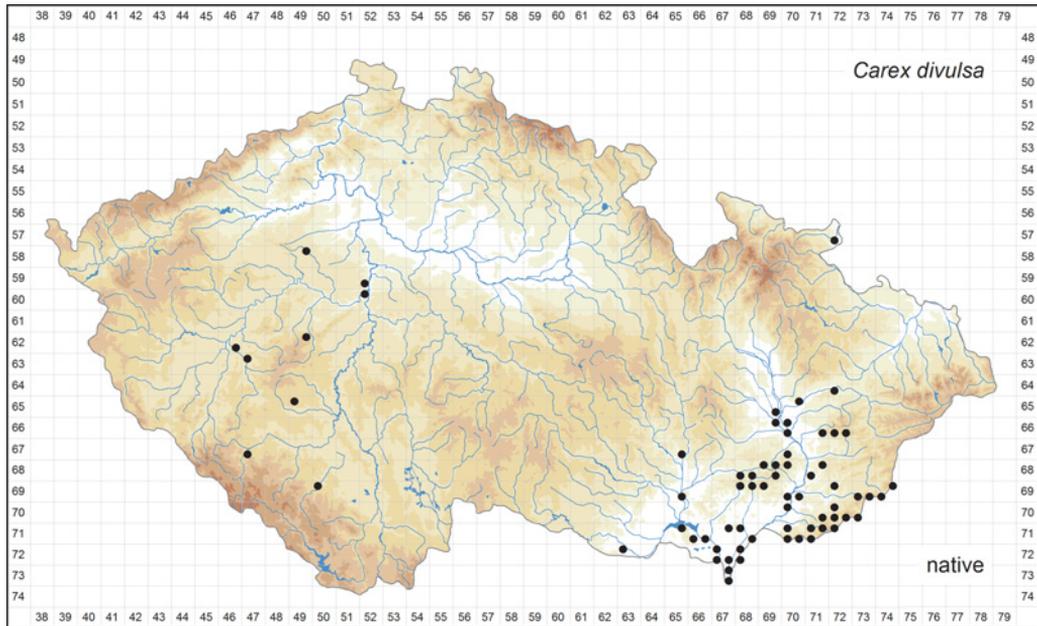
Fig. 23. – Distribution of *Carex divulsa* in the Czech Republic (67 occupied quadrants). Prepared by Radomír Řepka & Vít Grulich.
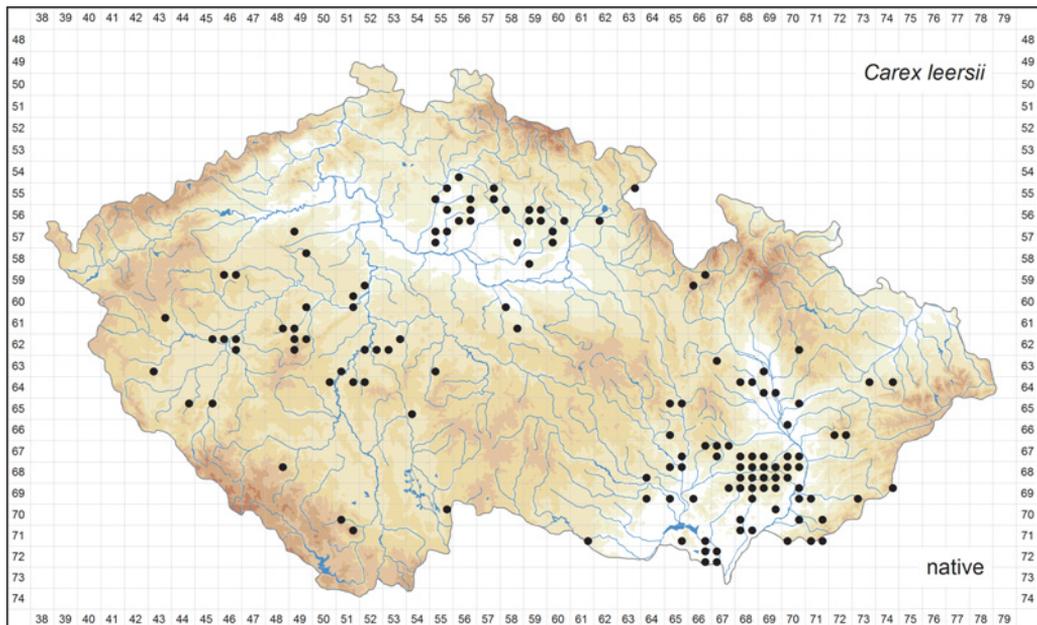

Fig. 24. – Distribution of *Carex leersii* in the Czech Republic (134 occupied quadrants). Prepared by Radomír Řepka & Vít Grulich.

damage by human intervention, such as excavation of peat and drainage. Some of the populations have been destroyed and nowadays only 6 or 7 still exist. The species is therefore classified as critically endangered (Grulich 2012).

Carex diandra (Fig. 21)

Carex diandra is a circumboreal species with a very large distribution range. It is common in northern and central Europe, towards the east reaching as far as the Far East via Siberia. Isolated occurrences are known from the Caucasus Mts, Anatolia, Kazakhstan, the Central-Asian mountains, Afghanistan and Pakistan. *Carex diandra* is common in the boreal zone of North America, extending southwards along the Rocky Mts to southern California. The records from the Canary Islands and New Zealand are erroneous (Hultén & Fries 1986, Egorova 1999). *Carex diandra* is a weak competitor very sensitive to eutrophication. It is most frequently found in poor fens, fen meadows, in marsh vegetation of tall sedges on the shores of fishponds and along ditches on fen soils with a slightly acidic to slightly alkaline pH. It is most abundant in middle altitudes, becoming less frequent towards the mountains. The species is frequent in southern Bohemia and in the Českomoravská vrchovina highlands. It is scattered in western and northern Bohemia and in parts of eastern Bohemia adjacent to Moravia. In Moravia this sedge occurs mainly in its central and northern parts, while being rare in the south. *Carex diandra* has declined considerably due to drainage and direct habitat destruction and it is now classified as endangered (Grulich 2012).

Carex disticha (Fig. 22)

The distribution range of *Carex disticha* extends from the British Isles in the west as far as the southern part of Siberia, Kazakhstan and China in the east. In Europe it occurs mainly in its central and western parts. Its southern range limit runs through northern Spain, southern France, central Italy, the countries of the former Yugoslavia and Bulgaria. There are also a few records from Anatolia and the Caucasus Mts (Meusel et al. 1965, Hultén & Fries 1986). In the Czech Republic *C. disticha* occurs in marsh vegetation of tall sedges, wet and alluvial meadows, and less frequently in fen meadows on heavy or sandy soils, usually rich in nutrients and with a high groundwater level. The species is frequent to scattered throughout the parts of the country with a warm and moderately warm climate; in contrast, it is very rare in the Českomoravská vrchovina and Drahanská vrchovina highlands, the Carpathian part of Moravia, Czech Silesia and generally in the mountains. Its altitudinal range is 150–980 m.

Carex divulsa (Fig. 23)

Carex divulsa is one of the eight taxa subsumed by Molina et al. (2008a) under *C. divulsa* agg., of which also *C. leersii* and *C. otomana* are present in central Europe. *Carex divulsa* is distributed mainly in western and central Europe from the British Isles in the west to Ukraine in the east. There are also records from southern Scandinavia and Latvia. It is common in the Carpathians. Outside Europe it is known from Anatolia, the Caucasus Mts and Turkmenistan (Molina et al. 2008a). In the Czech Republic *C. divulsa* is confined to forests with a closed or moderately closed canopy, in the same time showing rather

a strong affinity to disturbed places. It is usually found in hard-wood floodplain forests, oak-hornbeam and beech forests. It grows on soils that are rich in nutrients and humus, humid in the spring but may become dry during the summer. *Carex divulsa* is a rare species of the Czech flora. Most of its localities are found in southern and south-eastern Moravia along the Morava and Dyje rivers and in the Moravian Carpathians. The isolated occurrence in Czech Silesia is quite remote but it is situated not far from the closest localities in Poland. The dispersed records from Bohemia may relate both to indigenous populations (e.g. in the Brdy Mts) and accidental introductions (e.g. surroundings of the city of Plzeň), while status of other populations is uncertain. *Carex divulsa* was recorded at altitudes of 150–750 m. Some populations in southern and south-eastern Moravia approach *C. otomana* in morphological characters and could not be identified with certainty. Our experience from herbarium studies suggests that many literature records may be erroneous, based on misidentified specimens of *C. leersii* or *C. otomana*. That is why the distribution map is based only on revised herbarium specimens. *Carex divulsa* has been classified as endangered (Grulich 2012).

Carex leersii (Fig. 24)

Carex leersii occurs almost throughout the whole of Europe, from the British Isles in the west to the easternmost parts of Europe. It is most widespread in western and central Europe, including France, Germany and Austria. In its Mediterranean part it is replaced by *C. enokii*, in northern Europe by the recently described *C. nordica* and in south-western Europe by *C. magacis* (Molina et al. 2008a). Outside Europe it has been recorded in Anatolia. In the Czech Republic *C. leersii* grows mostly in oak-hornbeam forests, thermophilous oak forests and beech forests, usually in canopy openings, in forest edges, clearings, along forest paths and roads, in ditches and other disturbed open habitats such as road edges, railway embankments and city parks. In comparison with other species of *C.* sect. *Phaestoglochin* it seems to prefer base-rich soils developed above sandstone, limestone or marl. In the Czech Republic *C. leersii* grows mainly in eastern and eastern-central Bohemia and southern Moravia. It is rare elsewhere but may become locally abundant, for instance in the surroundings of the city of Plzeň. It is generally more frequent in Moravia, mainly in the Ždánický les, Litenčické vrchy and Chřiby hills. Its altitudinal maximum is 800 m. The distribution map is based solely on revised herbarium specimens.

Carex muricata (Fig. 25)

This species has a very large Eurasian distribution range reaching from the British Isles and Scandinavia in the west over southern Siberia as far as Mongolia and western China in the east; it is also found in the Caucasus Mts. In south-western Europe it occurs only in the Pyrenees, elsewhere being replaced by *C. pairae* and *C. omeyica*. There are further records from the Mediterranean area, where the species is confined to the mountains. Molina et al. (2008b) distinguish three subspecies, of which *C. m.* subsp. *muricata* and *C. m.* subsp. *cesanensis* are reported to occur in central Europe. However, the plants found in the Czech Republic may be assigned to any of the three subspecies based on the character states of a particular specimen; we therefore consider the subspecies to be of little taxonomic value. The most similar species is *C. pairae*, which was not distinguished from *C. muricata* in the Czech botanical literature until the late 1870s, and both species
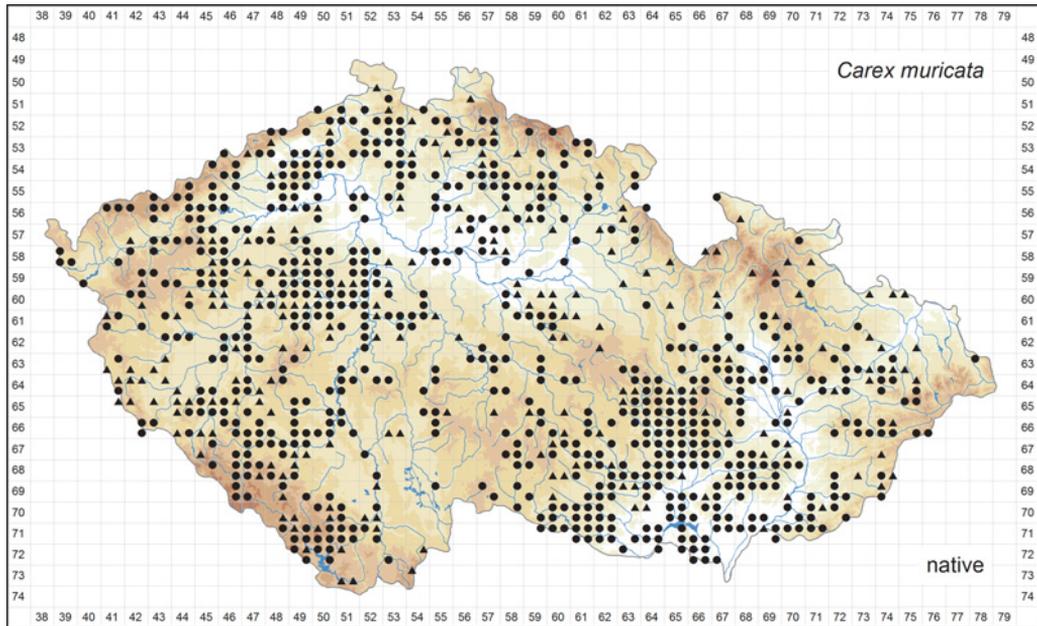
Fig. 25. – Distribution of *Carex muricata* in the Czech Republic: ● occurrence documented by herbarium specimens (672 quadrants), ▲ occurrence based on other records (217 quadrants). Prepared by Radomír Řepka & Vít Grulich.
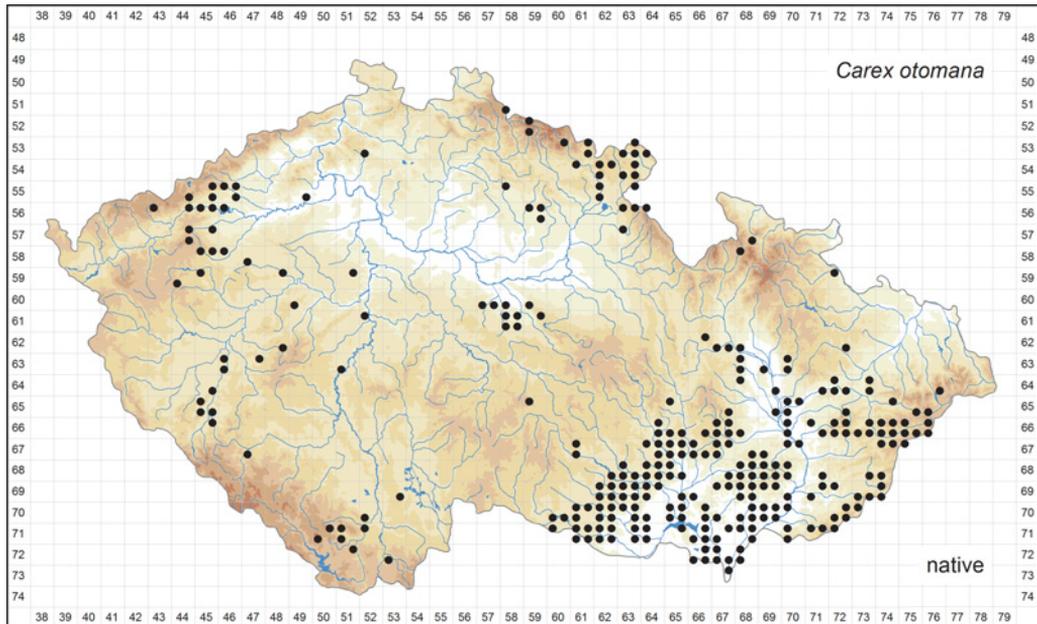
Fig. 26. – Distribution of *Carex otomana* in the Czech Republic (290 occupied quadrants). Prepared by Radomír Řepka & Vít Grulich.
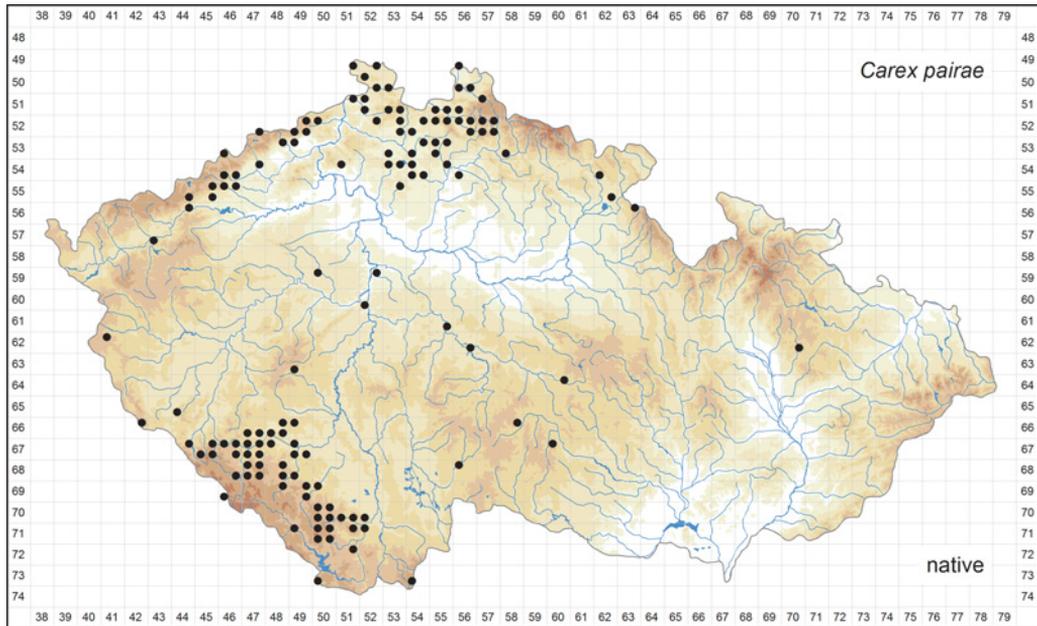
Fig. 27. – Distribution of *Carex pairae* in the Czech Republic (133 occupied quadrants). Prepared by Radomír Řepka & Vít Grulich.
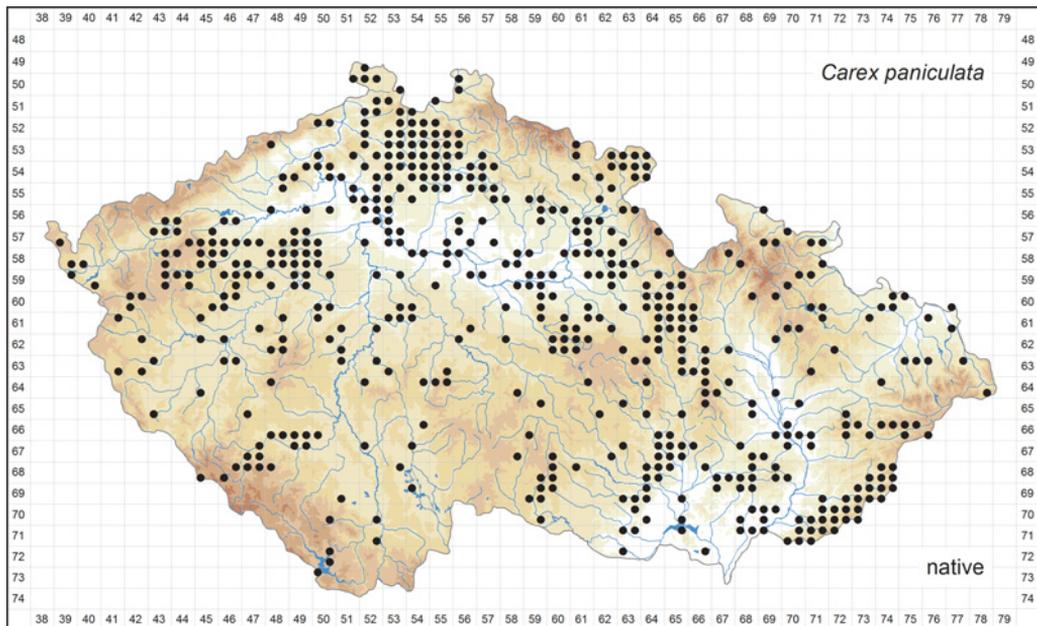
Fig. 28. – Distribution of *Carex paniculata* in the Czech Republic (539 occupied quadrants). Prepared by Vít Grulich & Radomír Řepka.

were referred to under the former name. This renders all earlier literature records of little use. In the Czech Republic *C. muricata* usually occurs in oak-hornbeam and beech forests, less frequently in Norway spruce plantations, black locust groves and other types of forests. It is further found in shrubby slopes, abandoned orchards, field boundaries, pastures, meadows and rocky hillsides, only rarely also in ruderal nitrogen-rich habitats. It prefers moderately shaded places with nutrient-rich soils, mostly above basic substrates (limestone and marl, but often also above crystalline bedrock) and it does not tolerate a high groundwater level. Like other species of the section it prefers slightly disturbed sites such as clearings and edges of forest roads. In the Czech Republic *C. muricata* is quite frequent in warm and moderately warm parts of the country, while being rare in the mountains. After *C. spicata*, this is the second most common species of the *C.* sect. *Phaestoglochin* in the country. It is common in well-preserved semi-natural deciduous forests but rare elsewhere, which explains its rarity in the Českomoravská vrchovina highlands, western Bohemia and the South Bohemian pond basins. It avoids also dry deforested areas. The species' altitudinal maxima in Czech mountain ranges are 1090–1120 m.

Carex otomana (Fig. 26)

Carex otomana was described from the western part of the Tian-Shan Mts in Central Asia. It is reported to occur also in Iran, Transcaucasia, Anatolia, Greece and Bulgaria (Molina et al. 2008a). According to our own findings, it is common in central Europe, in the west probably reaching Alsace in France. Still, its distribution is not yet known in detail. Formerly the species was recognized in the Czech botanical literature as *C. chabertii* (Řepka 1988). However, this name is no longer applicable because it was typified by a specimen taxonomically corresponding to *C. leersii* (Loos 1996). *Carex otomana* is remarkably variable over its distribution range but this variation is difficult to separate from habitat modifications; for instance, specimens from shaded habitats often resemble *C. divulsa*. In the Czech Republic *C. otomana* grows most frequently in oak-hornbeam forests, dry oak forest and black locust groves. Sometimes it is found in mixed stands of Scots pine and its plantations. It prefers semi-shaded places, such as forest edges, verges of forest roads and young tree plantations in clearings. It is found on loamy or sandy soils that are well supplied with nutrients. In the Czech Republic it is almost continuously distributed in southern, central and eastern Moravia. In contrast, it is much rarer and occurs only locally in Bohemia without any clear phytogeographical pattern. It grows from the lowlands to a maximum altitude of 900 m; however, it was also collected as accidentally introduced at 1335 m a.s.l. in the Krkonoše Mts. Because of frequent misidentifications, the distribution map is based solely on revised herbarium specimens.

Carex pairae (Fig. 27)

Carex pairae occurs mainly in south-western and western Europe, including the British Isles, the Iberian Peninsula, France and the Benelux countries. It is rare in southern and central Scandinavia and scattered over central Europe, where it occurs in Germany, the Czech Republic and Poland (Molina et al. 2008b). According to these authors it is also found in a large part of the Mediterranean area and the Balkans but we have not seen any specimens from there. In the Czech literature this species had been merged with *C. muricata* until the 1870s. However, the two taxa differ by a set of morphological and

phenological characters as well as by their ecological requirements (Hylander 1966, Hartvig 1987, Řepka 2003). In the Czech Republic it most frequently grows in pastures and dry meadows, on grassy and shrubby slopes, in field boundaries, road verges and along footpaths, but not in strongly eutrophicated habitats. Less frequently it occurs also in deciduous and coniferous forests, forest edges, clearings and along forest roads. It is usually found in sunny or semi-shaded habitats on sandy or loamy acidic soils, developed above acidic rocks, rarely also above basalt or limestone. In the Czech Republic *C. pairae* has a distribution pattern typical of Subatlantic floristic elements, with localities concentrated in the moderately warm parts of the western half of Bohemia, namely in northernmost Bohemia, the Krušné hory Mts and at the foothills of the Šumava Mts. There are only individual records from other parts of the country, the easternmost one from the Nízký Jeseník hills. *Carex pairae* is found in the mountains more often than the other members of the *C. muricata* aggregate, reaching altitudinal maximum at 1065 m in the Šumava Mts. Because of frequent misidentifications, the distribution map is based solely on revised herbarium specimens.

Carex paniculata (Fig. 28)

Carex paniculata has been recorded in almost all European countries, from the British Isles in the west to the Baltic countries and the western part of European Russia in the east. In Scandinavia it reaches central Sweden and south-western Finland. It is rare in southern Europe, where it has been recorded mainly in the mountains, reaching the southern margin of its distribution range on the continent in central Italy and northern Greece, and also occurring in Anatolia and the Caucasus Mts (Meusel et al. 1965, Hultén & Fries 1986). Plants from the south-western part of the distribution range are usually treated as a separate subspecies (see Chater 1980). In the Czech Republic *C. paniculata* grows mainly in wet meadows and tall sedge communities, sometimes forming even monodominant stands. It is often found around meadow springs, on shores of reservoirs, banks of streams, rarely in willow and alder carrs and fens. It is a competitively strong species, often forming conspicuous robust tussocks. *Carex paniculata* usually grows on gley soils rich in nutrients and with neutral to slightly alkaline pH, often above carbonate bedrock, less frequently on nutrient-poor acidic soils, but always with a high groundwater level. In the Czech Republic *C. paniculata* occurs almost throughout the country, but with varying frequency. Most of its localities are situated in the moderately warm regions, while being less frequent in the areas with a warm climate and rare in cold mountains. It is abundant in northern and eastern Bohemia, the Českomoravské mezihoří highlands and in the Bílé Karpaty Mts. In contrast, *C. paniculata* is almost missing from other parts of the Czech Republic such as the Bohemian side of the Českomoravská vrchovina highlands. In the Rýchory Mts it was recorded at an altitude of 950 m, reaching there its altitudinal maximum.

Carex spicata (Fig. 29)

Carex spicata has a wide Eurasian distribution range centred in the temperate zone. It grows in almost all of Europe from the British Isles and Iceland in the west as far as Lake Baikal in Siberia. In Europe it is missing from the boreal zone north of the 64th parallel in Scandinavia and in the northern part of European Russia. It is very rare in the warmest part of the Mediterranean area and in northern Africa, where it grows only in the moun-
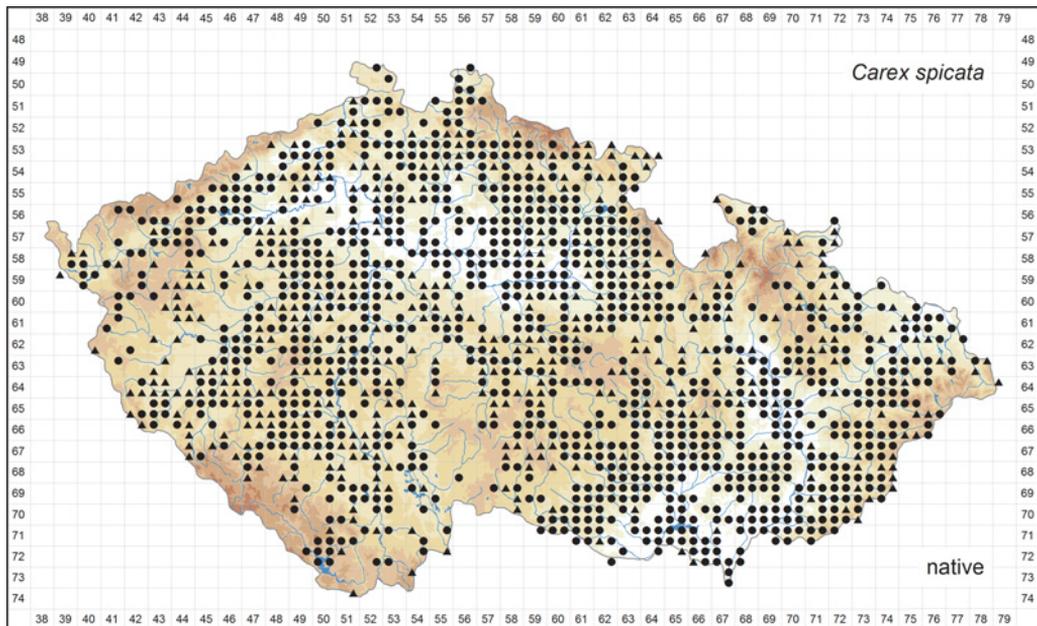
Fig. 29. – Distribution of *Carex spicata* in the Czech Republic: ● occurrence documented by herbarium specimens (1047 quadrants), ▲ occurrence based on other records (343 quadrants). Prepared by Radomír Řepka & Vít Grulich.
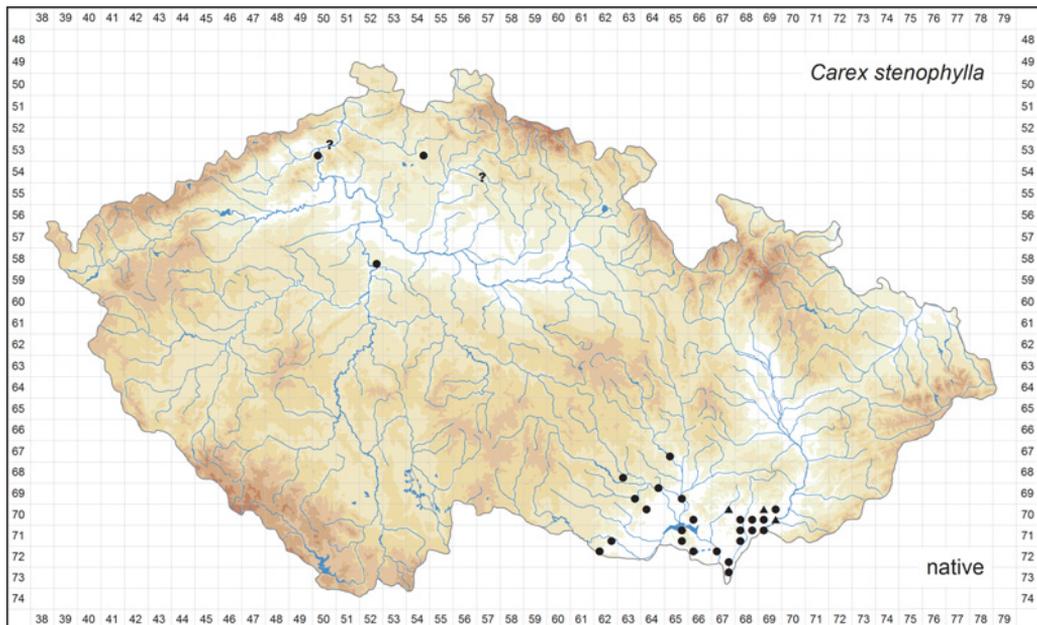
Fig. 30. – Distribution of *Carex stenophylla* in the Czech Republic: ● occurrence documented by herbarium specimens (26 quadrants), ▲ occurrence based on other records (3 quadrants). Prepared by Vít Grulich & Radomír Řepka.
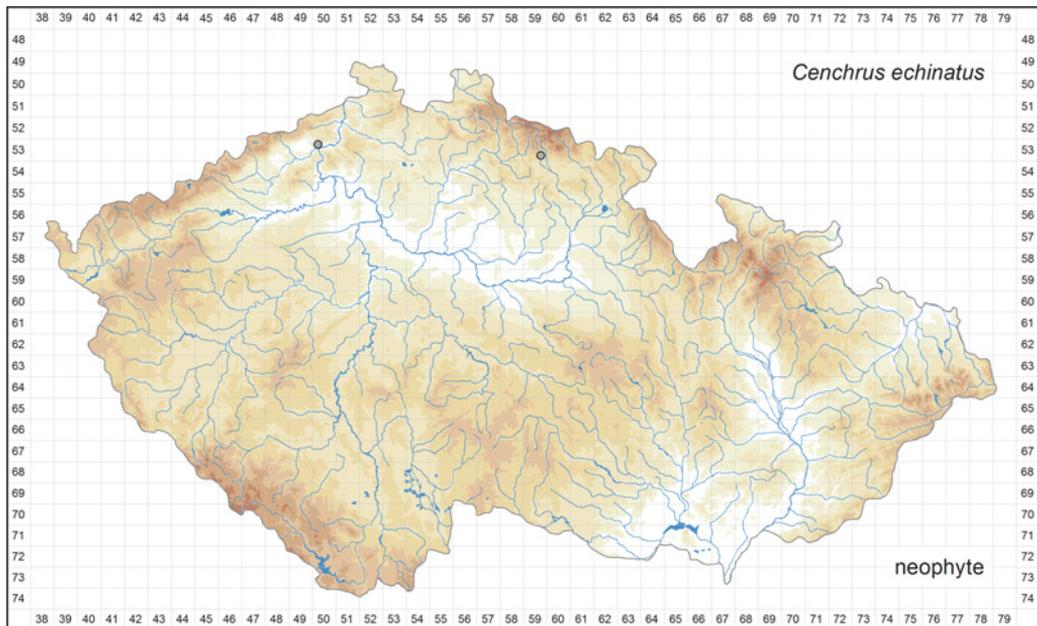
Fig. 31. – Distribution of *Cenchrus echinatus* in the Czech Republic (2 occupied quadrants). Prepared by Jitka Štěpánková.
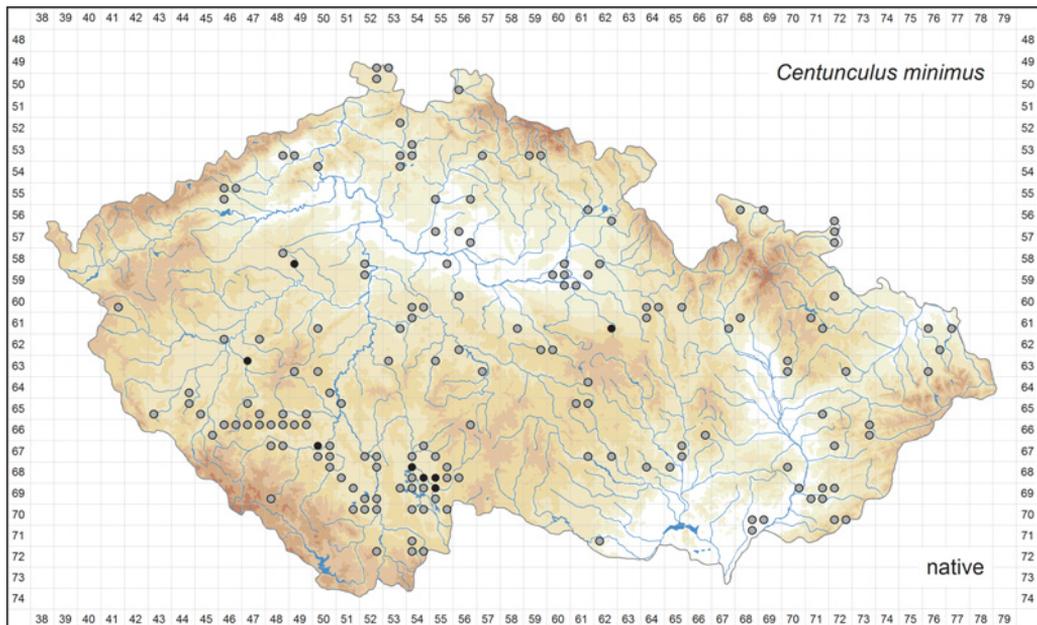
Fig. 32. – Distribution of *Centunculus minimus* in the Czech Republic: ● at least one record in 2000–2016 (8 quadrants), ◉ pre 2000 records only (156 quadrants). Prepared by Jan Prančl.

tains. In Asia the southern limit of its distribution range runs through Anatolia, the Caucasus Mts, northern Iran and Central Asia. It has also been recorded in Madeira and the Canary Islands. In Spain Molina et al. (2008b) distinguish *C. s.* subsp. *andresii*, whereas plants in the remaining part of the distribution range belong to the variable type subspecies. In the Czech Republic *C. spicata* has the widest ecological amplitude of all members of *C.* sect. *Phaestoglochin*, preferring mostly non-forest, secondary habitats and rarely occurring in semi-natural vegetation. Its most frequent habitats are ruderal sites, landfills, roadsides, railway embankments, wet, mesic and dry meadows and pastures, urban lawns, banks of ponds, clearings, black locust groves and rocky hillsides. This sedge has an obvious affinity to wet, rather warm and nutrient-rich soils, growing on soils with a high nitrogen content. Still, it also occurs on dry and sandy, sometimes even saline soils. It can withstand frequent mowing. In the Czech Republic *C. spicata* is the most common species of the *C. muricata* complex. It is widespread in warm and moderately warm parts of the country up to the altitude of 500 m, being absent from or scarce in western Bohemia and southern Bohemia east of the Vltava River; however this may be also due to underrecording. The species is missing from most of the mountains apart from (perhaps casual) introductions recorded along roads and walking paths up to a maximum altitude of 1100 m. *Carex spicata* can be easily distinguished from other species of *C.* sect. *Phaestoglochin* (Řepka 2003, Molina et al. 2008b) and we therefore accept non-documented field records for the map, being aware that some may still be erroneous.

Carex stenophylla (Fig. 30)

The *Carex stenophylla* group (Egorova 1999) includes about 5 taxa distributed in central and eastern Europe, the entire Siberian steppe zone, China, the Far East and continental North America. In Europe *C. stenophylla* occupies its continental and subcontinental parts, with the westernmost locality situated in northern Italy and Slovenia. It occurs in the Czech Republic, Austria, Slovakia, Hungary, Poland, northern part of the Balkan Peninsula, Ukraine and European Russia. In Asia it grows in Kazakhstan and the Altai Mts in western Siberia (Meusel et al. 1965, Hultén & Fries 1986, Korniak 1987). In the Czech Republic *C. stenophylla* is a facultative psammophyte growing mainly on road verges and in short lawns in human settlements. It has rarely been found also in dry grasslands over limestone and granite; formerly it also occurred in saline habitats that were partly flooded in spring. It is tolerant of mowing and other types of mechanical disturbance. In contrast, it is a weak competitor, and some populations are endangered by the encroachment of nitrophilous broad-leaved species. Its presence on gravel, sand and sandstone in Bohemia may be attributable to the Sarmatian plant migration as are the localities in Poland (Korniak 1987). In the Czech Republic *C. stenophylla* occurs only in its warmest regions, rarely elsewhere. In Bohemia it was collected in a small number of sites near the towns of Ústí nad Labem and Mimoň in northern Bohemia and in Prague, all other records being erroneous. All Bohemian populations have been extirpated. In Moravia *C. stenophylla* is found in the warmest parts of the province, being most frequent on wind-blown sands near the town of Hodonín. Its local distribution range may be roughly delimited by the lines connecting the city of Brno with the town of Znojmo in the southwest and with the town of Hodonín in the southeast, with the northernmost site situated at the northern edge of Brno. The local altitudinal maximum is at 400 m. The south

Moravian sites are situated not far from localities in Lower Austria, Slovakia and Hungary, which suggests its relationship with the Pontic-Pannonian flora (Podpěra 1930, Řepka 1983). *Carex stenophylla* is classified as endangered (Grulich 2012) but there is no immediate threat by humans as it occurs in sandy places trampled and strongly disturbed by man and no decline has been observed.

Cenchrus echinatus (Fig. 31)

Cenchrus echinatus is native to the tropical parts of both Americas and it has been introduced to most tropical countries of the World, rarely also to the warm part of the temperate zone, usually with wool shoddy, bird seeds or soya waste (DeLisle 1963). In Europe it has been recorded in the British Isles, Spain, France, Hungary and Greece (Holm et al. 1991, Greuter 2006). In the Czech Republic *C. echinatus* was first collected in 1908 in Trutnov next to a woollen factory, for the second time in 1968 in a goods railway station in Ústí nad Labem. It is classified as a casual neophyte (Pyšek et al. 2012b).

Centunculus minimus (Fig. 32)

Centunculus minimus is an amphi-atlantic species, occurring mainly in temperate zones of Europe and North America (Meusel et al. 1978, Hultén & Fries 1986). In Europe it is distributed across most of the continent, extending from Ireland and Portugal eastwards to temperate regions of European Russia, but appears to be rare in the Mediterranean area and absent from northern parts of Scandinavia, not exceeding 63°N there. It also occurs in the Azores, northernmost Africa and Ethiopia (Meusel et al. 1978), and rarely in Asia, being reported only from the Russian Far East, India and Taiwan (Tsvelev 1980, Hultén & Fries 1986, Hsu et al. 2009). It has been introduced to South America and south-eastern Australia (Zuloaga & Morrone 1999, Walsh 2003). *Centunculus minimus* is a competitively weak wetland annual, confined to open nutrient-poor habitats, mainly on sandy or gravelly substrates with acidic soil reaction (Popiela 1998). It prefers habitats such as exposed pond littorals, abandoned sand-pits, lightly managed arable fields (often stubble fields), edges of sandy tracks, disturbed sites in pastures, ditches and other wet places with sparse vegetation cover. In the Czech Republic *C. minimus* has probably never been common, although it has undoubtedly been overlooked due to its diminutive habit and late phenology. In the past it was sparsely distributed across the country, being most frequent in the fishpond landscapes of southern Bohemia. It has been recorded at altitudes 180–900 m, most frequently at middle elevations. *Centunculus minimus* has markedly declined since World War II. It has completely vanished from fishponds as a result of intensification of fish farming, especially fertilizing and restriction of summer drainage. The causes of its disappearance are probably similar to those of *Radiola linoides* (cf. Šumberová 2013c). Recently, the drainage of fishponds is not applied as systematically as in the past and most often only for a shortened period in the first half of the growing season. *Centunculus minimus* as a late growing species, flowering and fruiting mostly from July to October, is usually unable to complete its life cycle under these conditions. High amount of fertilisers and lime in fish farming causes eutrophication and supports more competitive tall growing species. The disappearance of the species from fields and pastures is associated with the decline of grazing, abandonment of lightly managed sandy crofts or their conversion to more productive cropland. *Centunculus minimus* has been

observed at only eight sites since 2000 and vanished completely from Moravia. It is currently classified as critically endangered (Grulich 2012). Several non-native populations on the sand-pits in the Třeboňská pánev basin, originating from recent rescue cultivations (A. Kučerová, in litt.), were not included in the map.

Convallaria majalis (Fig. 33)

Convallaria majalis is native to most of Europe, being absent from Iceland, northernmost Scandinavia and north-eastern Russia, and in the south from most of the Mediterranean area, Pannonian Basin and the Ukrainian and Russian steppe zone; the plants from the Crimea and Caucasus Mts are sometimes separated as *C. transcaucasica* but the variety rank seems to be more appropriate (Meusel et al. 1965, Hultén & Fries 1986, Kupriyanova 1986). In the Czech Republic *C. majalis* grows in open broadleaved and mixed forests, alluvial forests and on shrubby slopes. It is a rhizomatous geophyte preferring humid loamy soils rich in nutrients, basic to moderately acid. In dry, nutrient-poor soils and in forests with dense canopy it forms large patches of sterile plants. It is scattered or locally frequent in the wooded areas of the country, avoiding native spruce forests; above the timberline in the Krkonoše and Hrubý Jeseník Mts it is found only in glacial cirques. It occurs from the low to middle altitudes, reaching its altitudinal maximum of 1400 m in the Velká kotlina glacial cirque in the Hrubý Jeseník Mts. To the Czech Republic only *C. m.* var. *majalis* is native. However, plants cultivated in the gardens, parks and cemeteries belong to various taxa or cultivars of uncertain origin. Plants from the Caucasian region of Abkhasia were introduced in the 1970s to the garden of the Klatovy hospital in western Bohemia, became naturalized there and subsequently escaped to a nearby forest-park. They may be assigned to *C. m.* var. *transcaucasica* (Čížek & Král 2009).

Crocus heuffelianus (Fig. 34)

Crocus heuffelianus is distributed in the Carpathians, mountains of the Balkan Peninsula and very likely also in northern Italy (Harpke et al. 2015). Plants from the Western Carpathians (Slovakia, Poland and Czech Republic) are sometimes treated as a separate species, *C. discolor*. However, except for different number of chromosomes and slightly narrower leaves in the Western Carpathian plants, there is no clear line of distinction between the two variants and thus the broader species concept is adopted here. *Crocus heuffelianus* is found in regularly mown meadows, pastures, stream ash-alder woods and occasionally also in margins of mountain forests. In the Czech Republic it is native to eastern Moravia and Silesia, recently confirmed at five localities, each harbouring one to several populations. Isolated occurrences in the Orlické hory Mts and Žďárské vrchy Mts are most likely of secondary origin. In the Krkonoše Mts in north-eastern Bohemia, plants slightly different from those found in the Carpathians (but still included here in *C. heuffelianus*) have escaped from cultivation and become locally naturalized. *Crocus heuffelianus* occurs at altitudes about 320–950 m in eastern Moravia and Silesia and occasionally up to 1390 m in the Krkonoše Mts. Due to its rarity and possible habitat loss (abandonment of meadows and development of rural areas) it is classified as critically threatened (Grulich 2012).

Crocus vernus (Fig. 35)

Crocus vernus is a correct name for the species recently treated as *C. albiflorus* (Peruzzi et al. 2013). It is a European mountain species distributed in the Pyrenees, Alps, mountains in the north of the Balkan Peninsula and some adjacent uplands (Harpke et al. 2015). In the Czech Republic it reaches the northern limit of its distribution range. It may be native to the Šumava Mts and Novohradské hory Mts in southern Bohemia and in the Smolinka stream basin near the town of Valašské Klobouky in eastern Moravia. In contrast, there is reliable evidence for deliberate planting at other localities, e.g. the village of Slunečná near the town of Česká Lípa and near the town of Malá Skála in northern Bohemia. Scattered occurrences in the Krkonoše Mts in north-eastern Bohemia, mostly at meadows close to chalets and houses (the species is locally common in the easternmost part of the mountains) seem to have originated from either deliberate planting in the wild or as garden escapes. *Crocus vernus* grows in regularly mown meadows, pastures, occasionally also forest margins and shrubs at altitudes up to 1030 m. It is somewhat endangered by land-use changes, particularly by abandonment of meadows. Despite doubts about its indigenous status the species is classified as endangered (Grulich 2012).

Cryptogramma crispa (Fig. 36)

Cryptogramma crispa is an autotetraploid species distributed in high mountains of Europe eastwards to Scandinavia, the Carpathians and the Balkan Peninsula, and in adjacent western Asia, with an outpost in the Ural Mts (Hultén & Fries 1986). In the Czech Republic *C. crispa* inhabits mainly screes and crevices of siliceous rocks usually in glacial cirques in the mountains. It still occurs at several sites in the central part of the Krkonoše Mts and at the last site in the glacial cirque of the Mt Jezerní hora in the Šumava Mts. In the past *C. crispa* also occurred at several other sites in the Šumava Mts and also in the Novohradské hory Mts; an isolated occurrence was recorded in Kamenec hill near the village of Starý Šachov in the České středohoří Mts. It is classified as critically threatened (Grulich 2012).

Cyperus flavescens (Fig. 37)

Cyperus flavescens is a wetland annual species with a large but rather disjunct distribution range involving temperate to tropical zones of Eurasia, Africa, North, Central and South America and (probably secondarily) also Australia (Meusel et al. 1965, Lampe 1996). The only larger area with more or less continuous occurrence extends throughout the southern part of central Europe and central and eastern part of the Mediterranean area (Lampe 1996). High summer temperatures and moderately high precipitations (mainly from autumn to spring) are typical of areas with the occurrence of *C. flavescens*, which belongs to the thermophilous species, in central Europe not germinating before mid-May (Lampe 1996). In the Czech Republic *C. flavescens* was in the past frequently scattered in lowland and colline areas throughout the country, with the largest concentrations of localities in the Bohemian Cretaceous Basin, fishpond landscapes of southern Bohemia, and some parts of southern and central Moravia and the Carpathians. It is able to grow on a broad range of substrates, both calcareous and non-calcareous, e.g. wet sand, loam and clay (including salty type), and peaty and fen soils (Lampe 1996). The range of its potentially
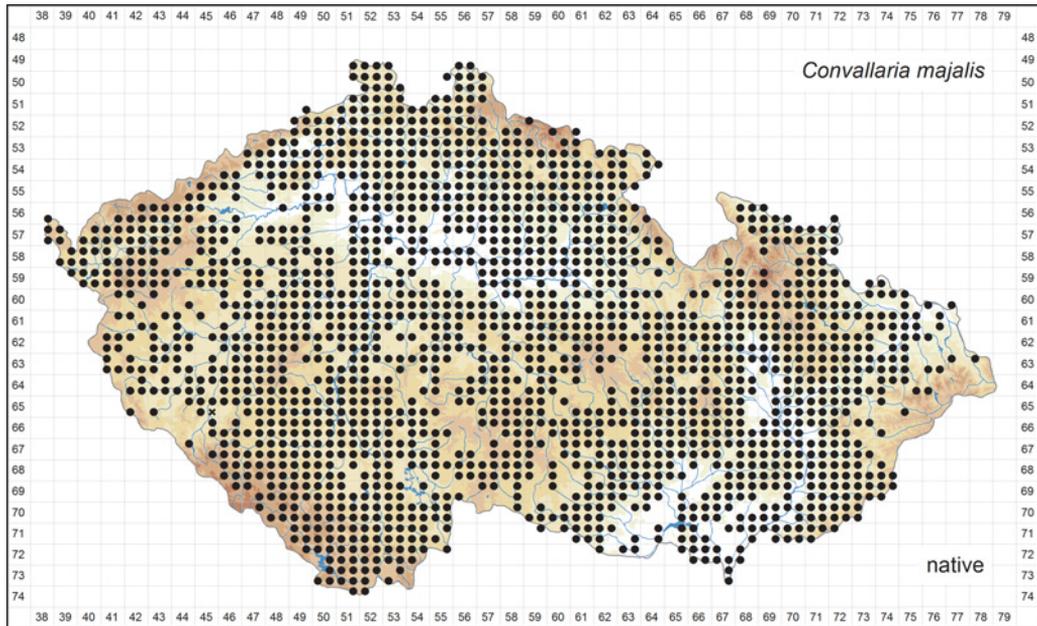
Fig. 33. – Distribution of *Convallaria majalis* in the Czech Republic: ● *C. m.* var. *majalis* (1807 quadrants), ✕ both *C. m.* var. *majalis* and alien *C. m.* var. *transcaucasica* (1 quadrant). Prepared by Jiří Zázvorka.
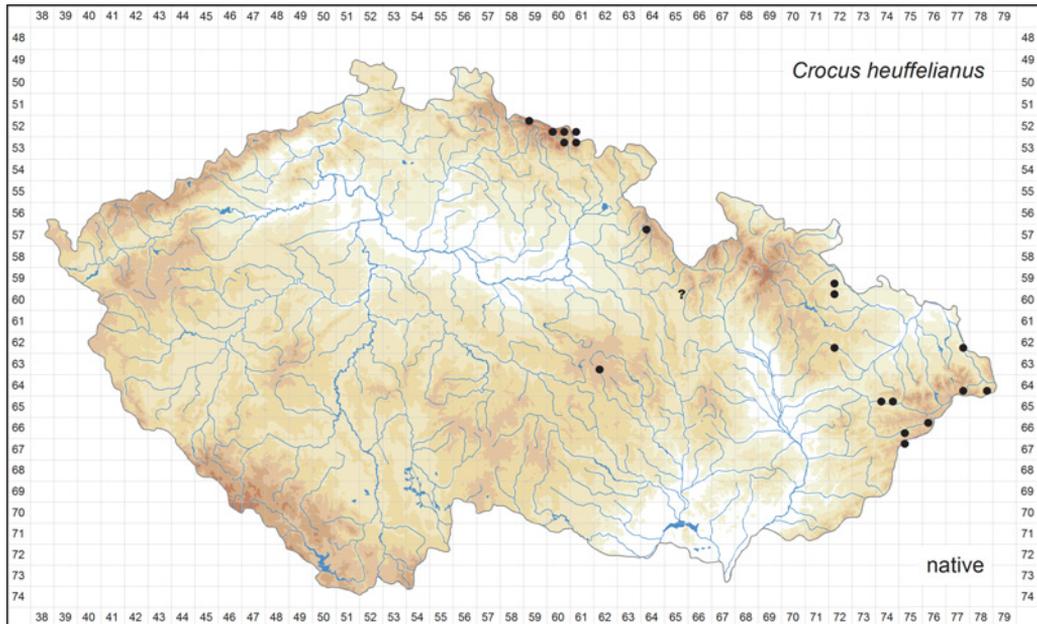
Fig. 34. – Distribution of *Crocus heuffelianus* in the Czech Republic (19 occupied quadrants). Prepared by Jindřich Chrtek Jr.
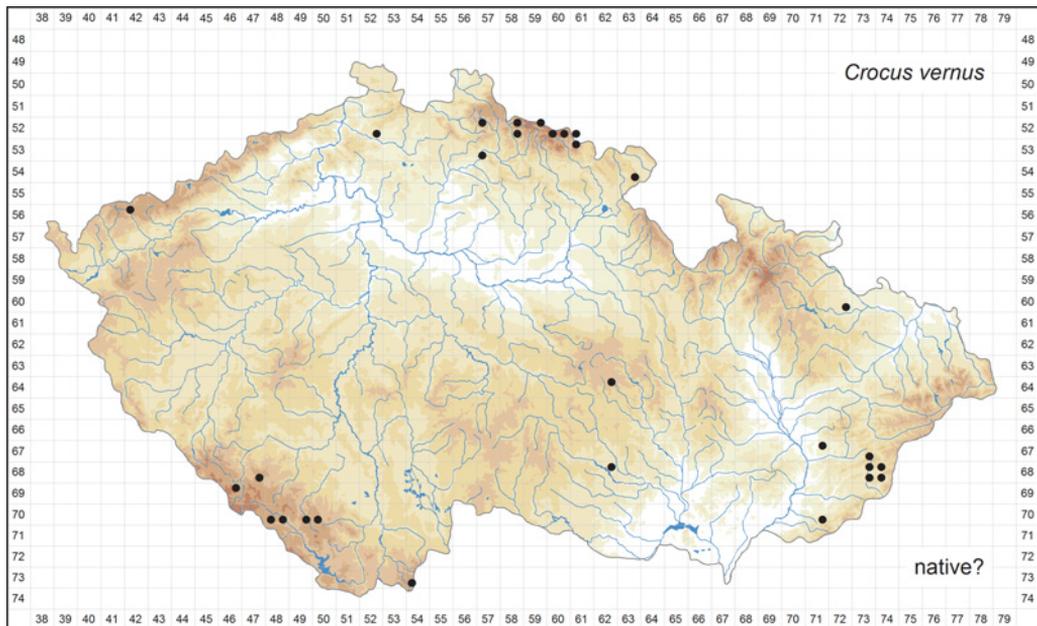
Fig. 35. – Distribution of *Crocus vernus* in the Czech Republic (29 occupied quadrants). Prepared by Jindřich Chrtek Jr.
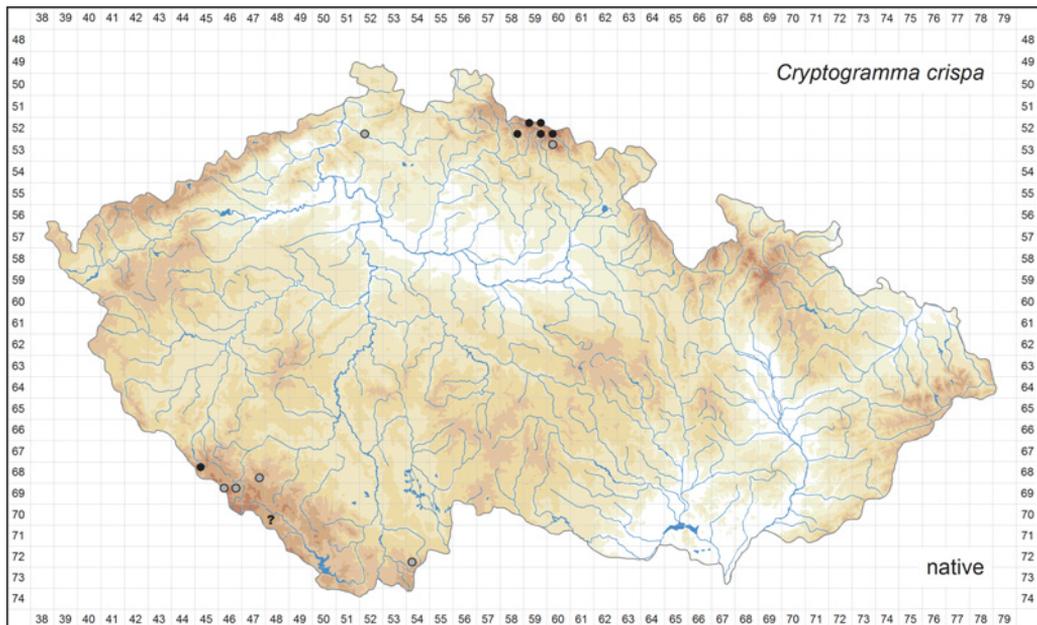
Fig. 36. – Distribution of *Cryptogramma crispa* in the Czech Republic: ● at least one record in 2000–2016 (6 quadrants), ◉ pre 2000 records only (6 quadrants). Prepared by Libor Ekrt.
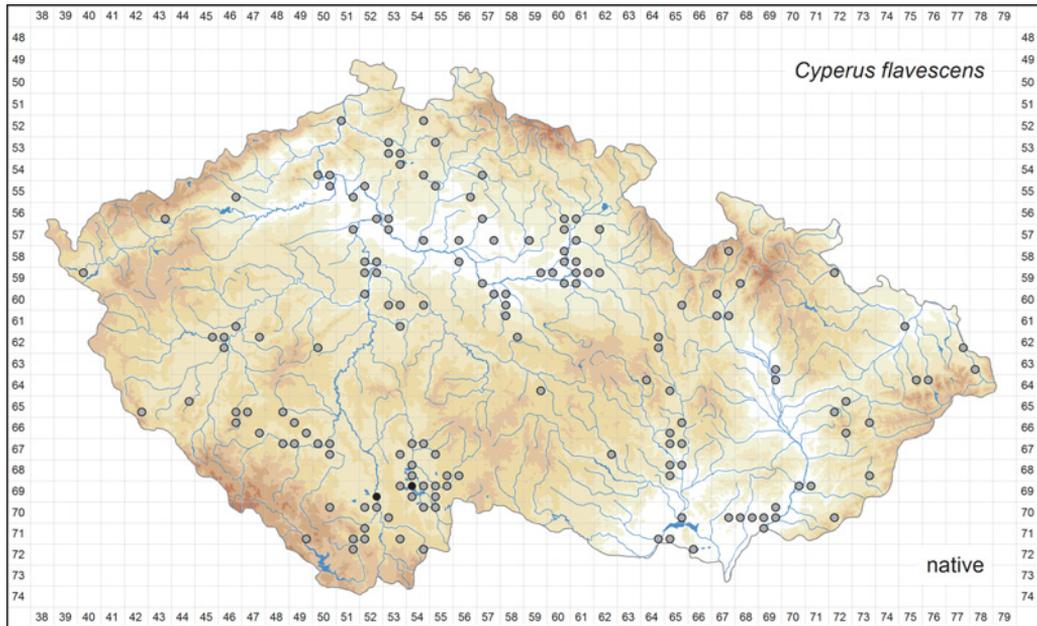
Fig. 37. – Distribution of *Cyperus flavescens* in the Czech Republic: ● at least one record in 2000–2016 (2 quadrants), ◉ pre 2000 records only (152 quadrants). Prepared by Kateřina Šumberová & Pavel Dřevojan.
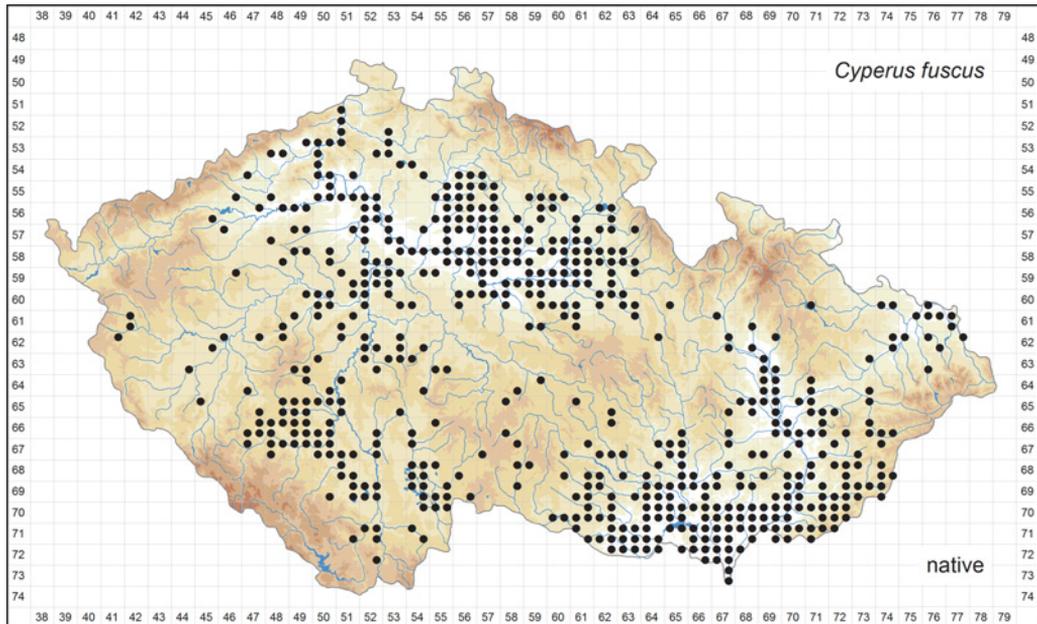
Fig. 38. – Distribution of *Cyperus fuscus* in the Czech Republic (576 occupied quadrants). Prepared by Kateřina Šumberová, Pavel Dřevojan, Zdenka Hroudová & Pavel Kúr.
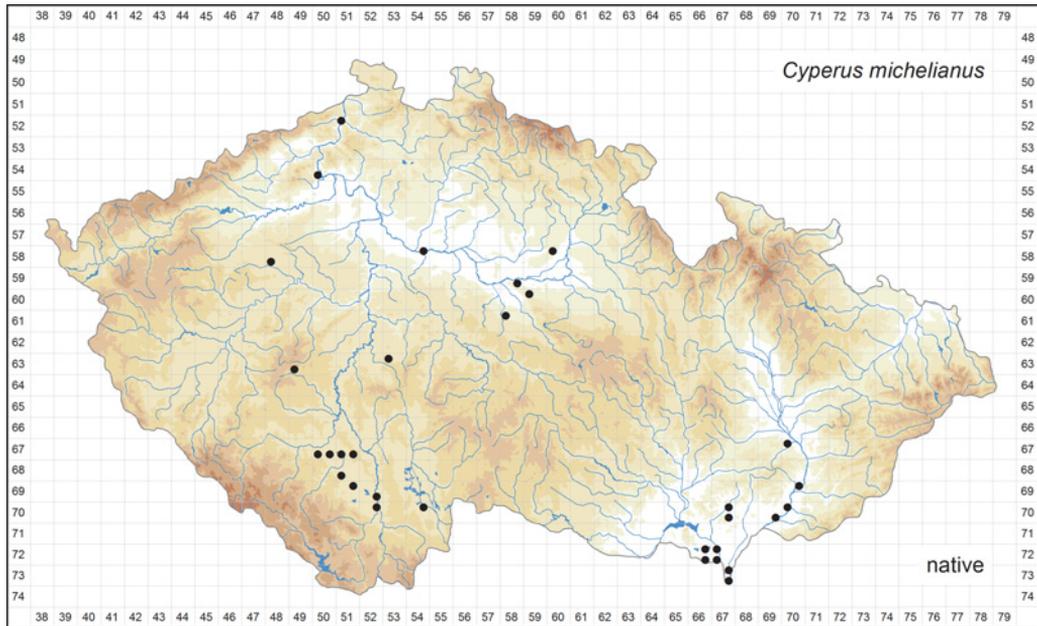
Fig. 39. – Distribution of *Cyperus michelianus* in the Czech Republic (31 occupied quadrants). Prepared by Kateřina Šumberová & Pavel Dřevojan.
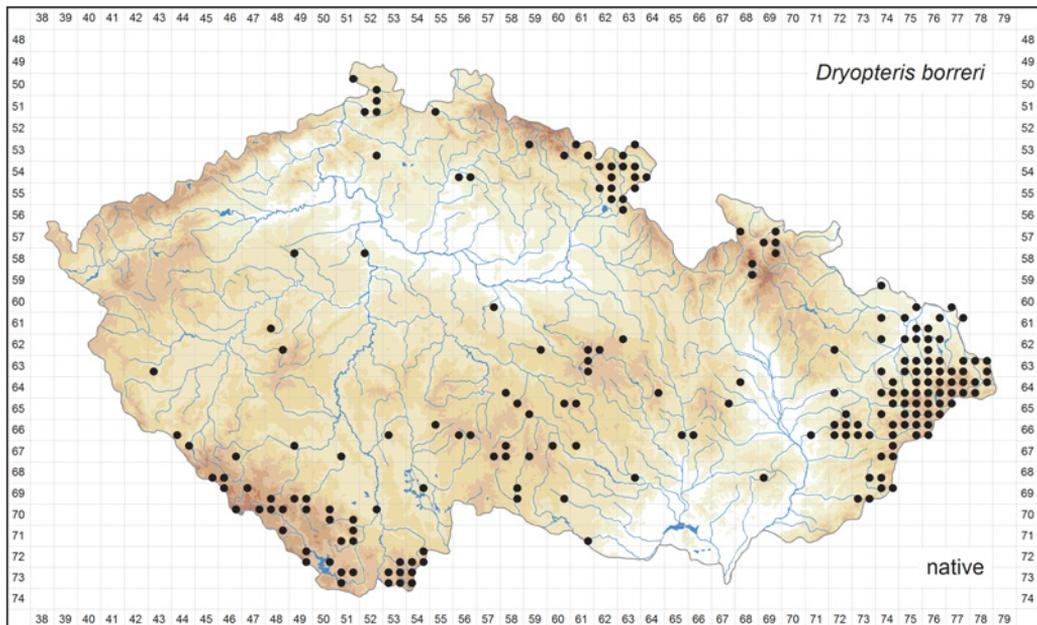
Fig. 40. – Distribution of *Dryopteris borreri* in the Czech Republic (200 occupied quadrants). Prepared by Libor Ekrt.

suitable habitats includes wet arable fields, pastures, disturbed springs and fens, fishpond margins, fish storage ponds, wet ditches, etc. Despite its large tolerance to variation of abiotic conditions, the species had already started to decrease by the late 19th century, probably as a consequence of large-scale abandonment of low-intensity grazing and overall intensification of agriculture. The last habitats in which the species managed to survive were sandy fishpond littorals, from which *C. flavescens* disappeared during the 1960s–1980s due to fish-farming intensification, in particular intensive fertilization and summer drainage restriction. Small-scale and moderate but regular disturbances are probably the key to *C. flavescens* survival in the landscape; patches without dense vegetation are indispensable for its successful germination and reproduction. Nowadays only two spontaneous populations of the species are known in the Czech Republic, both located in South Bohemian fish storage ponds. Some other populations of *C. flavescens* in the surroundings of the town of Třeboň in southern Bohemia have originated from intentional sowing in abandoned sand pits within rescue cultivations (A. Kučerová, in litt.) and they are not displayed in the map. *Cyperus flavescens* is classified as critically threatened (Grulich 2012).

Cyperus fuscus (Fig. 38)

Cyperus fuscus is a Eurasian wetland annual plant distributed mainly in warm temperate and Mediterranean parts of Europe, northern Africa and western Asia, with its northern distribution limit in southern Scandinavia. Eastwards it is scattered throughout the temperate zone of continental Asia (at high altitudes reaching the subtropical zone), being more frequent in floodplains of large rivers and around lakes. It is also known from North America where it is considered as introduced (Lampe 1996). *Cyperus fuscus* prefers mineral-rich calcareous soils, especially in northern parts of its distribution range (Hejný 1960). In the Czech Republic it is mainly confined to the Bohemian Cretaceous Basin, southern Moravia and some parts of the Carpathians (e.g. Bílé Karpaty Mts). *Cyperus fuscus* is a typical component of the vegetation of temporarily exposed bottoms of various water bodies, growing over a broad range of habitats, including fishponds, fish storage ponds, oxbows, river beds, sand pits, wet depressions in arable fields and disturbed places in wet meadows. In areas formed by acidic, mineral-poor bedrock, such as fishpond landscapes of southern Bohemia, the species was reported as rare until the 1950s, occurring mainly in eutrophic water bodies in settlements (Hejný 1960, Šumberová 2013a). As a consequence of overall eutrophication and soil chemistry changes associated with fish farming intensification, in particular combined fish and duck farming and intensive liming of some ponds, the number of records has considerably increased since then (Šumberová 2003, 2013a). Despite the loss of some populations due to habitat destruction in river alluvia, the species has recently had many hundreds of localities and is classified only as vulnerable (Grulich 2012).

Cyperus michelianus (Fig. 39)

Cyperus michelianus is a wetland annual plant with a disjunct distribution in temperate to tropical zones of Eurasia, Africa and Australia (Lampe 1996). In Europe the species reaches its northern distributional limits on the Elbe river in eastern Germany and on the Oder river in south-western Poland (Schultze-Motel 1980, Lampe 1996); however, it

meets optimal conditions in regions with warm and dry summers. *Cyperus michelianus* grows on exposed sandy or muddy substrates in river beds, oxbows, sand pits, fishponds and similar habitats, usually on mineral-rich and relatively nitrate-rich, sometimes slightly saline soils (Hejný 1960). It is able to persist in the soil seed banks and to re-appear after 15–20 years, although suitable conditions usually occur several times within the given period (Lampe 1996, Hejný 1999). In the Czech Republic and elsewhere in the northern part of its geographic range, *C. michelianus* usually occurs in warm lowland and colline areas. It used to be most frequent in the wetland-rich parts of southern Bohemia and southern Moravia. During the last two decades, *C. michelianus* started to be regularly observed both in areas of its former occurrence and elsewhere. However, most of its former occurrences have not been confirmed recently. Some of them were probably temporary (e.g. in the Brdy Mts). On the one hand, the changes in land-use and landscape management, such as river regulations and elimination of fishpond drainage over the growing season, restricted the number of suitable *C. michelianus* habitats. On the other hand, the changing climate, in particular repeated summer heat waves and drought periods, support more frequent occurrence and successful species' reproduction on existing sites. The species is listed as critically threatened (Grulich 2012).

Dryopteris borreri (Fig. 40)

Dryopteris borreri is a triploid member of the *D. affinis* group (Fraser-Jenkins 2007). This solely apomictic and polyploid complex includes four species in central Europe of which triploids *D. borreri* and *D. cambrensis* are present in the Czech Republic (Ekrt et al. 2009, 2010). The closely related diploid *D. affinis* and the newly discovered triploid *D. lacunosa* are in central Europe restricted to its western and southern parts (Jessen et al. 2011). *Dryopteris affinis* has not been reliably recorded for the Czech Republic yet but it is found in Germany not far from the border with Bohemia (S. Jessen, in litt.). *Dryopteris borreri* is a polymorphic species with assumed origin from the crossing between two diploids, *D. affinis* and *D. caucasica* (Widén et al. 1996). It is a species of European sub-Atlantic and sub-Mediterranean distribution, ranging from south-western Norway to northern Africa and from Macaronesia to the coast of the Caspian Sea in Iran (Fraser-Jenkins 2007). In the Czech Republic it grows mainly in mountain beech, spruce or ravine forests, at middle altitudes being confined to humid and rather cold valleys. In many sites it occurs only in moist places along margins of forest roads. It is particularly frequent in eastern Moravia and adjacent Silesia. It is scattered in the sandstone landscapes of northern and northeastern Bohemia and the mountains of southern Bohemia, and rare elsewhere, mainly in the Českomoravská vrchovina and Drahanská vrchovina highlands (Ekrt et al. 2010). There are no records from the westernmost parts of the country. *Dryopteris borreri* was not reliably distinguished from the similar *D. filix-mas* in the past. The distribution map is based on revised herbarium specimens and a few finds documented by photographs or plants personally inspected but discarded; consequently, many new sites are likely to be discovered in the future. The species is classified as vulnerable because of its limited distribution (Grulich 2012).

Dryopteris cambrensis (Fig. 41)

Dryopteris cambrensis is a triploid member of the *D. affinis* group (see comments under *D. borreri*) with assumed origin from crossing of the diploid *D. affinis* and *D. oreades* (Widén et al. 1996). It occurs throughout the western half of Europe except for its northernmost parts, ranging from the British Isles in the west as far as Bulgaria in the east (Fraser-Jenkins 2007). The Czech Republic is situated close to the north-eastern limit of the species' distribution range, where it is rare. It usually occurs as individual plants (and not rich populations) in spruce, beech, fir or pine forests and in ditches along forest roads. *Dryopteris cambrensis* was not distinguished from similar *D. filix-mas* and *D. borreri* until recently (Ekrt et al. 2009). It appears to be a rare plant: until now it has been found in northern and southern Bohemia, the Českomoravská vrchovina highlands, Czech Silesia and (based on old herbarium specimens) also north of the city of Brno in the karst area of Moravský kras (Ekrt et al. 2010). It is certainly under-recorded and its exact distribution is a topic for further field research. Only revised herbarium specimens were included in the distribution map. *Dryopteris cambrensis* is classified as critically threatened because of its rarity (Grulich 2012).

Dryopteris carthusiana (Fig. 42)

Dryopteris carthusiana is allotetraploid, probably derived from the crossing between the diploid *D. intermedia* and an extinct or as-yet undiscovered taxon (Stein et al. 2010, Sessa et al. 2012). It is a holarctic, mostly boreal-temperate species, being frequent in Europe (except its Mediterranean part) and the adjacent western Siberia, and also occurring in the central and eastern part of North America (Hultén & Fries 1986, Rünk et al. 2012). In the Czech Republic it is most frequent in humid alder forests along streams and springs, in alder carrs, willow scrubs and birch mire forests, and less frequently in beech, spruce, oak-hornbeam and ravine forests, forest margins and open peat bogs. It is widespread throughout the country, particularly at middle and higher elevations, but it tends to be rare or even missing at low altitudes due to absence of suitable humid forest sites. Most of the gaps in the map, mainly at middle altitudes, are due to under-recording rather than true absences of the species.

Dryopteris cristata (Fig. 43)

Dryopteris cristata is allotetraploid, probably derived from the crossing between the diploid *D. ludoviciana*, endemic to the south-eastern USA, and an extinct or as-yet undiscovered species, the same putative parent as that of *D. carthusiana* (Stein et al. 2010). *Dryopteris cristata* occurs mainly in Europe (except its northernmost, westernmost and southernmost parts), in adjacent western Siberia and in the central and eastern part of North America (Hultén & Fries 1986). In the Czech Republic *D. cristata* inhabits mainly peat bogs and treeless marshes. Still, it is able to persist in various succession stages of wetland tree and scrub vegetation such as alder carrs, birch mire forests and willow scrub, or even reed beds, which usually encroach upon this type of wetlands. Most of its localities are found in the Třeboňská pánev basin in southern Bohemia and around the towns of Doksy and Česká Lípa in northern Bohemia. Several other sites are known elsewhere in Bohemia, some of which were discovered only recently. *Dryopteris cristata* is classified as critically threatened (Grulich 2012).

Dryopteris dilatata (Fig. 44)

Dryopteris dilatata is a tetraploid species of as-yet unresolved origin with possible explanations that include several polyploidization events from the diploid *D. expansa* (Juslén et al. 2011) or allopolyploidy from a hybrid of the diploid *D. intermedia* and *D. expansa* (Gibby & Walker 1977, Sessa et al. 2012). It is almost exclusively a European species, being most frequent in western, central Europe and the Carpathians, and scattered in western Russia and western foothills of the Caucasus Mts. It is absent from most of the Mediterranean area, surroundings of the Black Sea areas and central and northern Scandinavia (Hultén & Fries 1986, Rünk et al. 2012). In the Czech Republic it occurs mainly in humid spruce, beech, alder and ravine forests and moist sandstone areas, less frequently in more humid types of oak and oak-hornbeam forests. It is widespread throughout the country but tends to be rare in or missing from low altitudes due to absence of suitable forest habitats. Most of the gaps in the map, particularly those at the middle altitudes, are due to under-recording rather than true absences of the species.

Dryopteris expansa (Fig. 45)

Dryopteris expansa is a diploid species distributed in Europe particularly in Scotland, Scandinavia and the adjacent part of European Russia, and in mountain areas of western, central and eastern Europe. Outside Europe it has been recorded in the Caucasus Mts and throughout subboreal Siberia as far as the Russian Far East and and in temperate and subboreal areas of North America (Hultén & Fries 1986, Rünk et al. 2012). In the Czech Republic it is found in humid sites in beech, spruce, alder and ravine forests, in *Pinus mugo* scrub, subalpine tall-fern vegetation or gorges in sandstone areas and semi-shaded moist screes. It occurs mainly in highlands and mountains up to the subalpine vegetation belts. In low elevations it is missing or very rare, confined to suitable humid microhabitats (Ekrt et al. 2013). It is classified as lower risk – near threatened (Grulich 2012). *Dryopteris expansa* was not distinguished or was frequently misidentified in the past. The map was therefore based solely on revised specimens and is inevitably incomplete.

Dryopteris filix-mas (Fig. 46)

Dryopteris filix-mas is an allotetraploid taxon, probably derived from hybridization of the diploid *D. oreades* and *D. caucasica* (Fraser-Jenkins 1976). It is a holarctic species with a mostly boreal-temperate disjunct range in Eurasia and northern North America (Hultén & Fries 1986). In the Czech Republic *D. filix-mas* inhabits humid spruce, beech, oak-hornbeam and ravine forests, and open stony screes. It is widespread throughout the country, and particularly frequent from the uplands to the mountains. At low altitudes it tends to be rare or missing due to the absence of suitable humid forest sites.

Dryopteris remota (Fig. 47)

Dryopteris remota is a triploid apomictic species, the origin of which is still not reliably resolved (Juslén et al. 2011). *Dryopteris remota* is a subatlantic and subalpine species of the European mountains, being most frequent in the Pyrenees and the adjacent southern part of France, in the Alps and their foothills, the Carpathians and the western part of the Caucasus Mts. Northwards it extends up to Ireland and Scotland, central Germany, the
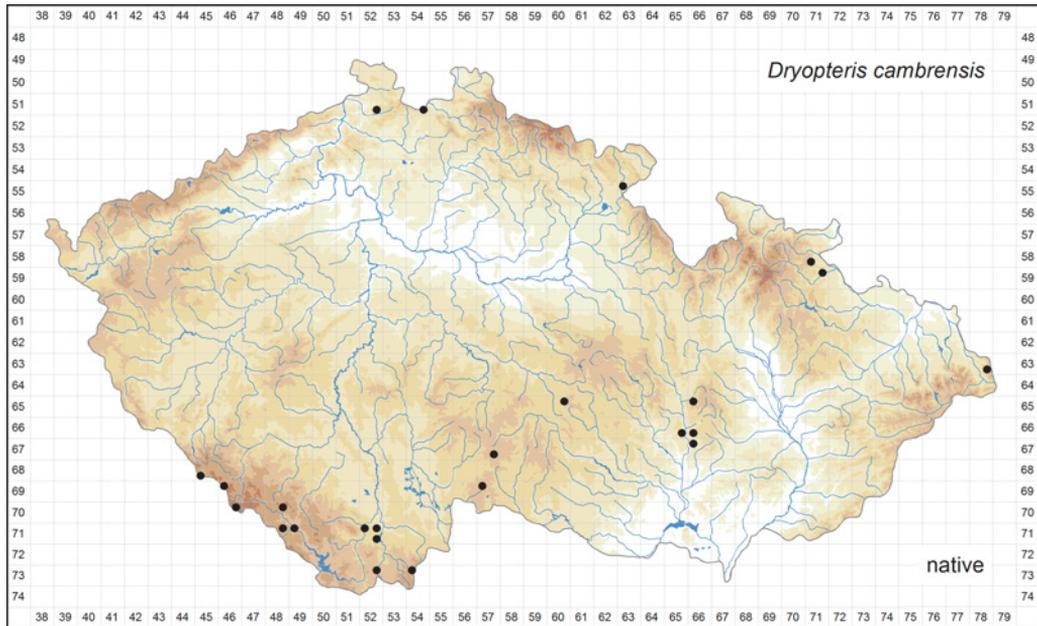
Fig. 41. – Distribution of *Dryopteris cambrensis* in the Czech Republic (24 occupied quadrants). Prepared by Libor Ekrt.
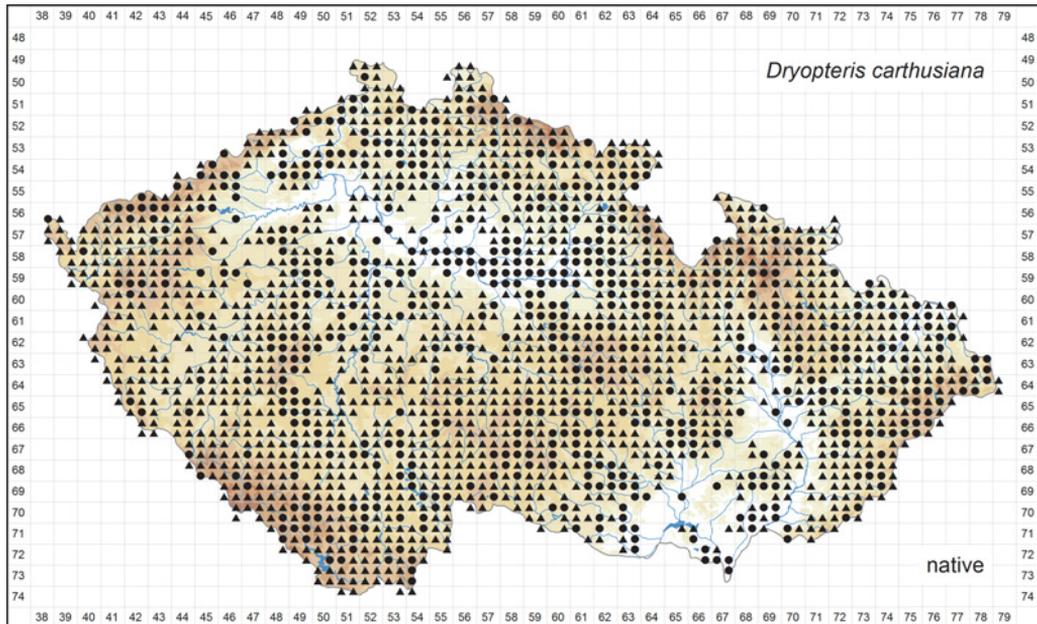
Fig. 42. – Distribution of *Dryopteris carthusiana* in the Czech Republic: ● occurrence documented by herbarium specimens (607 quadrants), ▲ occurrence based on other records (1376 quadrants). Prepared by Libor Ekrt.
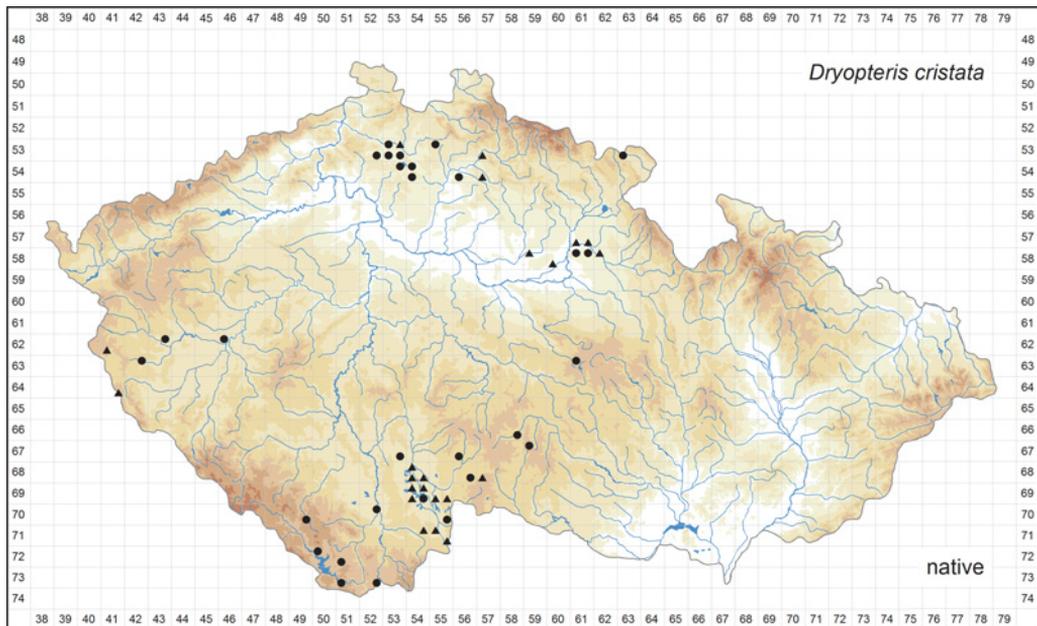
Fig. 43. – Distribution of *Dryopteris cristata* in the Czech Republic: ● occurrence documented by herbarium specimens (29 quadrants), ▲ occurrence based on other records (22 quadrants). Prepared by Libor Ekrt.
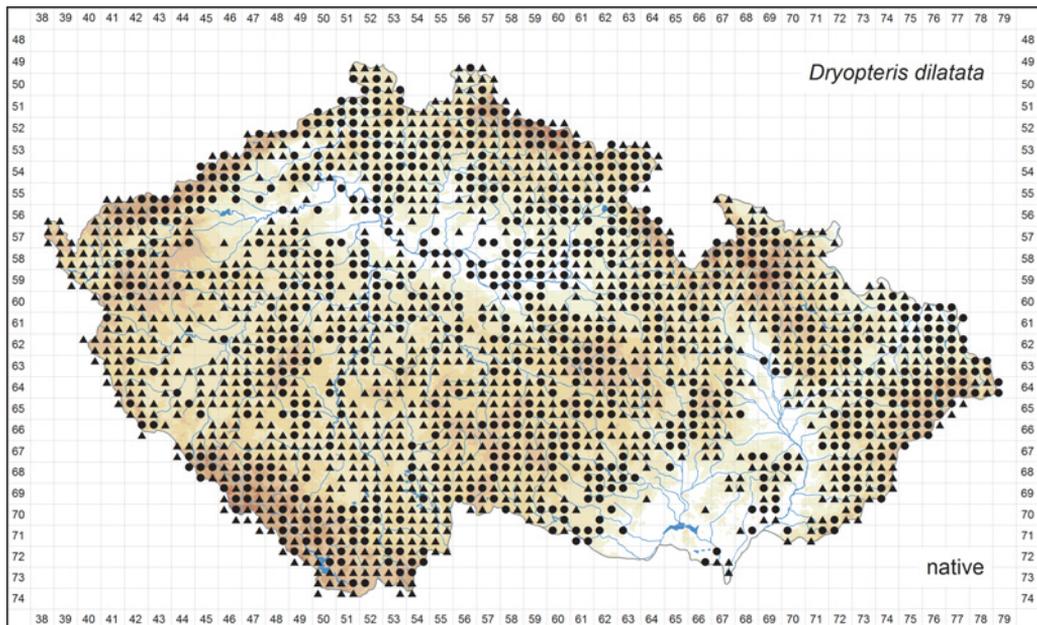
Fig. 44. – Distribution of *Dryopteris dilatata* in the Czech Republic: ● occurrence documented by herbarium specimens (752 quadrants), ▲ occurrence based on other records (1206 quadrants). Prepared by Libor Ekrt.
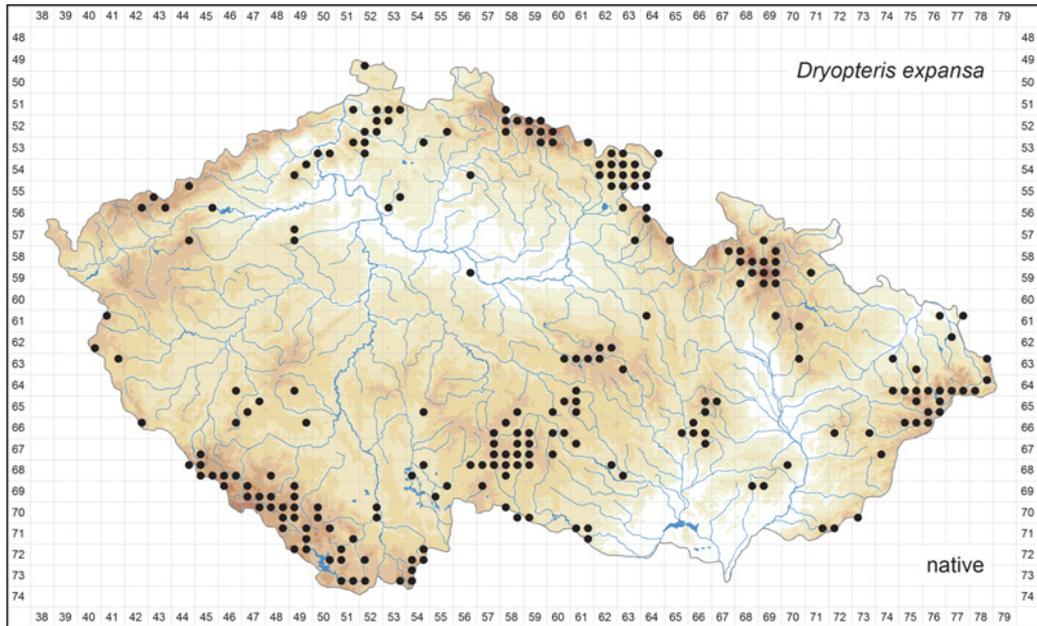
Fig. 45. – Distribution of *Dryopteris expansa* in the Czech Republic (225 occupied quadrants). Prepared by Libor Ekrt.
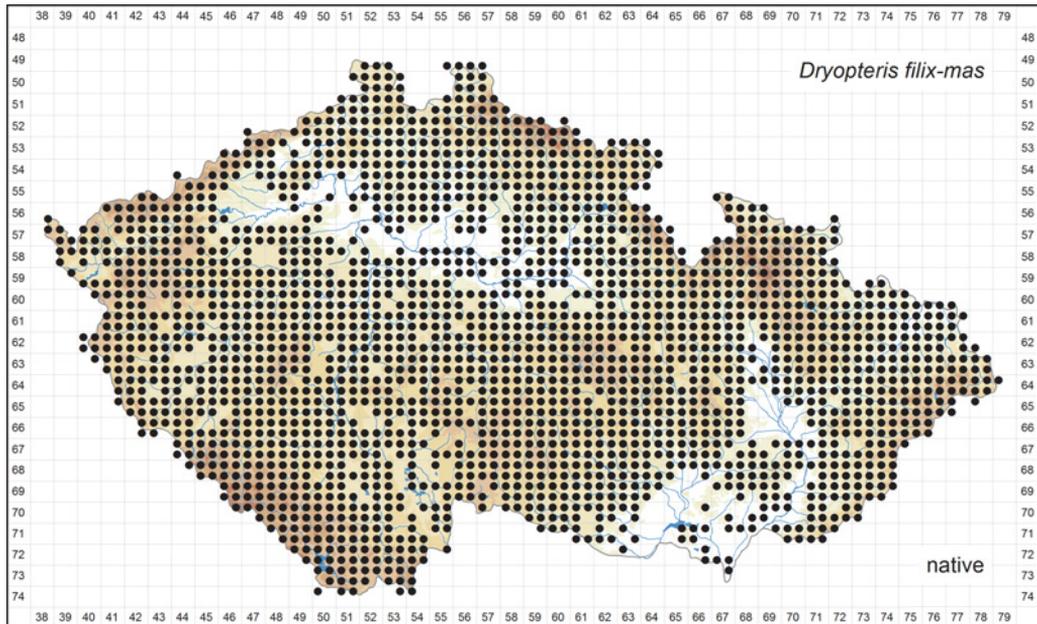
Fig. 46. – Distribution of *Dryopteris filix-mas* in the Czech Republic (2137 occupied quadrants). Prepared by Libor Ekrt.
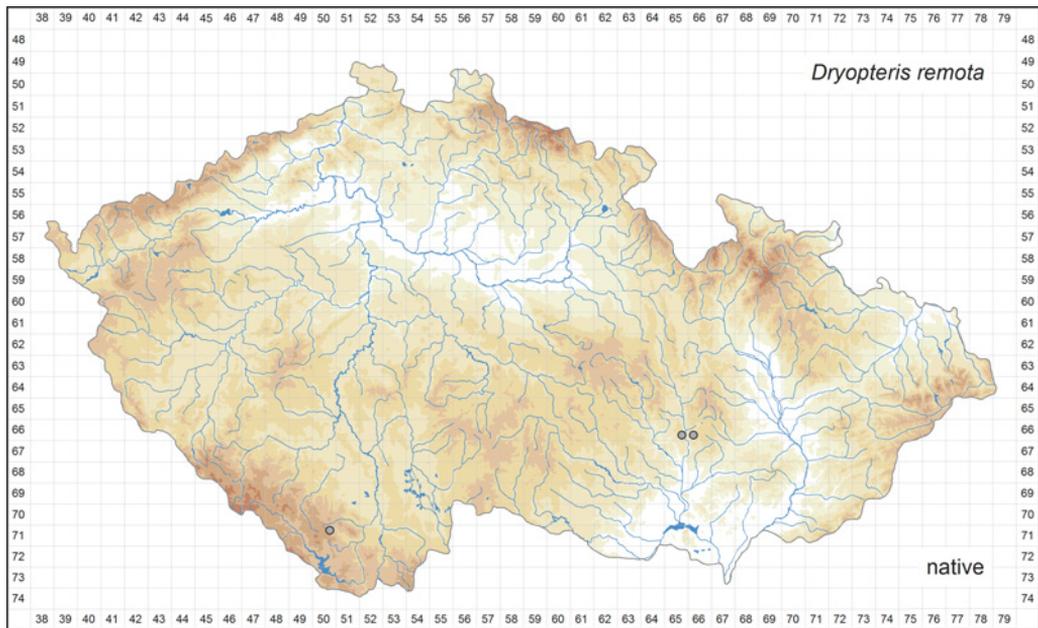
Fig. 47. – Distribution of *Dryopteris remota* in the Czech Republic (3 occupied quadrants). Prepared by Libor Ekrt.
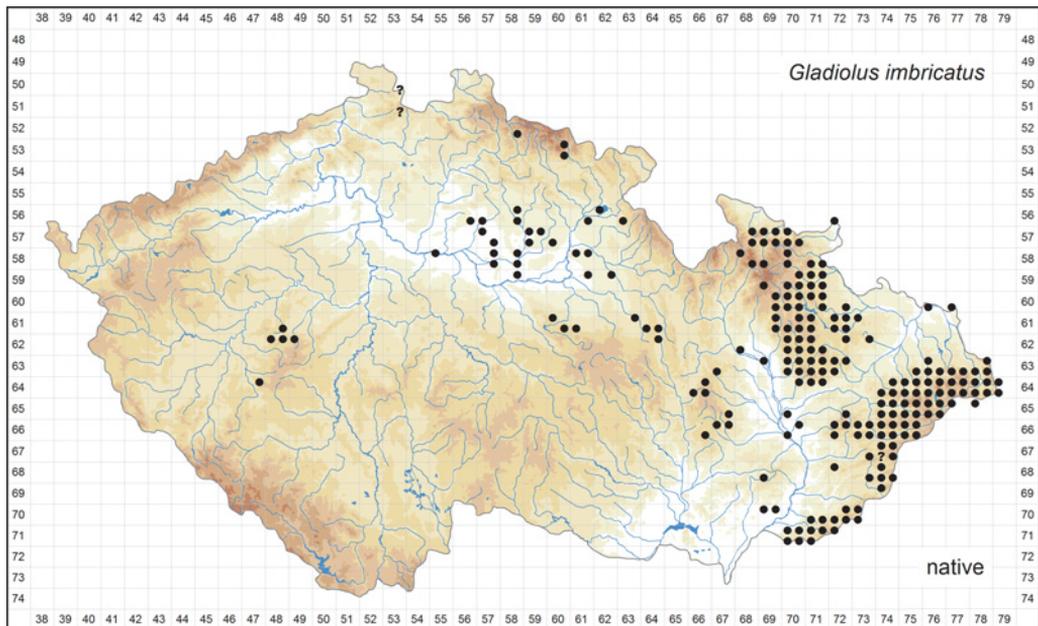
Fig. 48. – Distribution of *Gladiolus imbricatus* in the Czech Republic (211 occupied quadrants). Prepared by Jindřich Chrtek Jr.

Czech Republic, south-eastern Poland and south-western Ukraine (Ekrt et al. 2007). The Czech Republic is situated outside its continuous distribution range and *D. remota* represents there the Alpine migration element. It was discovered there in 1929 at several sites in the karst area of Moravský kras (Bílý 1931, 1937). The second find dates back to 2002 when it was found on Mt Malý Plešný near the village of Ktiš in southern Bohemia (Ekrt et al. 2007). Only a single plant was present there, growing on a rock in the ecotone between shrubs of *Ribes alpinus*, *Rosa pendulina* and *Lonicera nigra*, and open spruce-beech-fir forest. This occurrence has not been confirmed recently. All records shown in the map are based on revised herbarium specimens. Considering its rarity, the species was classified as critically threatened in the last Red List (Grulich 2012), but at present it is actually extirpated.

Gladiolus imbricatus (Fig. 48)

Gladiolus imbricatus is distributed in central and eastern Europe, reaching westwards to the French foothills of the Western Alps, and Thuringia and Lusatia in Germany, northwards to Poland and north-western Russia, southwards to Greece and eastwards as far as the Ural Mts. Outside Europe it has been recorded in western Siberia, the Caucasus Mts, Transcaucasia and Iran (Meusel et al. 1965, Hultén & Fries 1986). In the Czech Republic it inhabits mesic, intermittently wet and fen mown meadows, marshes, meadows springs, open-canopy forests; occasionally it may occur also as a weed in corn and clover fields. *Gladiolus imbricatus* is scattered throughout the Moravian Carpathians and Eastern Sudetes (Nízký Jeseník hills and lower elevations of the Hrubý Jeseník Mts), with occasional extensions to adjacent lowlands, and it is uncommon in eastern Bohemia. Isolated localities are known at foothills of the Brdy Mts near the border between south-western and central Bohemia, in central Bohemia, in the Krkonoše Mts in north-eastern Bohemia, and in the Drahanská vrchovina highlands north of Brno. It occurs at altitudes about 190–1210 m. *Gladiolus imbricatus* declined mainly during the 1950s and 1960s, mostly due to drainage of wetlands and fen meadows. Although many populations are protected in nature reserves nowadays, the species suffers from changes in land-use, mainly abandonment of meadows. It is therefore classified as endangered (Grulich 2012).

Gladiolus palustris (Fig. 49)

Gladiolus palustris is a European species with a disjunct distribution range reaching eastern France in the west, Poland and Lithuania in the north, Belarus, western Ukraine and Bulgaria in the east, and central Italy and Albania in the south (Meusel et al. 1965). In the Czech Republic *G. palustris* is found in intermittently wet meadows, semi-dry grasslands and lowland oak forests. It is very rare, confined to the south-western part of the Bílé Karpaty Mts and to the Hodonínská Dúbrava wood in south-eastern Moravia, and to several sites in the Bohemian Cretaceous basin in central and eastern Bohemia. It occurs in the planar, colline and supracolline vegetation belts with an altitudinal maximum at 430 m in the Čertoryje National Nature Reserve in the Bílé Karpaty Mts. *Gladiolus palustris* is a declining species, since 2000 only confirmed near the village of Velenka in central Bohemia and in south-eastern Moravia at a few sites in the Hodonínská Dúbrava wood and at a single site in the Čertoryje reserve in the Bílé Karpaty Mts. It is therefore classified as critically threatened (Grulich 2012).

Gratiola neglecta (Fig. 50)

Gratiola neglecta is a wetland annual species of North-American origin with the main distribution in the catchment basin of the Mississippi and Missouri rivers in the eastern USA (Pennell 1935). Since 1919 it has been recorded as an alien in various places of Europe: Alsace in France (Simon 1960, Soriano & Romero 2008), southern Finland (Suominen 1984, Soriano & Romero 2008) and north-western Germany (Raabe 2007). It is also reported from Japan (Ohwi 1965). Both in the primary and the secondary range the species colonizes muddy river beds, exposed pond and lake bottoms, wet arable fields and similar wet habitats with open vegetation (Pennell 1935, Šumberová & Ducháček 2009). In the Czech Republic *G. neglecta* was for the first time collected in 1941 near the town of Lázně Bohdaneč in eastern Bohemia but the specimen was misidentified as *G. officinalis* (Lustyk 2015). Recently it was found in fish storage ponds (probably the same site as of the find from 1941) and two small fishponds near the town of Lázně Bohdaneč in eastern Bohemia and in fish storage ponds in the town of Blatná in southern Bohemia (Šumberová & Ducháček 2009, Šumberová 2013b). Although its invasive potential in central Europe does not appear to be very high, it may persist for decades at one site. The manner of its first introduction to the Czech Republic remains unknown. Various dispersal modes, including anthropochory by means of vehicles used for fish transport or with fish-farming equipment (Šumberová et al. 2012b) are in question.

Gratiola officinalis (Fig. 51)

Gratiola officinalis is a species of mainly European distribution; isolated occurrences are also known from Anatolia, Central Asia and western Siberia (Hartl 1975, Meusel et al. 1978). In Europe it is known from the temperate and meridional zones where it follows large river floodplains (Burkart 2001), reaching its native northern distribution limit in the Baltic countries, the western limits in the Benelux countries and the Iberian Peninsula, the southern limits in Sardinia and Greece, and the eastern limits in the Volga and Kama river floodplains in Russia (Hartl 1975, Meusel et al. 1978). *Gratiola officinalis* has its optimum in lowland floodplain meadows with regular disturbances by flood, mowing and intensive fluctuations of soil moisture during the growing season (Burkart 2001). The species is able to grow also in other disturbed wet habitats such as ditches, flooded sand and gravel pits and fishpond banks. In the Czech Republic its past distribution involved lowland floodplains of large rivers, in particular of Morava, Dyje and Svratka in southern and central Moravia, and floodplains of the Labe river and its tributaries in north-western and central Bohemia. From there, the species reached lowlands in northern Moravia and Silesia and the colline belt in eastern and northern Bohemia; isolated occurrences were recorded in southern Bohemia. Old records outside the floodplains are probably due to garden escapes, related to the medical use of *G. officinalis* in the past (Skokan 1928, Hartl 1975). We accepted most of these records for the map, though some of them (mainly literature records) as uncertain. Records of plants intentionally planted outside gardens were excluded. The species has declined considerably since the beginning of the 20th century, probably as a consequence of land-use change (particularly the abandonment of low-intensity grazing) and the large-scale river regulations, in particular in the Labe river basin. During the 1960s to 1980s the drainage of meadows and their conversion to arable land, and abandonment of other wet grasslands
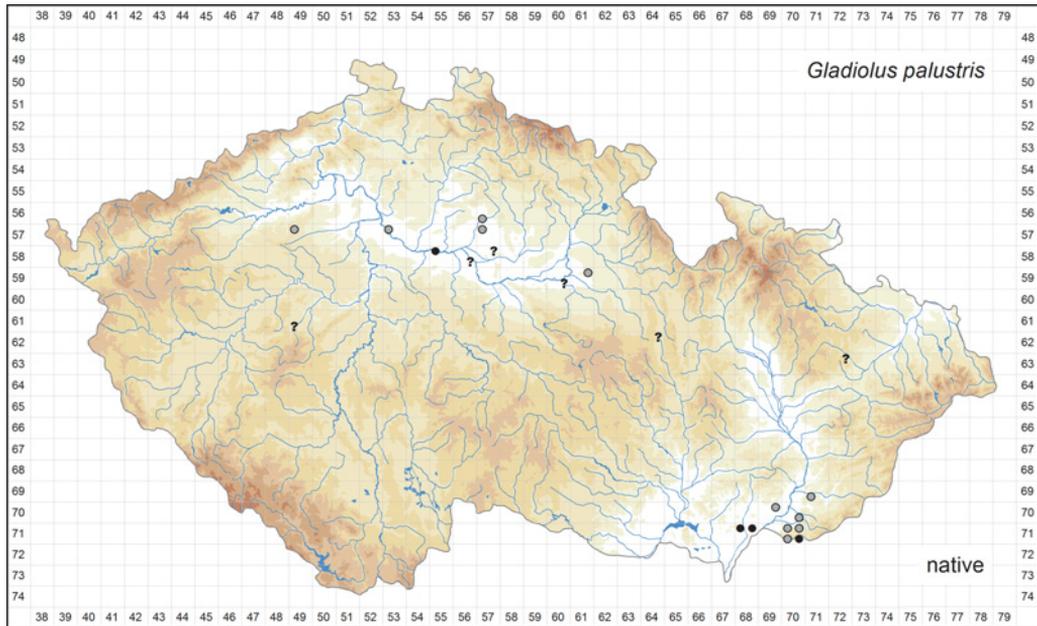
Fig. 49. – Distribution of *Gladiolus palustris* in the Czech Republic: ● at least one record in 2000–2016 (4 quadrants), ◉ pre 2000 records only (11 quadrants). Prepared by Jindřich Chrtek Jr.
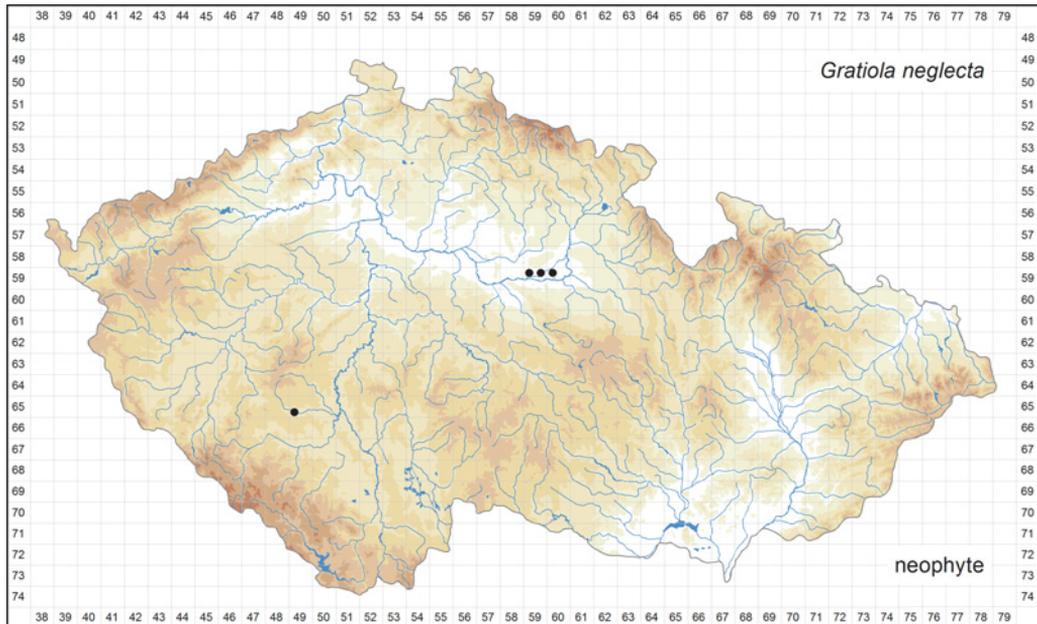
Fig. 50. – Distribution of *Gratiola neglecta* in the Czech Republic (4 occupied quadrants). Prepared by Kateřina Šumberová & Michal Ducháček.
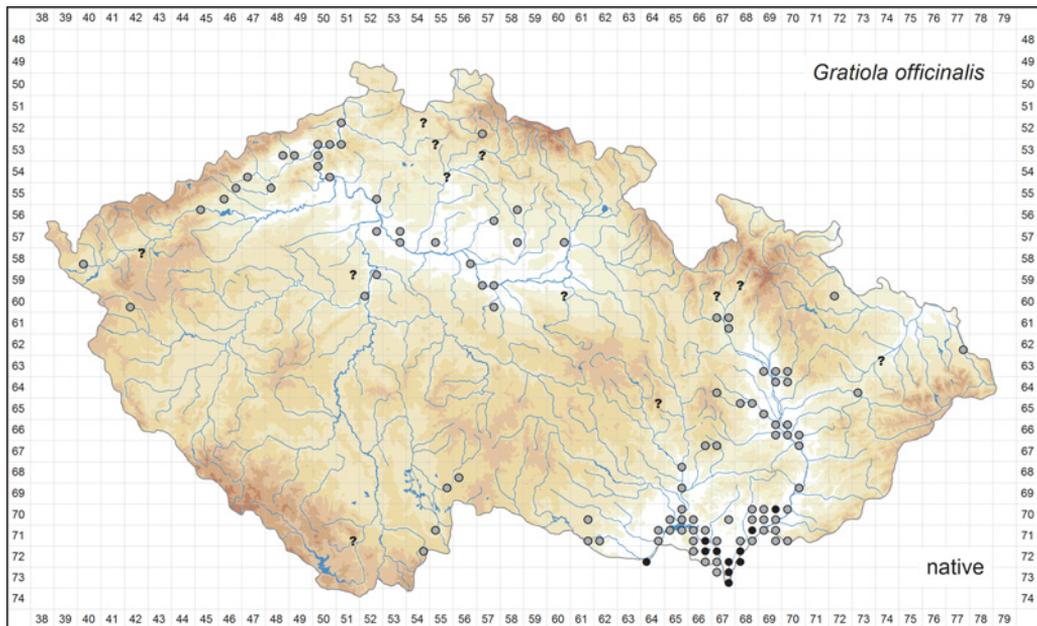
Fig. 51. – Distribution of *Gratiola officinalis* in the Czech Republic: ● at least one record in 2000–2016 (11 quadrants), ◉ pre 2000 records only (94 quadrants). Prepared by Kateřina Šumberová & Michal Ducháček.
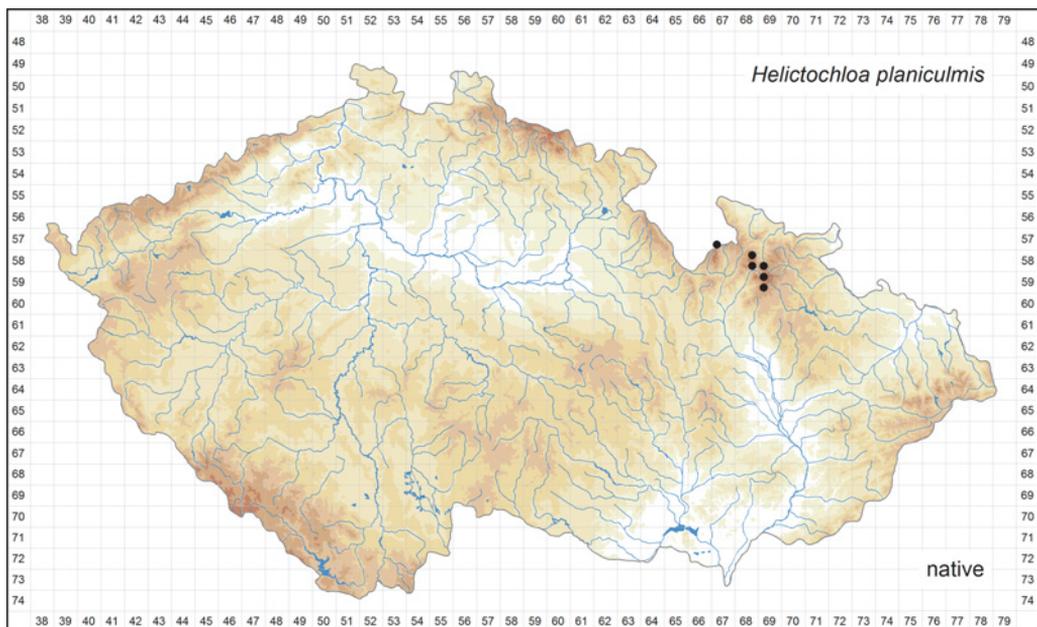

Fig. 52. – Distribution of *Helictochloa planiculmis* in the Czech Republic (6 occupied quadrants). Prepared by Jiří Zázvorka.

caused further losses of *G. officinalis* sites, in particular in Moravia. Recently the species has been recorded as a permanent resident only in the lower Morava and Dyje rivers floodplains in southernmost Moravia and it is listed as endangered (Grulich 2012).

Helictochloa planiculmis (Fig. 52)

Helictochloa planiculmis is distributed in the mountains of Europe, including the Eastern Sudetes in the Czech Republic, the Carpathians in Slovakia, Poland, Ukraine, Romania and Serbia, and Stara Planina, Vitoša, Rila and Rodopi Mts in Bulgaria. It has also been reported from central Poland from lower altitudes. Outside Europe it occurs in north-eastern Turkey (Röser 1996, Conert 1998). Two subspecies have been recognized: in the Czech Republic only the type subspecies occurs (the species was described by H. A. Schrader from Mt Králický Sněžník), while the plants from south-eastern part of the European distribution range are assigned to *H. p.* subsp. *angustior* (Romero-Zarco 2011). In the Czech Republic *H. planiculmis* is rare or locally scattered in subalpine grasslands of the Hrubý Jeseník Mts and Králický Sněžník Mts in the north-eastern part of the country. It is found in subalpine and supramontane vegetation belts at altitudes 1180–1450 m. It is classified as endangered (Grulich 2012).

Helictochloa pratensis (Fig. 53)

Helictochloa pratensis is a European sub-atlantic species, distributed mainly in western and central Europe, eastwards to south-western Finland, north-western Russia, Poland, the Czech Republic, Slovakia and Austria. In contrast, the literature records from western Ukraine, Transylvania and the Balkan Peninsula are uncertain (Conert 1998). *Helictochloa pratensis* occurs in various types of dry grasslands, meadows and pastures, rocky slopes, rarely in open pine or oak acidophilous forests, always in sunny, dry and warm habitats. It prefers acidic, neutral or slightly basic soils poor in calcium and nitrogen, usually over silicates, basaltic extrusive rocks, marlstone, serpentinite or limestone and even loess. In the Czech Republic *H. pratensis* is scattered in warm and rather dry parts of the country, predominantly at altitudes 200–500 m. It is found in north-western and central Bohemia and locally also in south-western Bohemia and in southern Bohemia between the towns of Písek and Protivín (Toman 1973, Soukup et al. 2010). In Moravia it occurs in its warmest southern and central parts east of the line connecting the towns of Třebíč and Vranov nad Dyjí, northwards almost reaching the city of Olomouc in central Moravia. In the Czech Republic two subspecies occur, differing in a few characters, but they are clearly delimited geographically. The Bohemian populations may be assigned to the type subspecies, which is replaced in Moravia by *H. p.* subsp. *hirtifolia*, present also in Slovakia and Lower Austria. *Helictochloa pratensis* is classified as lower risk – near threatened (subsp. *pratensis*) or lower risk – data deficient (subsp. *hirtifolia*) in the Czech Red List (Grulich 2012).

Helictotrichon desertorum subsp. *basalticum* (Fig. 54)

Helictotrichon desertorum is a Eurasian continental steppe plant distributed from central Europe as far as Transbaikalia in eastern Asia, and from Siberia southwards to the Altai Mts, the Pamir Mts, northern Mongolia and westernmost China. Four subspecies are

distinguished (Holub 1972): in the western part of the distribution range west of the Volga river only *H. d.* subsp. *basalticum* occurs; its distribution is discontinuous, consisting of many geographically isolated patches (Conert 1998, Holub 1958, 1962, Röser 1996). *Helictotrichon desertorum* is a perennial grass morphologically adapted to the climate of continental steppe. In the westernmost part of its distribution area it is considered a relict of early Holocene periglacial steppes. It grows on open grassy slopes of dry basalt rocks or their loess and marlstone edges, rarely on limestone (Kolbek & Boublík 2007). *Helictotrichon desertorum* reaches its western distribution limit in the Czech Republic where it is a rare species known from seven localities, six of them in the České středohoří Mts between the towns of Louny and Most in northern Bohemia and one in southern Moravia near the town of Mikulov. It occurs at altitudes from 230 m (Šibeničník hill near Mikulov) to 430 m (Oblík hill near Louny). It is classified as critically threatened (Grulich 2012).

Hierochloë australis (Fig. 55)

Hierochloë australis has a fairly small distribution range confined to Europe (Weimarck 1971). It is distributed mainly in central Europe, towards the east reaching southern Finland, Estonia, Latvia, Lithuania and Ukraine, southwards extending to northern Italy and Croatia; it is only scattered in Romania and Moldova (Meusel et al. 1965, Hultén & Fries 1986, Valdés et al. 2009). In the Czech Republic it grows usually on mineral-rich soils in habitats that are intermittently wet in the spring but dry out during the summer. It occurs mainly in thermophilous oak forests on volcanic hills of north-western Bohemia, in the Bohemian Cretaceous Basin of central and eastern Bohemia, and in deep river valleys of central and southern Bohemia and south-western Moravia. It is found at altitudes about 200–500 m, with the altitudinal maximum at about 700 m in Mt Milešovka in the České středohoří Mts. It is classified as vulnerable (Grulich 2012).

Hierochloë odorata (Fig. 56)

Hierochloë odorata is a member of the *H. odorata* aggregate, which is a widespread species complex with a circumpolar distribution (Meusel et al. 1965, Hultén & Fries 1986). In the Czech Republic two species are found: *H. odorata* growing only in Bohemia and *H. repens* limited to southern Moravia. *Hierochloë odorata* is distributed mainly in north-western Europe and north-eastern North America. In Europe it occurs from the British Isles and Netherlands northwards to Scandinavia and the Baltic countries; towards the east it reaches European Russia. In central Europe it is scattered in Germany, Poland, the Czech Republic and Switzerland, towards the south reaching south-eastern France. It has also been recorded in Iceland (Weimarck 1971). In the Czech Republic it usually grows on sandy soils in disturbed sites, such as roadsides and playgrounds, on the margins of pine woods and sandy shores of artificial lakes. Most of the localities of *H. odorata* are situated in the middle Labe river basin, particularly between the towns of Mělník and Lysá nad Labem. It is classified as critically threatened (Grulich 2012).
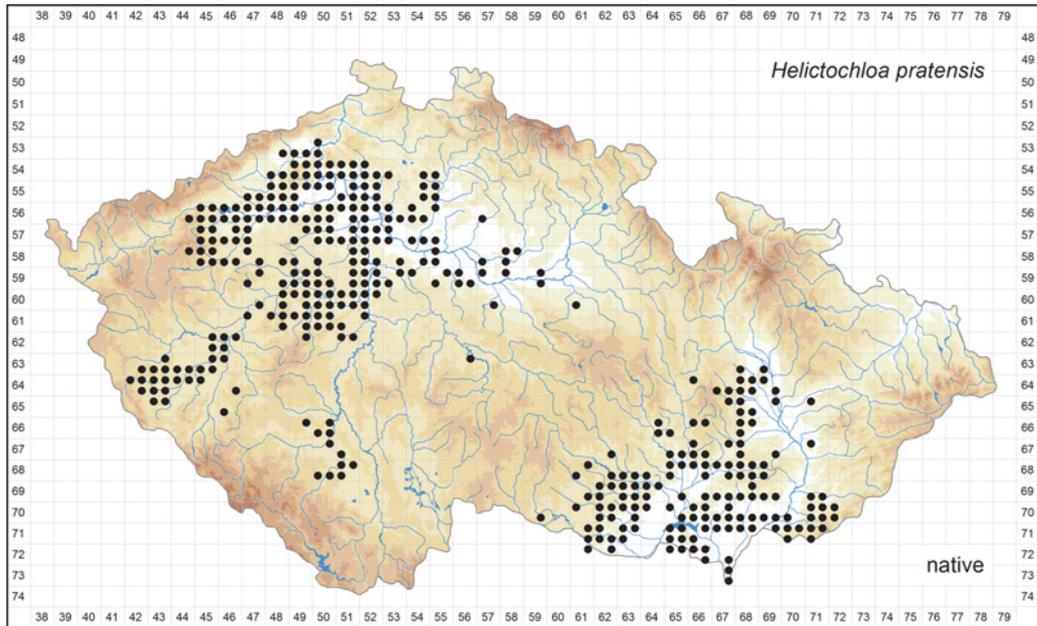
Fig. 53. – Distribution of *Helictochloa pratensis* in the Czech Republic (385 occupied quadrants). Prepared by Jiří Zázvorka.
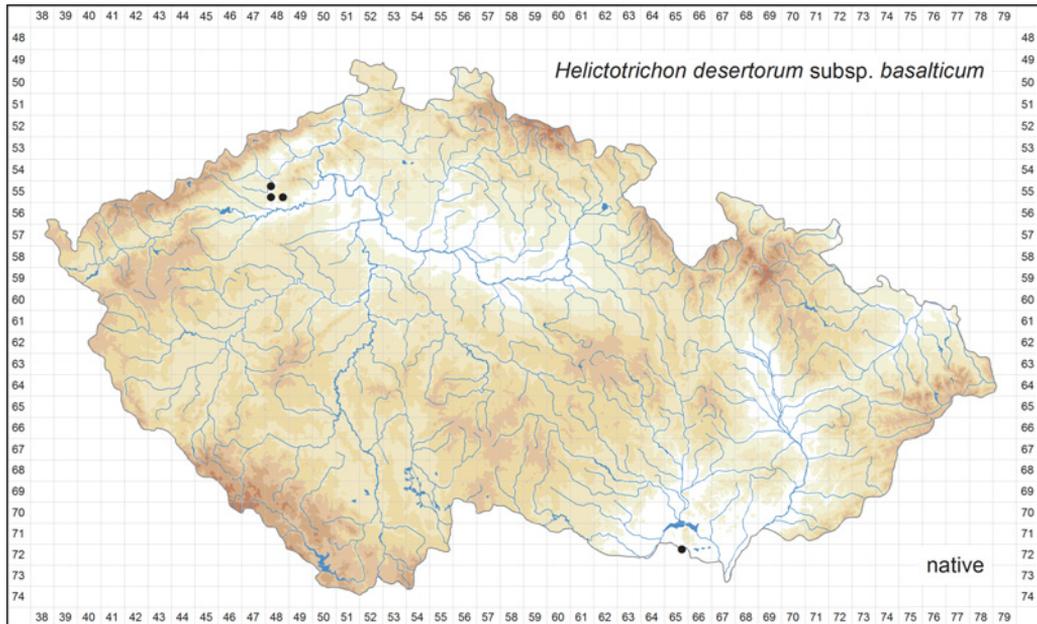
Fig. 54. – Distribution of *Helictotrichon desertorum* subsp. *basalticum* in the Czech Republic (4 occupied quadrants). Prepared by Jiří Zázvorka.
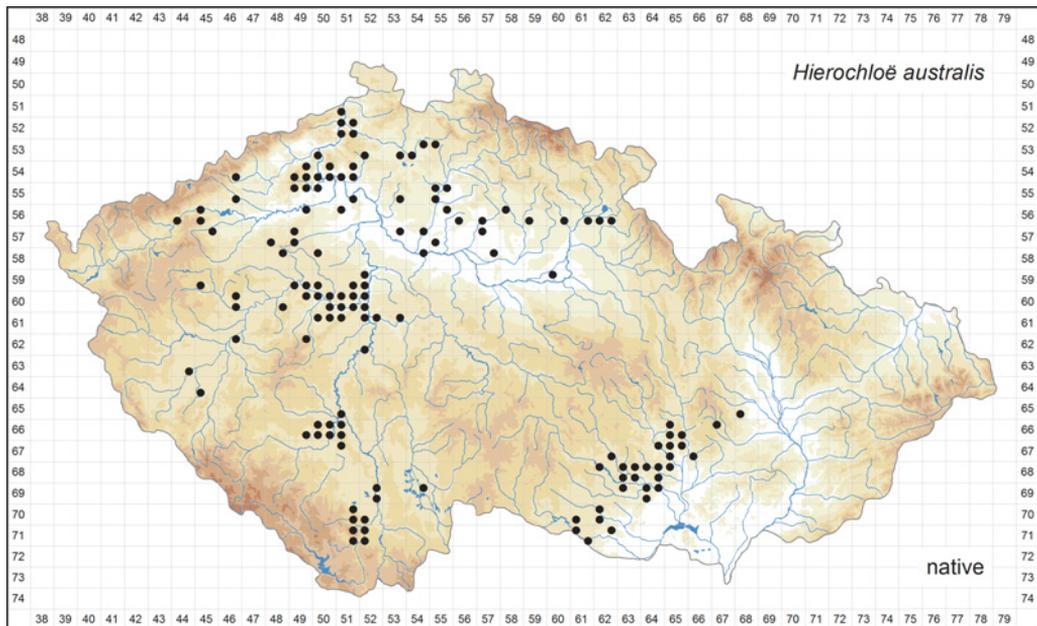
Fig. 55. – Distribution of *Hierochloë australis* in the Czech Republic (137 occupied quadrants). Prepared by Jitka Štěpánková.
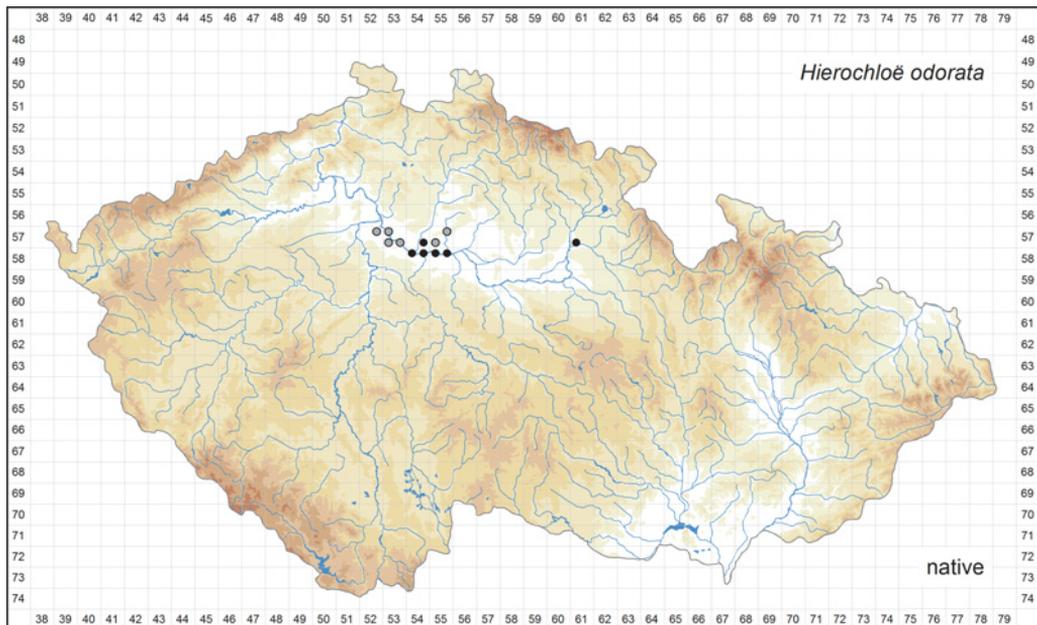

Fig. 56. – Distribution of *Hierochloë odorata* in the Czech Republic: ● at least one record in 2000–2016 (6 quadrants), ◓ pre 2000 records only (6 quadrants). Prepared by Jitka Štěpánková.

Hierochloë repens (Fig. 57)

Hierochloë repens, the other member of the *H. odorata* aggregate, is scattered throughout central and south-eastern Europe and adjacent south-western Asia. In Europe it occurs in the Czech Republic, Slovakia, Austria, Hungary, Romania, Bulgaria, Ukraine and south-eastern part of European Russia (Weimarck 1971). It grows on sandy soils in pine forests, along roads and railways; sometimes it occurs as a weed of arable fields or vineyards (Chrtek & Jirásek 1964, Weimarck 1971). In the Czech Republic it is confined to southern Moravia, where it is found in a few sandy sites south of the town of Lanžhot and between the towns of Hodonín and Veselí nad Moravou. It is classified as critically threatened (Grulich 2012).

Lindernia dubia (Fig. 58)

Lindernia dubia is a wetland annual species native to the basin of the Mississippi river in the USA; localities in other parts of the USA, Canada and South America are probably due to introductions (Pennell 1935, Meusel et al. 1978). The secondary range of the species also involves the temperate and meridional zones of Europe and temperate to tropical parts of Asia (Meusel et al. 1978). The first European records date back to the middle of the 19th century from France. Recently, the species has been reported from Spain, Portugal, Italy, Bulgaria, Romania, Slovenia, Serbia, Hungary, Slovakia, Germany, Poland, the Netherlands and Belgium, in many of the countries showing high invasive potential (Šumberová et al. 2012b, Hrivnák et al. 2016). Both in its primary and the secondary range the species grows mainly on muddy river banks, in lake and pond littorals and on exposed bottoms of water reservoirs and in ditches. In Italy and south-eastern Asia it also occurs in rice fields (Šumberová et al. 2012b). In the Czech Republic *L. dubia* was found for the first time in 1989 on the Lužnice river in southern Bohemia (Kurka 1990) and this locality was for more than a decade the only known site of the species in the country. Since 2000, a total of 11 new localities of *L. dubia* have been discovered in the basins of the Lužnice, Vltava and Otava rivers in southern Bohemia (Šumberová et al. 2012b). While the populations on the Lužnice river show a clear link to the very first record (Lepší & Douda 2005), the source of propagules for all the other localities was most probably the fish storage pond system in the town of Hluboká nad Vltavou. The species was dispersed by two ways: on rubber boots, fish farming equipment or vehicles among the individual fish-farming pond systems situated on various watercourses, and via water between the pond systems and the watercourses collecting their waters (Šumberová et al. 2012b). Further spread is very likely in already invaded catchments. In rivers the spread may remain unnoticed for a long time because suitable conditions for *L. dubia* growth occur irregularly during extremely dry summers, whereas in the years with average rainfall the species survives in the soil seed bank.

Lindernia procumbens (Fig. 59)

Lindernia procumbens is a wetland annual species with a Eurasian range. It is most frequent in eastern and south-eastern Asia with the summer monsoon climate, where the species occurs as a weed of rice fields. Outside this region, the species is scattered in floodplains of large rivers throughout the warm-temperate parts of Europe, western and

central Asia and western Siberia (Lampe 1996). In the Czech Republic *L. procumbens* colonises exposed river beds, oxbows, artificial channels, sand pits and exposed bottoms of fishponds and fish storage ponds. *Lindernia procumbens* has high temperature and moisture demands; in central Europe it germinates on wet substrates not before the end of May. Most of its localities in fishponds were lost probably due to summer drainage applied in spring and early summer which does not suit reproduction of *L. procumbens*, or due to competition of taller herbs on the fishponds with long summer drainage. *Lindernia procumbens* has been recorded in the floodplains of the Labe and Vltava rivers and their tributaries in central, northern and eastern Bohemia, the Lužnice river in southern Bohemia, the Dyje and Morava rivers in central and southern Moravia, and the Odra river in northern Moravia. Most of the extant populations are situated in fish storage ponds where tall vegetation is regularly eliminated by mowing, poultry grazing or occasional herbicide application (Šumberová et al. 2012b). The localities in river floodplains are, at least in some regions, still maintained too. Over the last few years with extraordinary hot and dry summers, i.e. in 2012 and 2015, new populations were found and its occurrence at some already known sites was confirmed (Šumberová et al. 2013). It is obvious that under favourable climatic conditions *L. procumbens* may emerge more frequently from the soil seed bank or after recent dispersal events (via water or waterfowl; Burkart 2001, Šumberová et al. 2012b). Although the species is listed as critically endangered (Grulich 2012), under recent climate conditions it has a potential to survive or even to spread to new localities (cf. Nobis et al. 2010).

Maianthemum bifolium (Fig. 60)

Maianthemum bifolium is a Eurasian boreal species with a large continental distribution range. It grows in the boreal forest zone from western Europe (but excluding most of the British Isles, where it is doubtfully native, and the Iberian Peninsula) as far as Japan in the Far East (Hultén & Fries 1986, Meusel et al. 1965). In the Czech Republic *M. bifolium* grows on humid or rather dry loamy or sandy acid soils in dense coniferous, broadleaved and mixed forests, alluvial forests, glacial cirques and mountain pine growths. It is frequent in forest-rich areas throughout the country, occurring from the planar to subalpine vegetation belts and reaching its altitudinal maximum at 1420 m in the Krkonoše Mts at the upper edge of the Kotelné jámy glacial cirque.

Myriophyllum alterniflorum (Fig. 61)

Myriophyllum alterniflorum is an amphi-atlantic species being most frequent in Atlantic western and northern Europe. It is widespread northwards to Iceland and the Scandinavian coast of the Arctic Ocean, southwards to the Portuguese Atlantic coast. It becomes rare towards the continental interior, eastwards reaching the Baltic countries, western Ukraine, central Europe, Italy and Greece (Meusel et al. 1978, Hultén & Fries 1986, Sarika-Hatzinikolaou et al. 1994). Outside Europe it occurs in northern and north-eastern parts of North America, in Greenland, the Azores, north-western Africa and China (Hultén & Fries 1986, Yu et al. 2002). In the Czech Republic it occurs predominantly in the upper reaches of rivers and bigger streams, rarely in standing water (especially in detached river arms). It prefers clear, oligo-mesotrophic to dystrophic, slightly acidic waters with sandy or gravelly bottom. The majority of *M. alterniflorum* localities in the
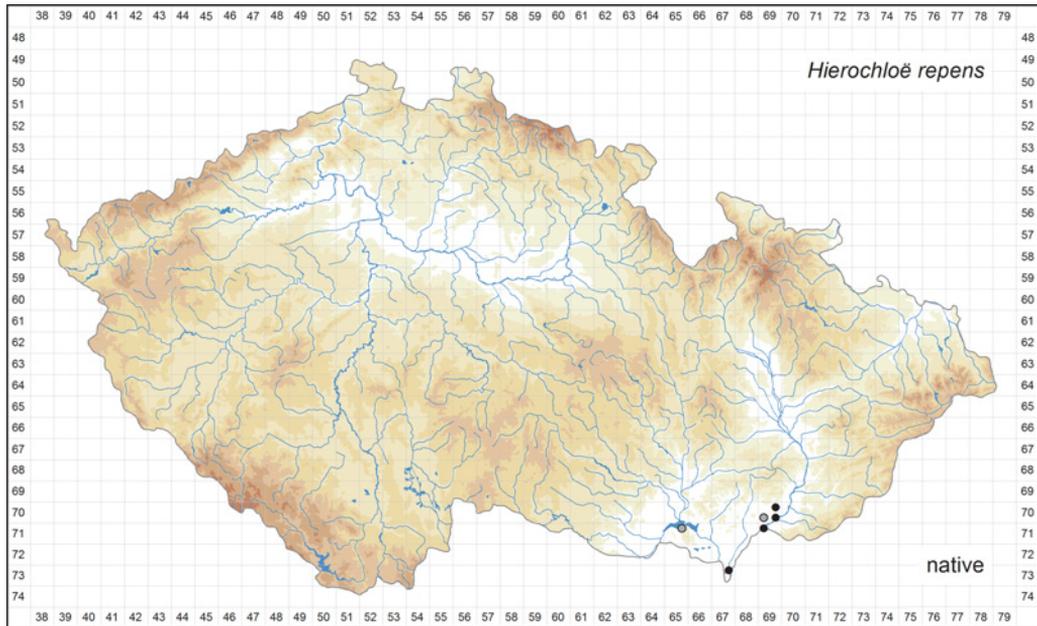
Fig. 57. – Distribution of *Hierochloë repens* in the Czech Republic: ● at least one record in 2000–2016 (4 quadrants), ◐ pre 2000 records only (2 quadrants). Prepared by Jitka Štěpánková.
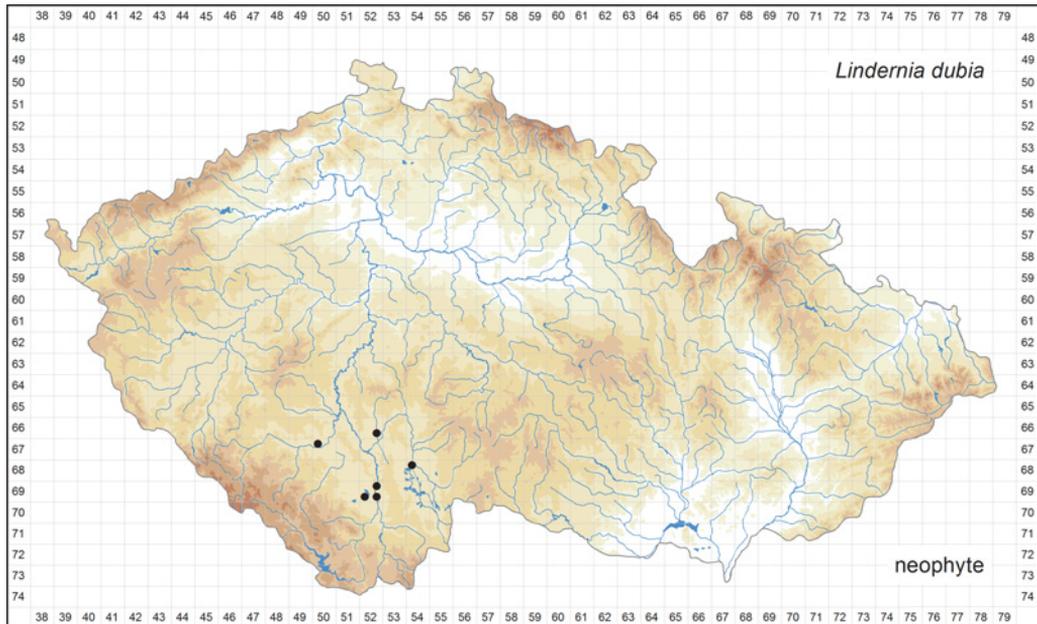
Fig. 58. – Distribution of *Lindernia dubia* in the Czech Republic (6 occupied quadrants). Prepared by Kateřina Šumberová & Michal Ducháček.
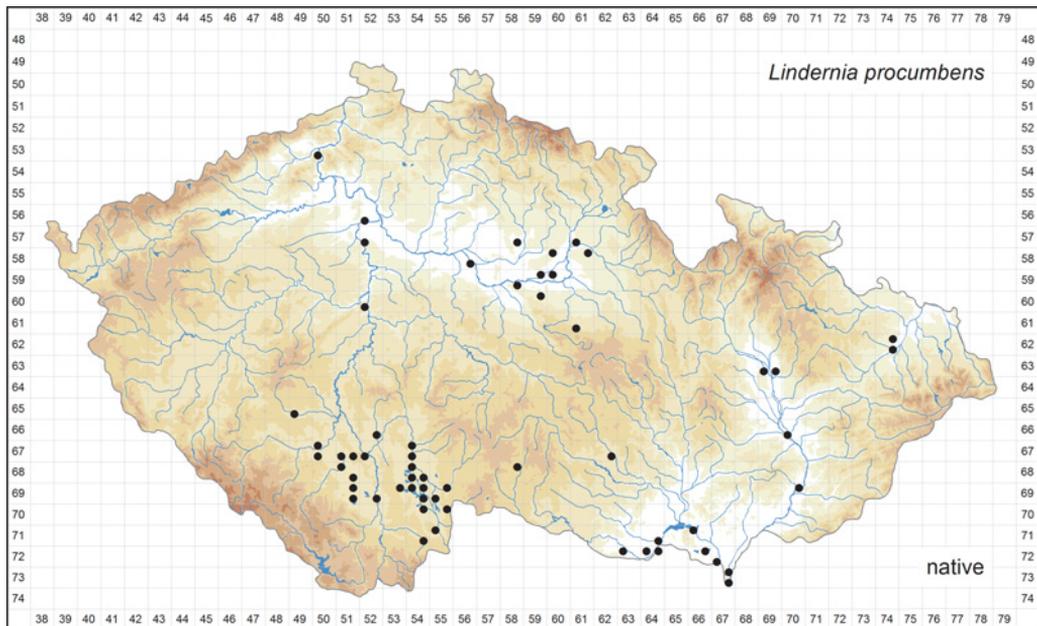
Fig. 59. – Distribution of *Lindernia procumbens* in the Czech Republic (58 occupied quadrants). Prepared by Kateřina Šumberová & Michal Ducháček.
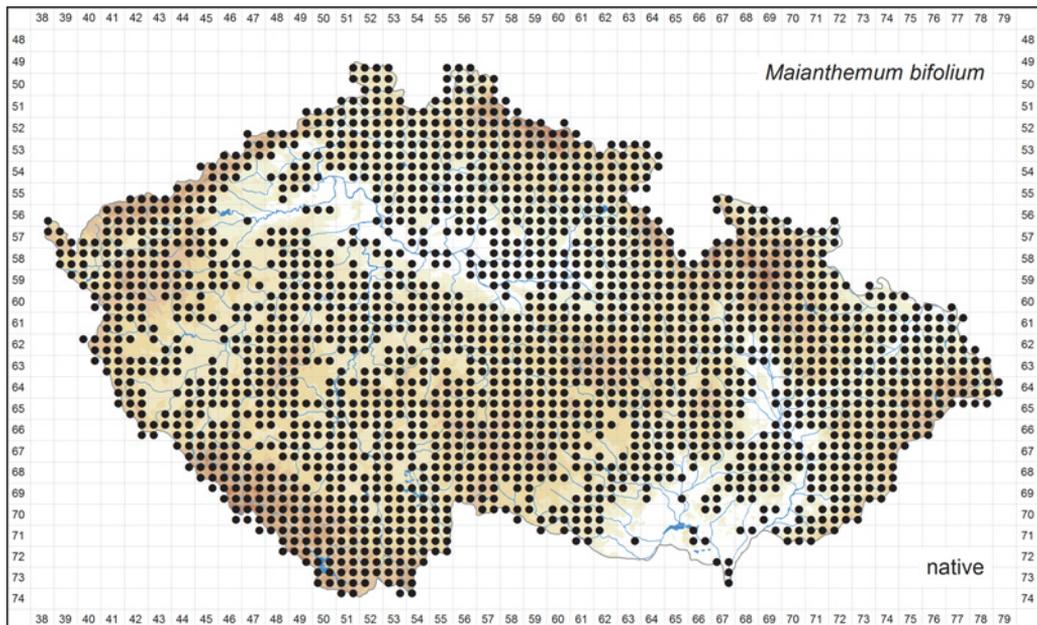
Fig. 60. – Distribution of *Maianthemum bifolium* in the Czech Republic (2064 occupied quadrants). Prepared by Jiří Zázvorka.
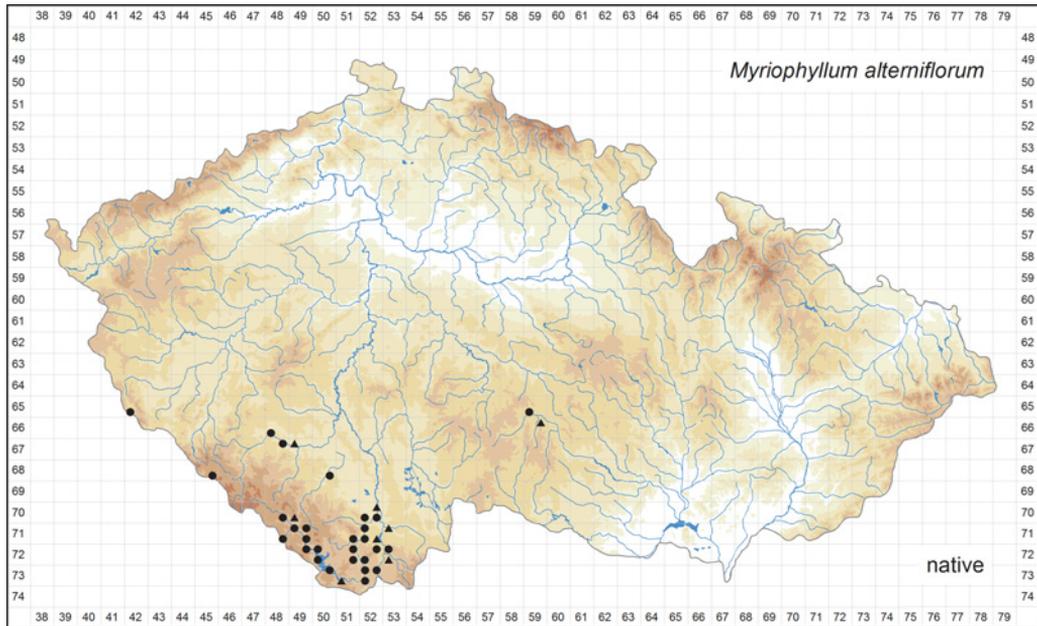
Fig. 61. – Distribution of *Myriophyllum alterniflorum* in the Czech Republic: ● occurrence documented by herbarium specimens (28 quadrants), ▲ occurrence based on other records (8 quadrants). Prepared by Jan Prančl.
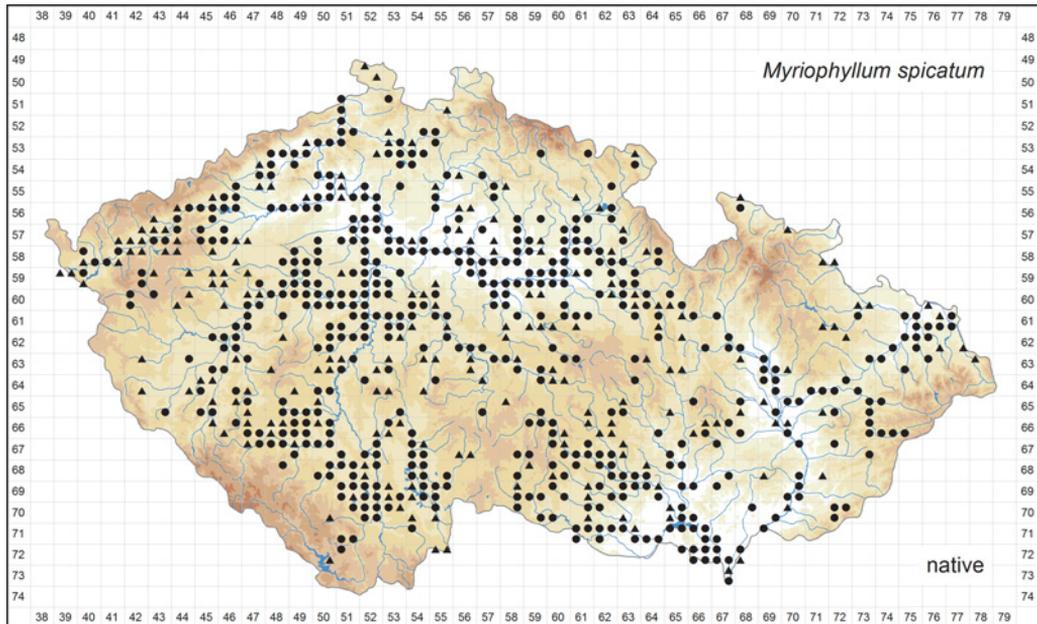
Fig. 62. – Distribution of *Myriophyllum spicatum* in the Czech Republic: ● occurrence documented by herbarium specimens (503 quadrants), ▲ occurrence based on other records (212 quadrants). Prepared by Jan Prančl.
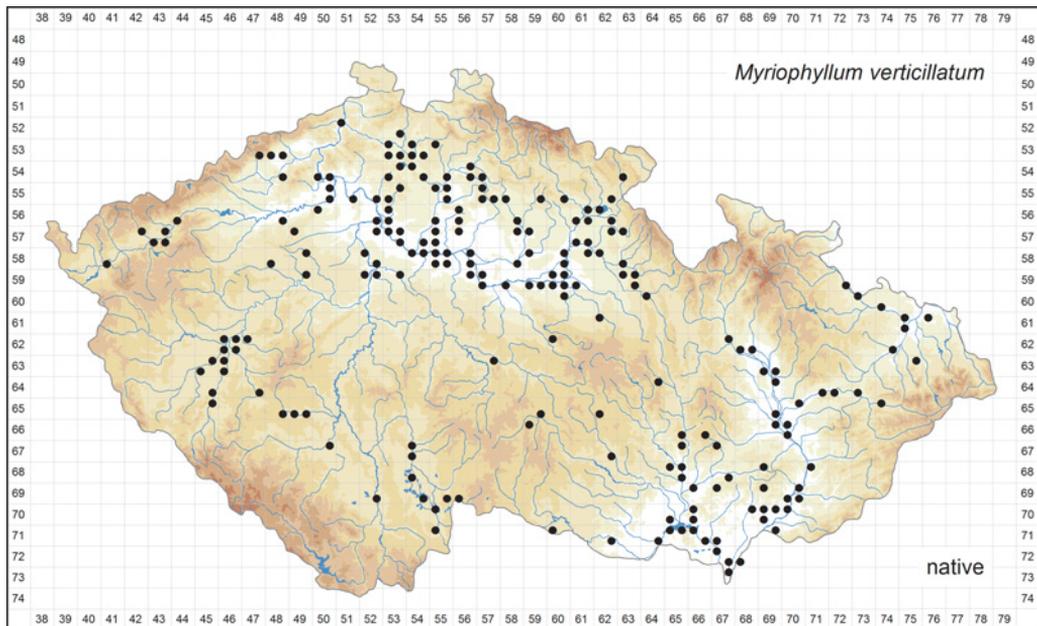
Fig. 63. – Distribution of *Myriophyllum verticillatum* in the Czech Republic (207 occupied quadrants). Prepared by Jan Prančl.
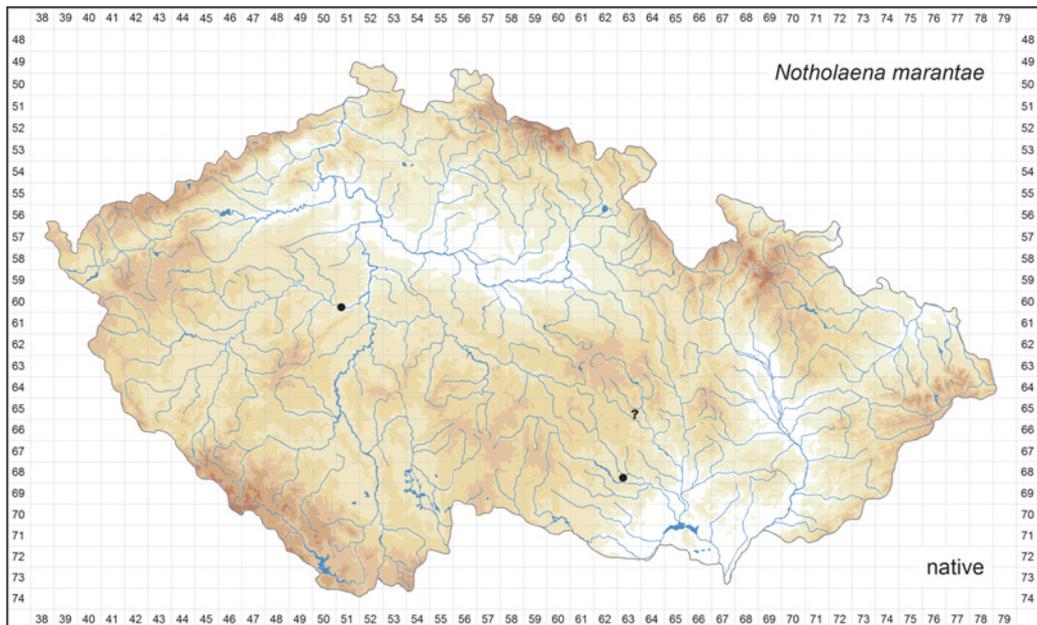
Fig. 64. – Distribution of *Notholaena marantae* in the Czech Republic (2 occupied quadrants). Prepared by Libor Ekrt.

Czech Republic are found in the Vltava river and several of its tributaries in the Šumava Mts and its foothills in southern Bohemia. It used to occur in but has vanished from other rivers in this area (Malše, Otava and Blanice) and from the Laka glacial lake. The north-ernmost Bohemian population in the Nemanický potok stream in the Český les Mts has been discovered only recently. The species was known to occur also in the Jihlava river in the Českomoravská vrchovina highlands (last record in 2003). *Myriophyllum alterni-florum* is a competitively weak species sensitive to water pollution and stream regulation, reaching the south-eastern limit of its local distribution in the Czech Republic. Therefore, it is classified as endangered (Grulich 2012). However, the populations in the Vltava river are abundant and not threatened.

Myriophyllum spicatum (Fig. 62)

Myriophyllum spicatum is widespread in most of Europe (although apparently rare in central and northernmost parts of Scandinavia) and the temperate regions of Asia. Eastwards it reaches Japan and the Russian Far East, southwards the Canary Islands, northern and eastern Africa, western Asia, the Himalayas, southern China and the Philip-pines (Meusel et al. 1978, Hultén & Fries 1986). It has become naturalized in most of North America, where it is considered an invasive and noxious weed; it has also been introduced to southern Africa (Scribailo & Alix 2014, Weyl & Coetzee 2014). In the Czech Republic *M. spicatum* shows a broad ecological amplitude, inhabiting various types of standing and running waters, such as rivers and bigger streams, fishponds, reser-voirs, sand-pits, drainage channels, alluvial and artificial pools. It grows in mesotrophic to strongly eutrophic water and prefers habitats in early succession stages or with regular disturbance. It is distributed almost throughout the country, from lowlands to middle ele-vations and only rarely exceeds the altitude of 600 m. It is most frequent along the middle and lower courses of large rivers and in flat fishpond-rich basins, where it is one of the most common aquatic plants. Under optimal conditions it is able to form large dense stands and become a noxious weed in farm fishponds. In contrast, it is rare in or locally missing from the driest lowlands with a lack of suitable habitats.

Myriophyllum verticillatum (Fig. 63)

Myriophyllum verticillatum is a circumpolar species, growing mainly in temperate regions of Europe, Asia and North America. The continuous distribution range extends from the British Islands and France to central Asia. Further eastwards the species becomes scattered, reaching Japan, the Russian Far East and China. In North America it is scattered throughout the temperate and boreal zones and also occurs in north-western Africa (Meusel et al. 1978, Hultén & Fries 1986, Scribailo & Alix 2014). In Europe it is widespread in its Atlantic, central and eastern parts but is rare in the Mediterranean area and in the coldest regions. *Myriophyllum verticillatum* grows in mesotrophic to naturally eutrophic, transparent, standing or very slowly running waters. It occurs in habitats in an advanced stage of terrestrialization with a thick layer of organic mud on the bottom, and frequently forms terrestrial stands on the deep muddy substrates. In the Czech Republic it grows most often in alluvial pools, oxbow lakes, lightly managed fishponds and chan-nels. It is a scattered species, with most records from basins of large rivers (especially in the Labe river basin and in southern Moravia) and in wet and relatively warm regions in

northern Bohemia (broader vicinity of Česká Lípa and Jičín towns), but rare elsewhere. It is largely a lowland species, only rarely exceeding the altitude of 450 m. *Myriophyllum verticillatum* is endangered by eutrophication, habitat destruction, river regulations and intensive fish farming. It has markedly declined during the last decades and is currently absent from Czech Silesia. Therefore it is classified as vulnerable (Grulich 2012). Because of frequent confusion of *M. spicatum* and *M. verticillatum*, the distribution map of the latter was based solely on revised herbarium specimens.

Notholaena marantae (Fig. 64)

Notholaena marantae has a highly fragmented distribution range. It is most frequent in the Mediterranean area, Macaronesia, the southern part of the Arabian Peninsula, Ethiopia and around the Himalayas (Pichi Sermoli 1979). It reaches the northernmost limit of its distribution in the Czech Republic (Kaplan 2012). Only two recent localities and one uncertain are known in the Czech Republic. *Notholaena marantae* was discovered in 1858 by C. Römer on the serpentines near the town of Mohelno in southern Moravia (Juratzka 1858). The population comprises of nearly 2000 clumps, growing on dry and sunny serpentine rocks and in rocky steppe with southern to south-eastern aspect at altitudes 270–370 m (Ekrt 2015). The second population was discovered about 10 years ago in the karst area of Český kras near the village of Hlásná Třebaň in central Bohemia. This site hosts several clumps growing on ultrabasic picrite rocks (Šprynar 2004). In the past another locality of *N. marantae* was reported to exist in the serpentines at the Spálený mlýn mill in the valley of the Nedvědička stream near the village of Pernštejn in western Moravia (Formánek 1884). However, this record is uncertain, because the occurrence was not confirmed there later and no herbarium voucher has been found. *Notholaena marantae* is classified as critically threatened because of its rarity (Grulich 2012).

Nymphoides peltata (Fig. 65)

Nymphoides peltata is native to Europe and northern Asia. In Europe it reaches the British Isles, southern Scandinavia and the Baltic countries in the north and the Iberian Peninsula, Italy and Greece in the south. In Asia it extends through its temperate zone eastwards to China, the Russian Far East and Japan (Meusel et al. 1978, Hultén & Fries 1986). It has become naturalized in North America (Stuckey 1974) and has been introduced to Australia (Randall 2007) and New Zealand (Howell & Sawyer 2006). In the Czech Republic *N. peltata* grows in fishponds, slow-moving rivers and adjacent oxbow lakes. It occurs in 50–150 cm deep water, but tolerates drops of the water table down to the bottom and even requires such drops for seed germination. It grows in water bodies with a mineral bottom, therefore it is common in early succession stages but it declines with accumulation of organic sediments (Šumberová 2011a). *Nymphoides peltata* was most frequent in fishpond landscapes of south-western and southern Bohemia. It occurred very locally also in central Bohemia and in south-western, central and north-eastern Moravia, and only rarely elsewhere. It has declined during the 20th century due to terrestrialization of alluvial pools and changes in fishpond management, particularly in its intensification and elimination of regular summer drainage. As a decorative plant, *N. peltata* has become available in garden stores and plant nurseries since the 1990s. It is being occasionally cultivated in garden pools and sometimes intentionally planted for
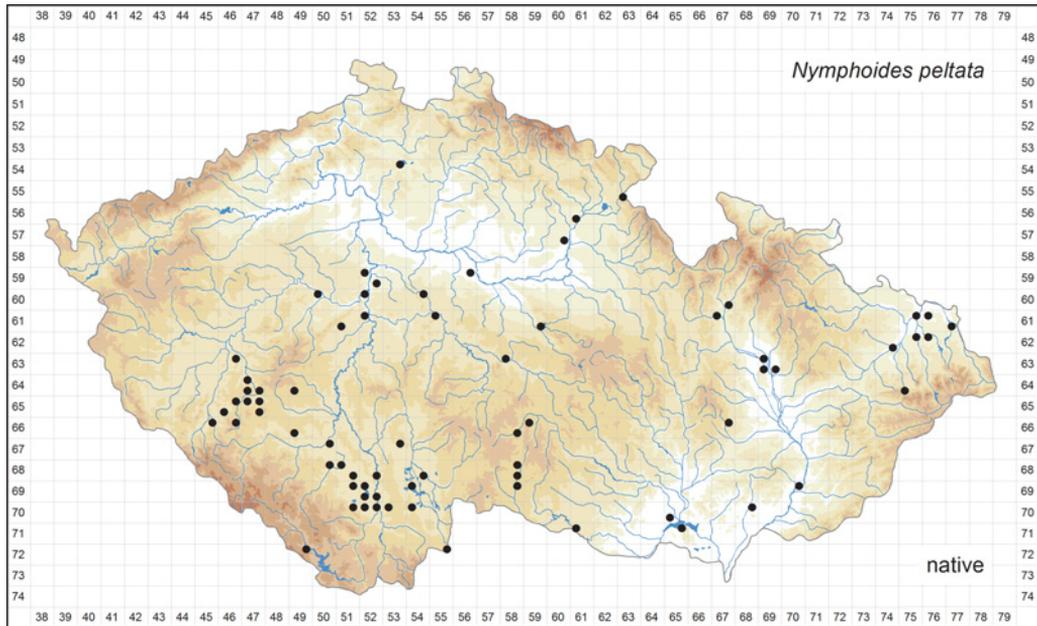
Fig. 65. – Distribution of *Nymphoides peltata* in the Czech Republic (70 occupied quadrants). Prepared by Zdeněk Kaplan.
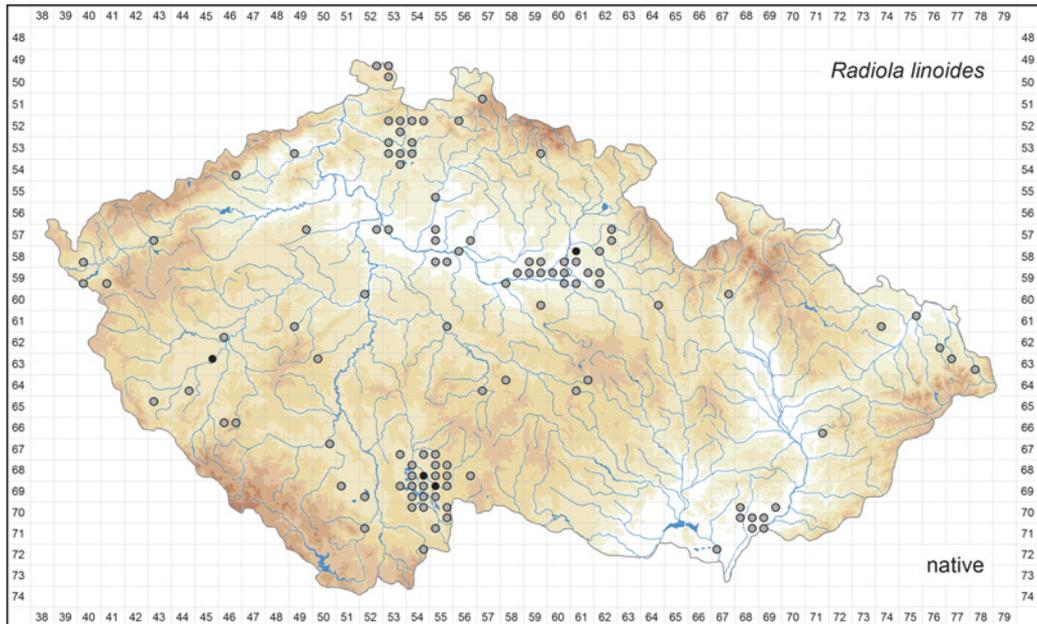
Fig. 66. – Distribution of *Radiola linoides* in the Czech Republic: ● at least one record in 2000–2016 (4 quadrants), ◉ pre 2000 records only (108 quadrants). Prepared by Jan Prančl.
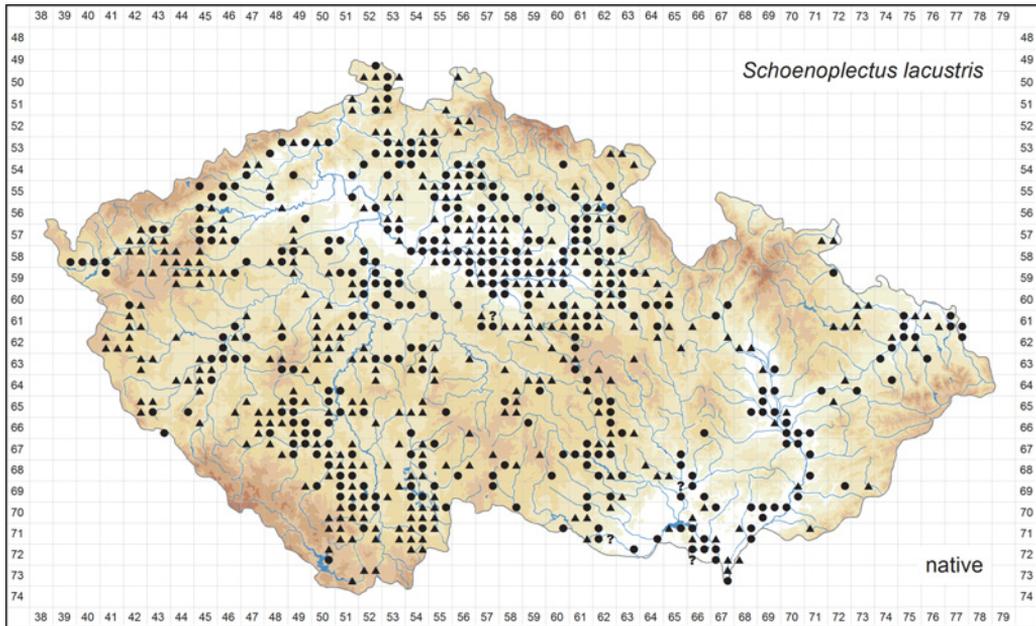
Fig. 67. – Distribution of *Schoenoplectus lacustris* in the Czech Republic: ● occurrence documented by herbarium specimens (325 quadrants), ▲ occurrence based on other records (353 quadrants). Prepared by Petr Filippov & Jiří Danihelka.
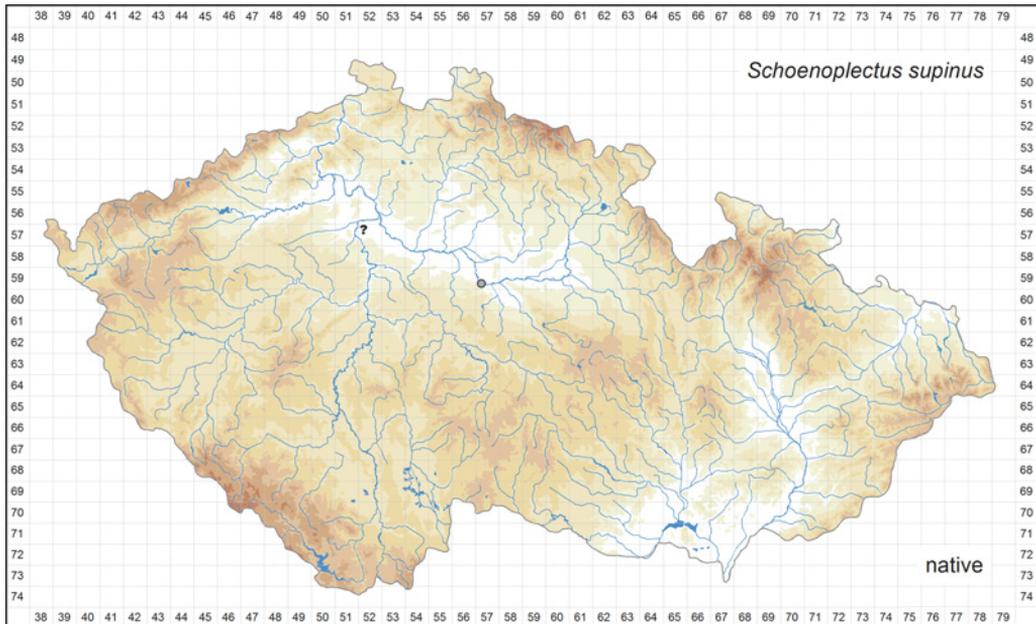
Fig. 68. – Distribution of *Schoenoplectus supinus* in the Czech Republic (1 occupied quadrant). Prepared by Petr Filippov & Jiří Danihelka.

ornamental purposes in fishponds and flooded abandoned quarries. These plants are of unknown, but probably distant origin and may cause genetic erosion of native populations. In many of the recently emerged localities populations of spontaneous origin cannot be distinguished from those deliberately established by planting. However, the fact that most of the new localities appeared in areas where the species was previously unknown indicates that the latter is more likely. The native populations are apparently rarer and more endangered than the number of currently known sites may indicate. The species is classified as critically threatened (Grulich 2012).

Radiola linoides (Fig. 66)

Radiola linoides is mainly a European species with a sub-Atlantic distribution. It is relatively widespread (at least historically) in western Europe, occurring from Shetland and southern Sweden to Spain, Italy and Crete. Eastwards it becomes more scattered, reaching western Russia and north-western Anatolia (Meusel et al. 1978, Hultén & Fries 1986, von Lampe 1996). Outside Europe it is reported from Lebanon, Macaronesia, northern Africa and as very rare from tropical Africa. Is has been also introduced to north-eastern part of the USA (Meusel et al. 1978, Hultén & Fries 1986). The species is a competitively weak wetland annual, growing mainly on sandy, nutrient-poor substrates with acidic soil reaction (Popiela 1998), rarely also on peat soils. It inhabits exposed pond bottoms, abandoned sand-pits, lightly managed arable fields (often stubble fields), disturbed sites in pastures, ditches, the edges of sandy tracks and other damp places with sparse vegetation cover. In the Czech Republic *R. linoides* used to grow mainly in flat areas with frequent open sandy habitats, such as the Třeboňská pánev basin in southern Bohemia, the broader vicinity of the town of Česká Lípa in northern Bohemia, the Labe river basin in eastern Bohemia and the surroundings of the town of Hodonín in southern Moravia, being rare elsewhere. *Radiola linoides* declined markedly after World War II. At present, it is extinct in Moravia and has been confirmed only at four sites in Bohemia since 2000. However, the species can be easily overlooked due to its diminutive habit and late phenology. Several populations in sand-pits in the Třeboňská pánev basin are not native, as they originated from recent rescue cultivations (A. Kučerová, in litt.; not included in the map). The species has totally vanished from fishponds and fields for the same reasons as did the ecologically and phenologically similar *Centunculus minimus* (Šumberová 2013c, and see above under that species). *Radiola linoides* is currently close to its extirpation from the Czech Republic and it is therefore classified as critically threatened (Grulich 2012).

Schoenoplectus lacustris (Fig. 67)

Schoenoplectus lacustris is found in most of European countries, northern Africa, the Caucasus Mts, Anatolia and southern Siberia (Hultén & Fries 1986, Jiménez-Mejías & Luceño 2011) but the eastern European and north Asian populations are sometimes treated as a separate subspecies or even species, *S. hypoliti* (Timohina 1990). In North America it is replaced by its sibling species *S. heterochaetus* (Smith 2002). In the Czech Republic *S. lacustris* occurs mainly in littoral zones of fishponds, sand pits, oxbow lakes and beds of lowland rivers, usually in mesotrophic to slightly eutrophic habitats. It is able to form submerged stands with ribbon-leaved forms, especially in rivers, and readily

colonizes recently disturbed or newly created water reservoirs (Šumberová 2011b). The species occurs scattered all over the country, being locally common in river basins, floodplains of lowland rivers and areas with many fishponds such as the Třeboňská pánev and Budějovická pánev basins. It is rare in dry parts of the country without large water bodies, such as north-west of Prague or south-west and north of Brno, and absent from the mountains. *Schoenoplectus lacustris* must have spread considerably with the establishment of fishponds during the Middle Ages. However, it has been declining for decades due to changes in fishpond management and eutrophication, and even longer due to river canalization and drainage. Being still quite common at some parts of the country, it is classified only as near threatened (Grulich 2012).

Schoenoplectus supinus (Fig. 68)

Schoenoplectus supinus is a cosmopolitan species found in most parts of the World apart from Antarctica and North America. The type subspecies occurs in northern Africa, south-western and central Asia and Europe (Liang et al. 2010), being absent from its northern and eastern part. The Czech Republic is situated at the northern limits of species' distribution range. *Schoenoplectus supinus* is generally rare and declining in central Europe (e.g. Fischer 2008), and only two records exists from the Czech Republic, both from central Bohemia. The species was discovered as new for the Bohemian flora in July 1881 in sandy wet places along a road north of the town of Kolín; this occurrence is documented by numerous herbarium specimens collected in 1881–1885. In contrast, we consider the other record, published under *Isolepis supina* (J. Rozum in Rohlena 1926), from the vicinity of the village of Úžice west of the town of Kralupy nad Vltavou somewhat doubtful. One would take it for granted that a herbarium specimen had been collected but we failed to locate it despite the targeted search both at PRC and PR. The habitat of the population found in 1881 was described in herbarium labels as the shores of a shallow pool or wet sandy places, whereas Rohlena (1926) referred to a destroyed saline meadow. These one or two temporary occurrences may be explained by long distance seed dispersal by waterfowl. Not seen for almost a century, the species is now considered extinct (Grulich 2012).

Schoenoplectus tabernaemontani (Fig. 69)

If *Schoenoplectus tabernaemontani* and *S. validus*, reported to occur in the Far East, North America and elsewhere, are considered conspecific (e.g. Smith 2002), the resulting variable taxon has an almost cosmopolitan distribution range (Meusel et al. 1965, Hultén & Fries 1986). It is found in most European countries, in western and northern Europe showing a clear affinity to sea shores. In the Czech Republic *S. tabernaemontani* grows in ditches, wet depressions in arable fields, pools in abandoned quarries, pits after open cast coal mining, remnants of salt marshes and littorals of fishponds, formerly also in the surroundings of mineral springs and around brackish lakes. Sometimes it forms large stands, sometimes only small colonies are found (Sádlo 2011). Its distribution in the Czech Republic is discontinuous, restricted mainly to the areas with substrates rich in nutrients and warm climate. It is found in north-western, central and eastern Bohemia, mainly in the basins of the Ohře and Labe rivers. Particularly remarkable is the population around mineral springs in the Soos National Nature Reserve near the town of Františkovy Lázně in western Bohemia. In the eastern parts of the country, it is scattered over central and southern Moravia,
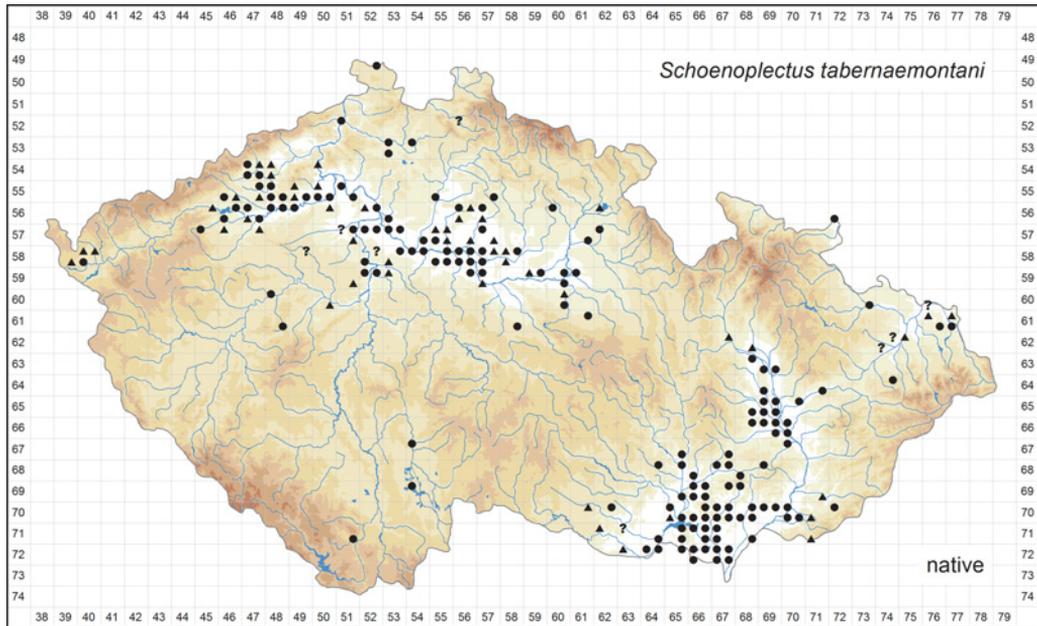
Fig. 69. – Distribution of *Schoenoplectus tabernaemontani* in the Czech Republic: ● occurrence documented by herbarium specimens (151 quadrants), ▲ occurrence based on other records (50 quadrants). Prepared by Petr Filippov & Jiří Danihelka.
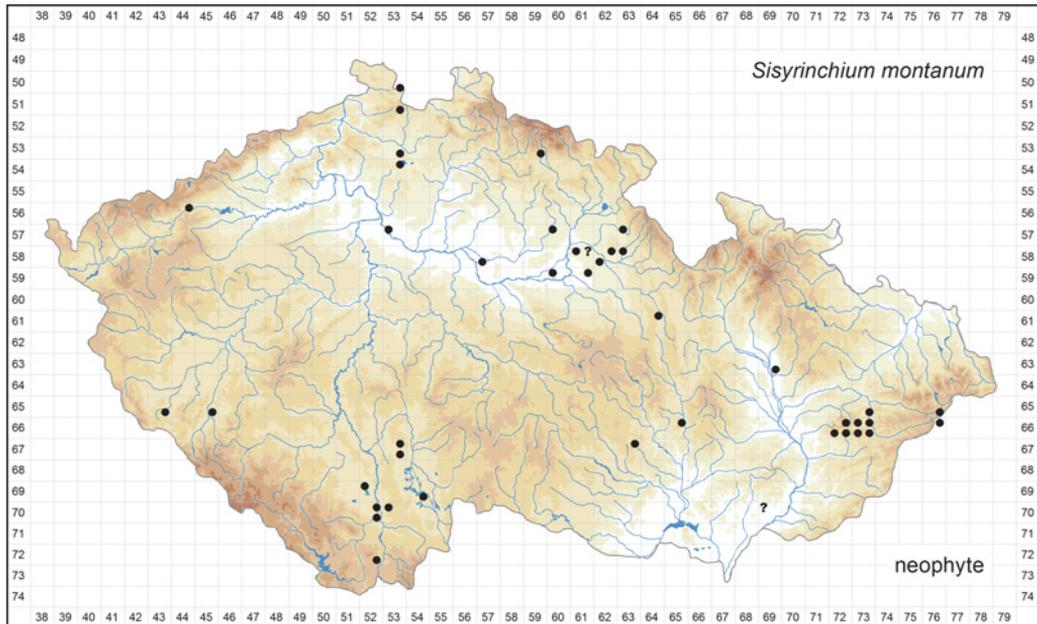
Fig. 70. – Distribution of *Sisyrinchium montanum* in the Czech Republic (40 occupied quadrants). Prepared by Jindřich Chrtek Jr.
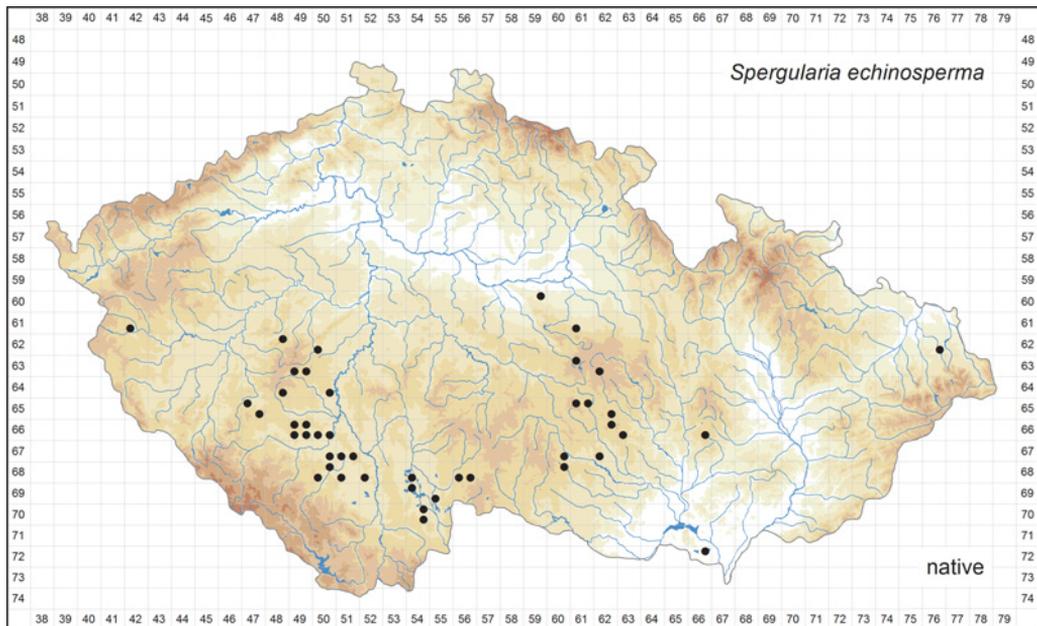
Fig. 71. – Distribution of *Spergularia echinosperma* in the Czech Republic (44 occupied quadrants). Prepared by Pavel Kúr.
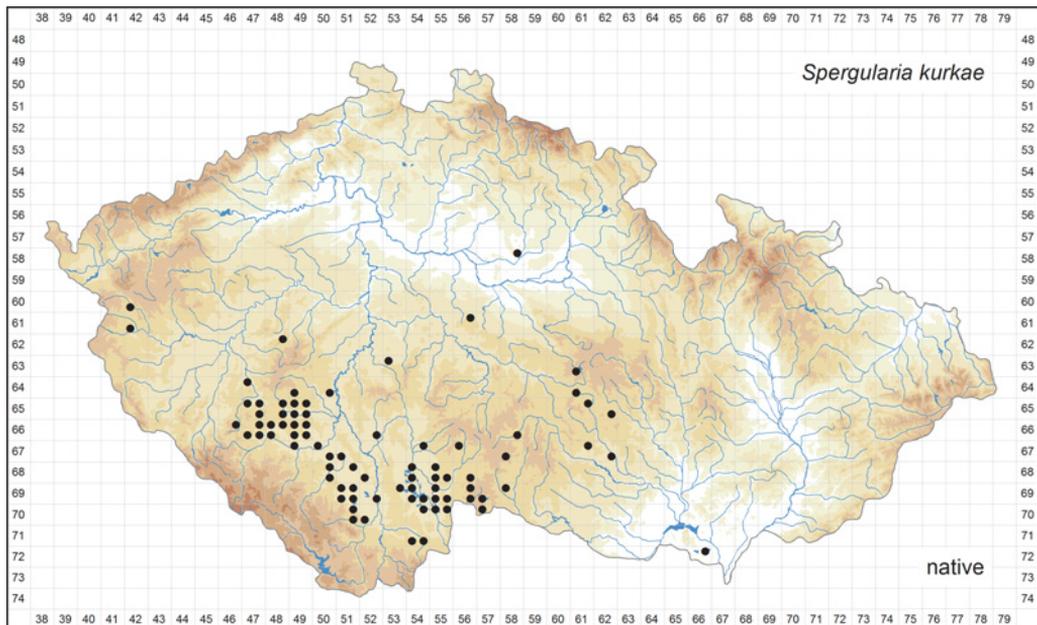
Fig. 72. – Distribution of *Spergularia kurkae* in the Czech Republic (80 occupied quadrants). Prepared by Pavel Kúr.

being more common in the areas where halophilous vegetation once used to be present. It was collected also elsewhere throughout the country, usually in secondary habitats, probably due to long-distance dispersal by waterfowl. Despite its colonization ability, it has generally declined and is therefore considered endangered (Grulich 2012).

Sisyrinchium montanum (Fig. 70)

Sisyrinchium montanum is native to North America but has been introduced to and locally naturalized in Europe (Hultén 1958). Grown as a garden ornamental, it used to escape to wet disturbed grassy places, wet depressions, ditches and waysides. In the Czech Republic it was first reported as escaped in the village of Boršov nad Vltavou near the city of České Budějovice in southern Bohemia in 1853. Since then, it rather occasionally escaped elsewhere. However, many occurrences were short-term as plants suffer from competition by grass species. It has become widely naturalized only in the Hostýnské vrchy Mts (first collected there at the beginning of the 20th century, scattered till now) in eastern Moravia. Less frequently it escaped as a casual in the surroundings of the city of Hradec Králové in eastern Bohemia (for the first time at the beginning of the 20th century, nowadays very rare) and in the vicinity of České Budějovice and the town of Veselí nad Lužnicí in southern Bohemia (nowadays very rare). It occurs from the planar to montane vegetation belts, with altitudinal maximum at 870 m.

Spergularia echinosperma (Fig. 71)

Spergularia echinosperma is a central-European endemic (Friedrich 1979, Dvořák 1990). A recent critical revision of herbarium collections (Kúr et al., in prep.) has confirmed its presence in the Czech Republic, Germany, Austria and Slovakia only. It is confined to vegetation of annual wetland herbs on periodically exposed bottoms of freshwater reservoirs. The primary habitat of *S. echinosperma* includes alluvial pools and sandy banks of rivers; the species, however, most frequently occurs in secondary habitats, mainly exposed bottoms of fishponds (Friedrich 1979, Dvořák 1990). In the Czech Republic *S. echinosperma* is most frequent in areas with many fishponds, i.e. southern, south-western and eastern Bohemia. The species prefers pond bottoms with lower trophic levels and a sandy substrate, which may be covered with a thin layer of mineral mud (Kúr et al., in prep.). It is currently threatened by the intensification of fishpond management and is classified as endangered (Grulich 2012). *Spergularia echinosperma* has unresolved taxonomy, and it probably comprises two intraspecific taxa. A taxonomic study of this species, employing molecular markers (Kúr et al. 2014), is currently in progress (Kúr et al., in prep.). Because of frequent misidentifications, the distribution map was based solely on revised herbarium specimens and our own field records.

Spergularia kurkae (Fig. 72)

Spergularia kurkae is a newly recognized species, which was described by Dvořák (1989) as a hybrid between *S. echinosperma* and *S. rubra* but has not been listed in any flora or checklist except for the Flora of the Czech Republic (Dvořák 1990) since then. Recent studies have proved that *S. kurkae*, although truly being of hybrid origin, is a stabilized, morphologically and ecologically well-separated species (Kúr et al. 2012, Kúr et

al., in prep.). The species occurs mainly in central Europe (Czech Republic, Germany and Austria), although outposts in Switzerland and France possibly exist (the taxonomic identity of these plants needs to be further investigated; Kúr et al., in prep.). It is confined to vegetation of annual wetland herbs on periodically exposed bottoms of freshwater reservoirs. The typical habitats of the species are alluvial pools, river banks, and, above all, fishponds and fish storage ponds. In the Czech Republic *S. kurkae* is most frequent in areas with many fishponds, i.e. southern, south-western and eastern Bohemia. The species has a wider ecological niche than *S. echinosperma* and can very rarely and for a short time survive outside pond bottoms (e.g. in pond sediment deposits). Its current threat level is unknown; herbarium records show that it is approximately twice as common as *S. echinosperma*. Because of frequent misidentifications, the distribution map was based solely on revised herbarium specimens and our own field records.

Spergularia marina (Fig. 73)

Spergularia marina is a nearly cosmopolitan halophilous species occurring in coastal and inland salt marshes of Europe, Asia, northern and southern Africa, North and South America, Australia and New Zealand (Hultén & Fries 1986, Meusel & Jäger 1992, Monnier & Ratter 1993, Hartman & Rabeler 2005, Adams et al. 2008). It is not clear in which parts of its distribution range the species is indigenous and where it has been introduced. In the Czech Republic *S. marina* used to grow relatively frequently in natural saline habitats in north-western Bohemia and southern Moravia. An isolated occurrence was around mineral springs in the Soos National Nature Reserve in western Bohemia. Since World War II the species has declined considerably as a result of habitat destruction and changes in landscape management. Today it survives at a few localities only (two sites in north-western Bohemia and about ten sites in southern Moravia). However, the species has been recently found to be rapidly spreading along roads that are treated by de-icing salts during the winter. In Austria and Germany the spread of *S. marina* on road verges has been known since the 1970s (Friedrich 1979, Hohla & Melzer 2003, Hetzel 2006). In the Czech Republic the species occurs most frequently along motorways, especially in colder areas where the application of de-icing salts is more intense, and is rare in warm and dry areas. There is also a noticeable decreasing gradient in the species' abundance from the west of the country to the east. The indigenous populations are currently classified as critically threatened (Grulich 2012). Because of frequent misidentifications, the distribution map was based solely on revised herbarium specimens and our own field records.

Spergularia media (Fig. 74)

Spergularia media is an obligate halophyte native to coastal and inland salt marshes of Eurasia and North Africa. It has been introduced to North and South America, Australia, New Zealand and southern Africa (Hultén & Fries 1986, Meusel & Jäger 1992, Monnier & Ratter 1993, Hartman & Rabeler 2005, Adams et al. 2008). In the Czech Republic *S. media* used to grow naturally in saline habitats in north-western Bohemia (three localities only) and southern Moravia (a few dozens of localities). It was also introduced to the ore yard of the ironworks in Polanka nad Odrou, north-eastern Moravia, in the 1960s (Kilián & Krkavec 1962; misidentified as *S. salina*). Since World War II the species has declined dramatically as a result of habitat destruction and changes in landscape management.
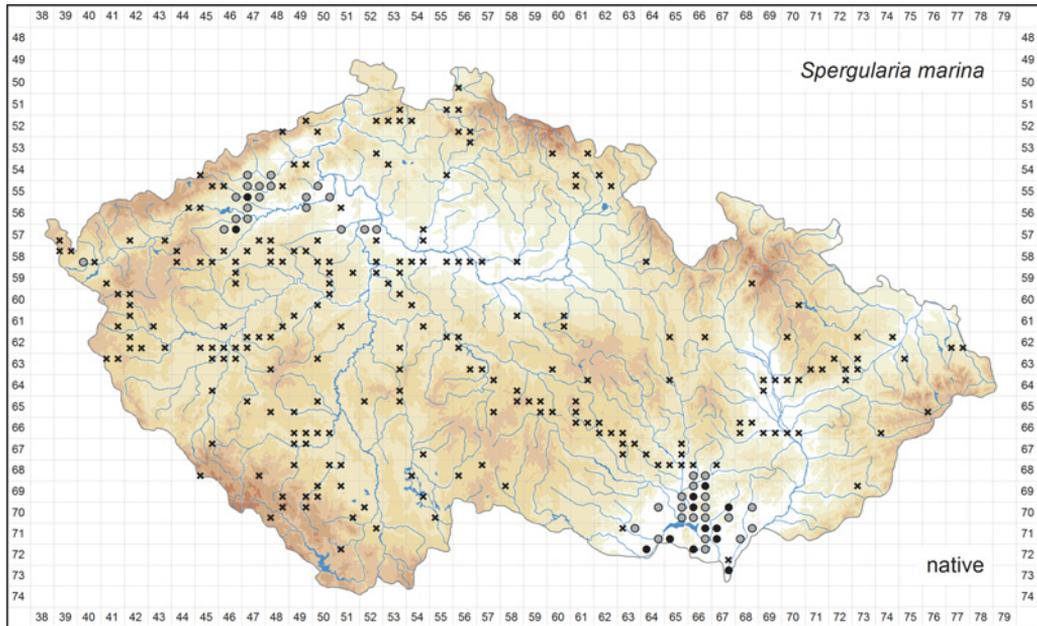
Fig. 73. – Distribution of *Spergularia marina* in the Czech Republic: ● native, at least one record in 2000–2016 (13 quadrants), ◉ native, pre 2000 records only (38 quadrants), ✖ alien (224 quadrants). Prepared by Michal Ducháček & Pavel Kúr.
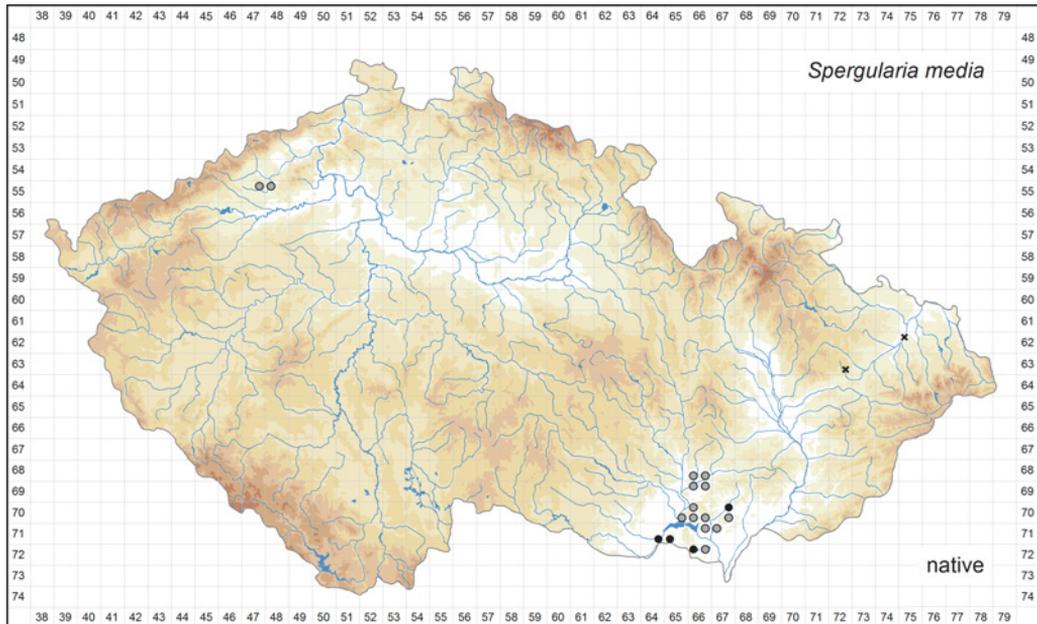
Fig. 74. – Distribution of *Spergularia media* in the Czech Republic: ● native, at least one record in 2000–2016 (4 quadrants), ◉ native, pre 2000 records only (14 quadrants), ✖ alien (2 quadrants). Prepared by Michal Ducháček & Pavel Kúr.
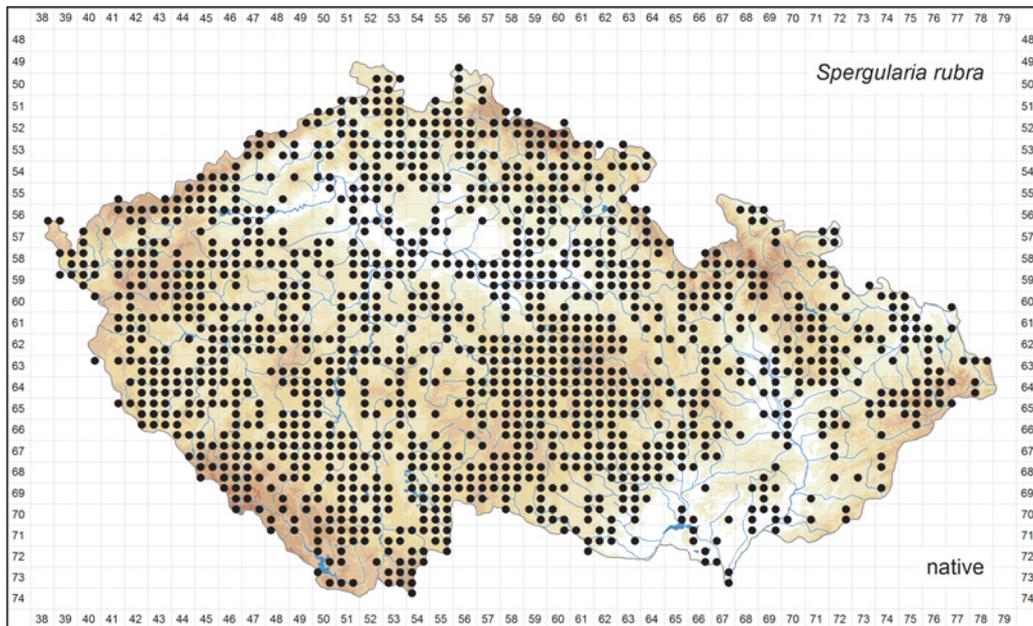
Fig. 75. – Distribution of *Spergularia rubra* in the Czech Republic (1395 occupied quadrants). Prepared by Pavel Kúr & Michal Ducháček.
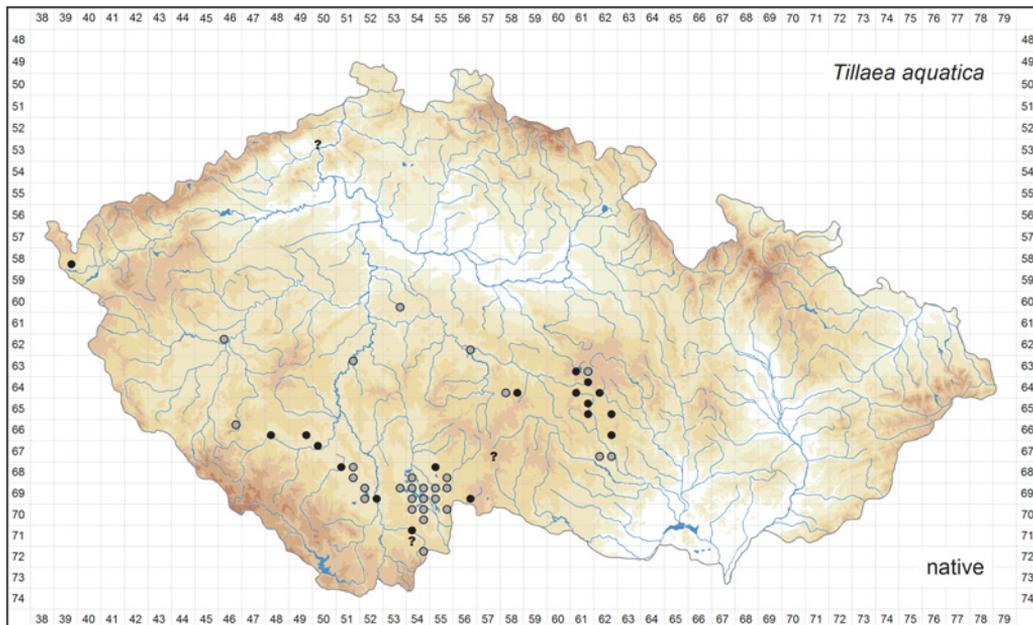

Fig. 76. – Distribution of *Tillaea aquatica* in the Czech Republic: ● at least one record in 2000–2016 (18 quadrants), ◉ pre 2000 records only (28 quadrants). Prepared by Kateřina Šumberová, Jan Prančl & Michal Ducháček.

Today it survives at four localities in southern Moravia only. *Spergularia media* has also been recently found at three sites on motorway verges (motorways D1 in north-eastern Moravia and D2 in southern Moravia). The species has been known from this type of habitat from Austria too (Hohla & Melzer 2003, Adler et al. 2008, Fischer et al. 2008), but its establishment and spread along road verges is slow as it is adapted to less disturbed habitats (Scott & Davison 1982). The species is currently classified as critically threatened (Grulich 2012). Because of frequent misidentifications, the distribution map was based solely on revised herbarium specimens and our own field records.

Spergularia rubra (Fig. 75)

Spergularia rubra is a cosmopolitan species native to Eurasia and introduced to North and South America, southern Africa, Australia and New Zealand (Hultén & Fries 1986, Monnier & Ratter 1993, Hartman & Rabeler 2005, Adams et al. 2008). Its assumed primary habitats are river banks and alluvial pools, but it has successfully spread to various types of secondary habitats. It prefers disturbed sandy sites, like footpaths, field margins or road verges, avoiding calcareous soils (Friedrich 1979, Hartman & Rabeler 2005). In the Czech Republic *S. rubra* is widespread throughout the country. Most of the gaps in the distribution map are due to under-recording but some may be true absences caused by the lack of suitable habitats or the dominance of base-rich and heavy soils.

Tillaea aquatica (Fig. 76)

Tillaea aquatica has a sub-oceanic circumpolar distribution. In Europe its localities are concentrated to Scandinavia with northernmost occurrences along the Arctic Circle, south- and eastwards it is scattered throughout western and central Europe and European Russia (Hultén & Fries 1986); in some parts of its range the species has vanished during the last decades (Šumberová et al. 2012a). Outside Europe, the species occurs in North America and eastern Asia (Hultén & Fries 1986). The species' distribution pattern shows its preference for acidic, non-calcareous substrates and a relatively cold and moist climate. *Tillaea aquatica* is a low-growing, competitively weak wetland annual. It is a typical component of the vegetation of temporarily exposed pond bottoms, growing on sandy or loamy littorals and bottoms of fishponds, fish storage ponds, water reservoirs and rarely also in river beds. The development of the plants is slow and therefore *T. aquatica* does not produce its first ripe seeds until some four months after the spring germination (Šumberová et al. 2012a). Its localities in the Czech Republic are at the southern limit of the species' distribution range. It has been recorded at several dozens of localities, particularly in fishpond landscapes of southern Bohemia and in the Českomoravská vrchovina highlands. Isolated records are known also from western and central Bohemia. Since the 1960s *Tillaea aquatica* vanished from majority of its former sites (Grulich 1985, Šumberová et al. 2012a). The restriction of the length of summer drainage and the long intervals between them, not enabling reproduction of the species in most of the fishponds, had probably the major impact. High amounts of fertilisers and lime applied in fishponds supported tall-growing species, which outcompeted *T. aquatica*. Recently, the species has been found in fishponds used for low-intensity fish farming and in fish storage ponds with long summer drainage and vegetation grazed by sheep or eliminated by glyphosate herbicides, i.e. the management selectively favouring *T. aquatica* (Šumberová et al.

2012a). Although about 20 populations have been recorded during the last two decades, only some of them have a chance of a long-time survival. Consequently, *Tillaea aquatica* is classified as critically threatened (Grulich 2012). Because of rather frequent misidentifications, including even specimens of *Callitriche* and *Elatine*, the map is based mainly on revised herbarium specimens, supplemented by our own finds and reliable literature records. Several populations in abandoned sand pits around the town of Třeboň in southern Bohemia have originated from rescue cultivations (A. Kučerová, in litt.) and they were not included into the map.

Veratrum album subsp. *album* (Fig. 77)

Veratrum album subsp. *album* is distributed in European mountains and on adjacent foothills from the Iberian Peninsula through the Alps and Carpathians to the central part of the Balkan Peninsula, northwards reaching southern Bohemia in the Czech Republic, north-eastern Austria and south-eastern Poland. In the Czech Republic, it is confined to the Novohradské hory Mts, southern part of the Šumava Mts and their foothills in southernmost Bohemia. It is a good example of biogeographical links between the Eastern Alps and the mountain ranges forming the southern border of Bohemia (Kaplan 2012). *Veratrum album* subsp. *album* occurs in hygrophilous forests, springs, fen meadows and pastures at altitudes about 530–1300 m. Its populations are stable and not threatened, individual plants can even survive in a vegetative state in spruce plantations. Because of its rarity, it is classified as endangered (Grulich 2012).

Veratrum album subsp. *lobelianum* (Fig. 78)

Veratrum album subsp. *lobelianum* is distributed in south-western, central and eastern Europe, reaching central France in the west, Poland and the coast of the Arctic Ocean in the north, central Italy and northern Greece in the south and the Ural Mts in the east. Outside Europe the range continues eastwards through Siberia as far as the Russian Far East and Japan, and southwards to the Tian Shan Mts, China and Mongolia (Meusel et al. 1965). In the Czech Republic *V. album* subsp. *lobelianum* occurs in subalpine grasslands and shrub communities, tall fern stands, springs, wet meadows and pastures, and deciduous and spruce forests. It is scattered to locally common over mountains in northern and north-eastern Bohemia, Silesia, and northern and north-eastern Moravia. Most populations are found in the subalpine and montane belts, with an altitudinal maximum at 1535 m. In northern and north-eastern Bohemia, it has locally spread into lower altitudes, especially in the understory of forests along rivers (mainly the Jizera river), reaching elevations of ca 330 m. In contrast, in eastern Bohemia, Moravia and Silesia it reaches areas more distant from mountain ridges, and lower elevations, the latter especially in the Morava river basin (near the town of Litovel, ca 230 m a.s.l.) and close to Czech-Polish border (near the town of Vidnava, ca 240 m). *Veratrum album* subsp. *lobelianum* is classified as lower risk – near threatened (Grulich 2012).
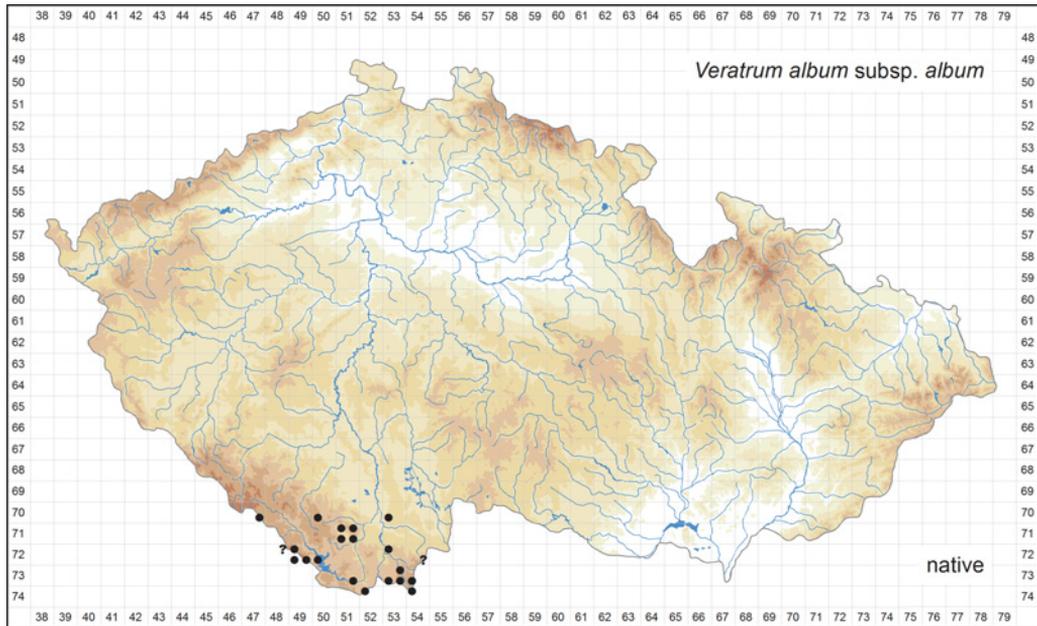
Fig. 77. – Distribution of *Veratrum album* subsp. *album* in the Czech Republic (19 occupied quadrants). Prepared by Jindřich Chrtek Jr.
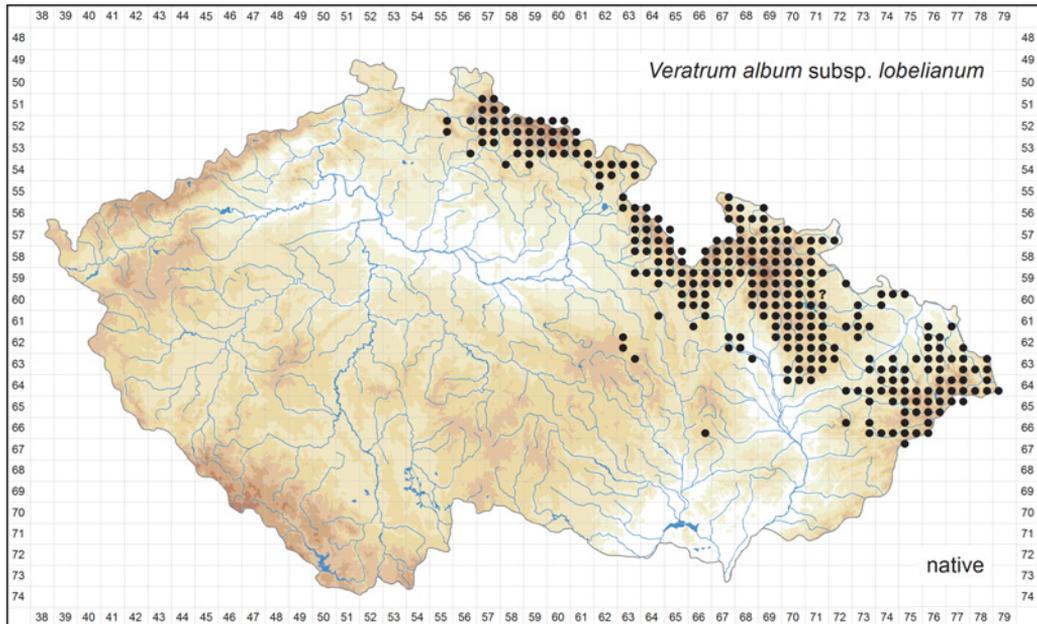
Fig. 78. – Distribution of *Veratrum album* subsp. *lobelianum* in the Czech Republic (288 occupied quadrants). Prepared by Jindřich Chrtek Jr.
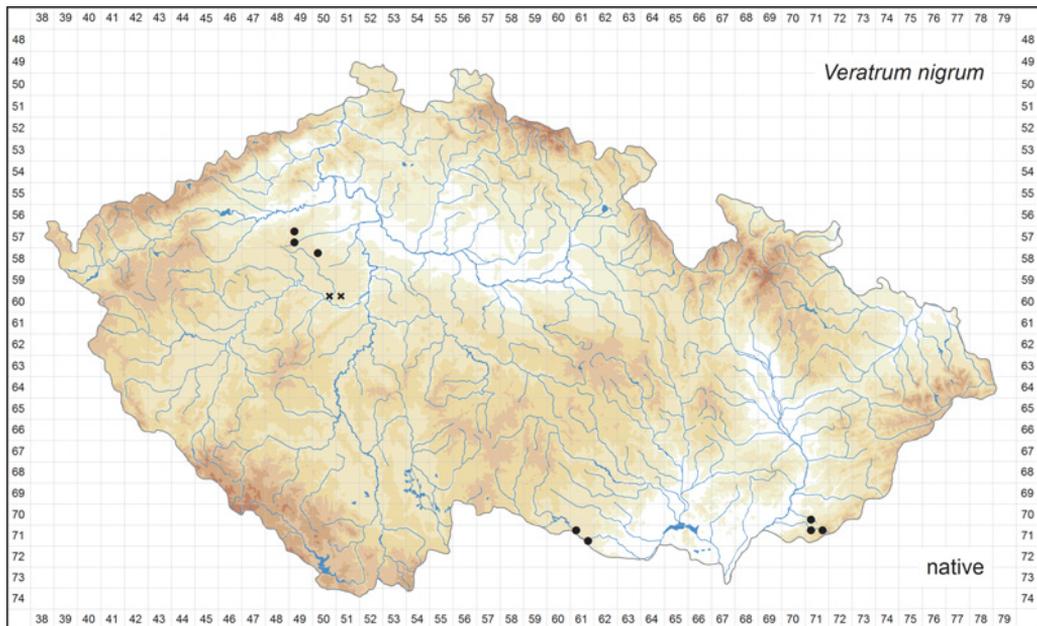
Fig. 79. – Distribution of *Veratrum nigrum* in the Czech Republic: ● native (8 quadrants), ✕ alien (2 quadrants). Prepared by Jindřich Chrtek Jr.
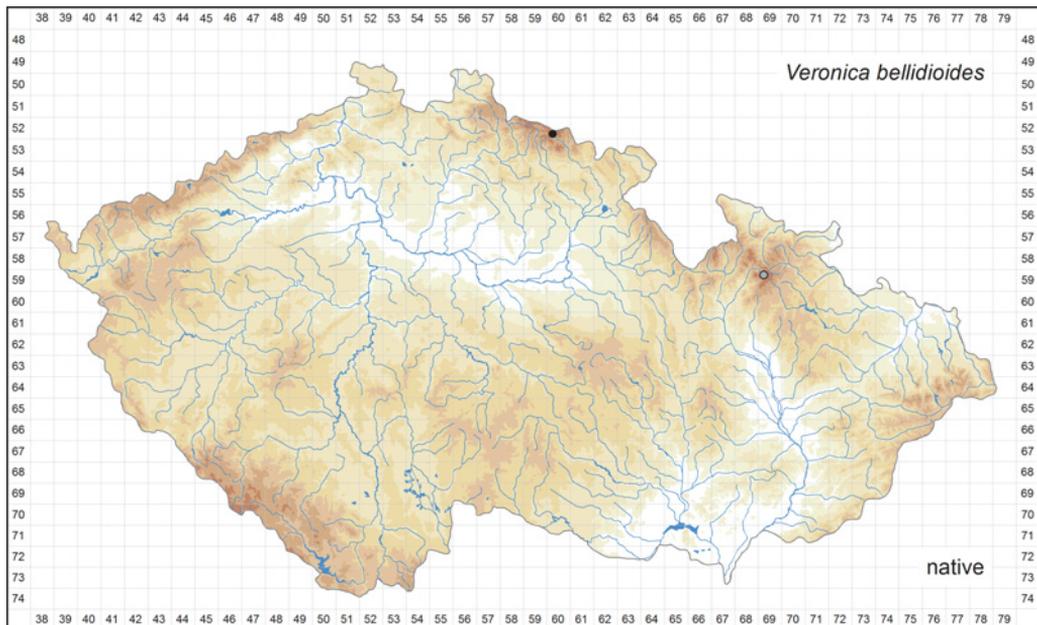
Fig. 80. – Distribution of *Veronica bellidioides* in the Czech Republic: ● at least one record in 2000–2016 (1 quadrant), ◉ pre 2000 records only (1 quadrant). Prepared by Jiří Danihelka.

Veratrum nigrum (Fig. 79)

Veratrum nigrum is a Eurasian species with disjunct European range reaching southern France in the west, Austria, the Czech Republic and southern Poland in the north, central Italy and northern Greece in the south and the Volga river in the east (Niklfeld 1971). The more or less continuous Asian part of the range includes Kazakhstan, southern Siberia (zone of hemiboreal forests), the Amur river basin, Mongolia and China (Nosova 1965). In central Europe it is considered to be a relict from earlier periods of the Holocene (Hájková et al. 2011, Roleček et al. 2015). In the Czech Republic it is found in open-canopy forests, clearings, semi-dry and mesophilous meadows at altitudes about 190–510 m. Its disjunct range in the country is split into three small parts; the first one is located in forests west of the village of Bílichov and near the town of Kladno in central Bohemia, the second one in the narrow valley of the Dyje river along the border between Moravia and Austria west of the town of Znojmo, and the third one in the vicinity of the town of Velká nad Veličkou in the south-western part of the Bílé Karpaty Mts. It was deliberately planted and still survives at Velká hora hill near Beroun in central Bohemia. *Veratrum nigrum* has not declined and does not seem endangered; closing of forest canopy affects flowering and the plants then survive as basal rosettes. Still, due to its rarity it is classified as critically threatened (Grulich 2012).

Veronica bellidioides (Fig. 80)

Veronica bellidioides is distributed in the high mountains of Europe, including the Pyrenees, Alps, Sudetes in the Czech Republic, Eastern Carpathians and the mountain systems of the Balkan Peninsula (Küpfer 1974). The localities in the Krkonoše Mts and the Hrubý Jeseník Mts are situated at the northern edge of the species' distribution range. In the Krkonoše Mts the species was discovered on the summit of Mt Sněžka as early as 1786 by Th. Haenke and repeatedly collected for herbaria, including two exsiccate collections, since then. It grows in species-poor alpine grasslands dominated by *Juncus trifidus*, *Festuca supina* and *Avenella flexuosa*. In 2001–2004, 150–200 plants were counted, growing in four patches (Chrtek et al. 2007). The other population existed in the Velká kotlina glacier cirque in the Hrubý Jeseník Mts. The plants were discovered in 1838 by J. Spatzier and once again collected by the same author 10 years later; the last observation may be that by F. Kolenati from summer 1859 (Kolenati 1860). Heinrich Grabowski (Grabowski 1843), who saw the plants there in summer 1839, reported *V. bellidioides* growing "among *Plantago montana*". It may be therefore assumed that the plants grew there on a rocky slope in a species-rich alpine grassland together with *Helianthemum grandiflorum*, *Poa alpina*, *Plantago atrata* subsp. *sudetica* and *Thymus pulcherrimus* (Bureš 2013). The species is now classified as critically endangered (Grulich 2012).

Veronica filiformis (Fig. 81)

Veronica filiformis is native to the Caucasus Mts and north-eastern Anatolia. It was first found in continental Europe in 1893 near Marseille in France as "plants being packed around the roots of vine shoots imported from Georgia" and since then repeatedly introduced for ornamental purposes because of its small but showy flowers. First records of escaped plants in western and central Europe date back to the period 1901–1930. Nowadays

it is fully naturalized in north-western, northern and central Europe, all areas with Atlantic and Subatlantic climates (Scalone & Albach 2012). It is now naturalized also in the east and west of North America (USDA, NRCS 2016) and New Zealand (Webb et al. 1988). In the Czech Republic the cultivation of *V. filiformis* in plant nurseries has been documented from 1930 onwards; the first escaped plants were recorded in the Lednice chateau park in southern Moravia as early as in 1938 (still present there) as well as in Prague and in the town of Smiřice in eastern Bohemia in 1941 (Jehlík 1998). In its secondary distribution area in Europe, *V. filiformis* is a strictly clonal plant, propagating only vegetatively because of strong self-incompatibility, which is combined with the presence of a limited number of clones. In already colonized sites it spreads by intense growth and rooting from almost all nodes of its creeping stems. If the stem is cut by a mowing machine or torn to pieces by raking, each of fragments produces adventitious roots from all its nodes within three days under standard conditions (Scalone & Albach 2012). These propagules are transported with garden waste or in streams and easily colonize new sites. It is also spread by deliberate planting. In the Czech Republic *V. filiformis* usually occurs in lawns and frequently mown meadows in settlements, gardens, chateau parks, around weekend houses and in alluvial meadows. It prefers humid soils rich in humus and nutrients. The current distribution of *V. filiformis* in the Czech Republic reflects its affinity to the Atlantic climate. It occurs mainly at middle elevations, less frequently in the mountains and lowlands. Its presence in warm and dry parts of the country is limited to floodplain meadows and sites with suitable mesoclimate. *Veronica filiformis* is currently classified as a naturalized neophyte (Pyšek et al. 2012b).

Veronica montana (Fig. 82)

Veronica montana is distributed mainly in the British Isles, western and central Europe, and the Carpathians, with some outposts in southern Scandinavia, the Iberian Peninsula, north Africa, the Apennine and the Balkan Peninsula, where it usually occurs in the mountains. The eastern distribution limit runs through the Baltic countries, Belorussia and Ukraine. Its general distribution is very similar to that of *Fagus sylvatica* (Meusel et al. 1978). In the Czech Republic it occurs usually in beech and beech-fir forests, alder and common ash floodplain forests, forest springs and in shady places on streamsides; rarely it is also found in hardwood floodplain forests. It requires humid to moist, neutral to slightly acidic loamy soils, usually developed over acidic to slightly alkaline bedrock. In Bohemia *V. montana* is distributed mainly in the mountain ranges along the country's border and also in highlands such as the Brdy hills and the Českomoravská vrchovina highlands. It is almost continuously distributed in northern Moravia and adjacent Silesia, as well as in the Carpathians. The occurrence in the floodplain forests of southernmost Moravia south of the town of Břeclav may be explained by propagule transport from the Carpathians during major floods. The species is found at altitudes from 151 m at the confluence of the Morava and Dyje rivers up to 1290 m in the Hrubý Jeseník Mts. The distribution map clearly demonstrates the species' affinity to areas with humid and moderately warm to cold climate, which is correlated with middle and high elevations. *Veronica montana* is easily identified; still, some of literature records may be wrong, based on misidentifications of vegetative shoots of *V. chamaedrys* and *Galeobdolon* sp. The species is classified as lower risk – near threatened (Grulich 2012).
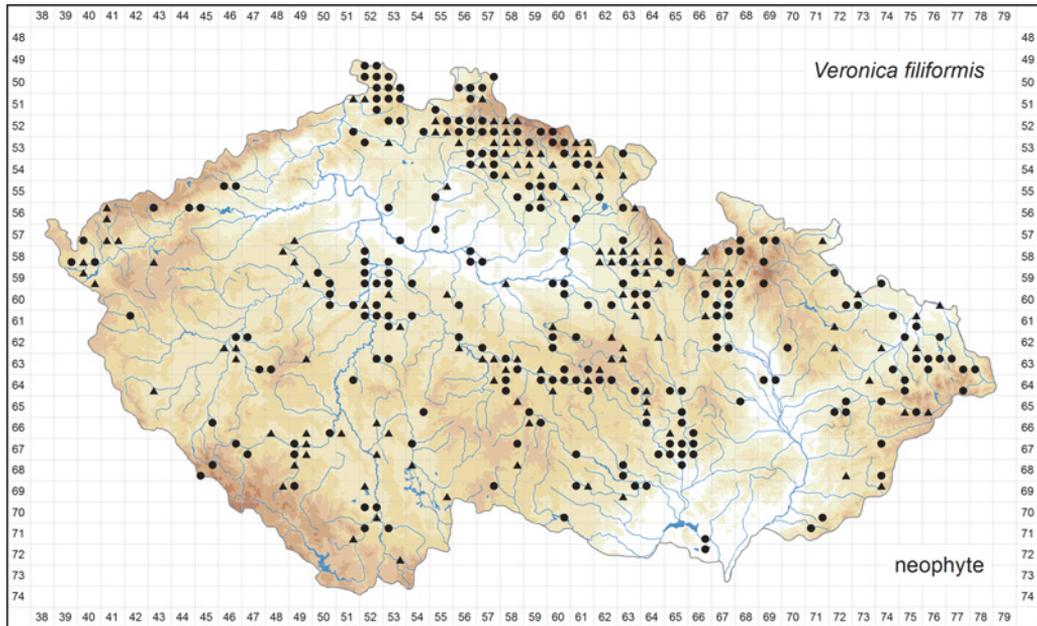
Fig. 81. – Distribution of *Veronica filiformis* in the Czech Republic: ● occurrence documented by herbarium specimens (227 quadrants), ▲ occurrence based on other records (132 quadrants). Prepared by Jiří Danihelka.
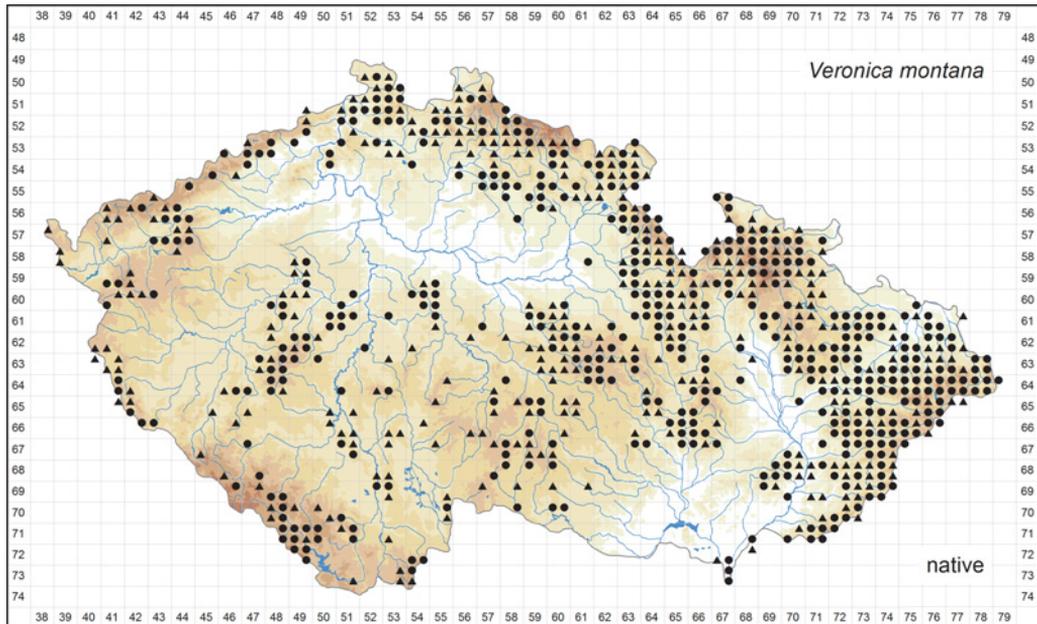
Fig. 82. – Distribution of *Veronica montana* in the Czech Republic: ● occurrence documented by herbarium specimens (448 quadrants), ▲ occurrence based on other records (334 quadrants). Prepared by Jiří Danihelka.
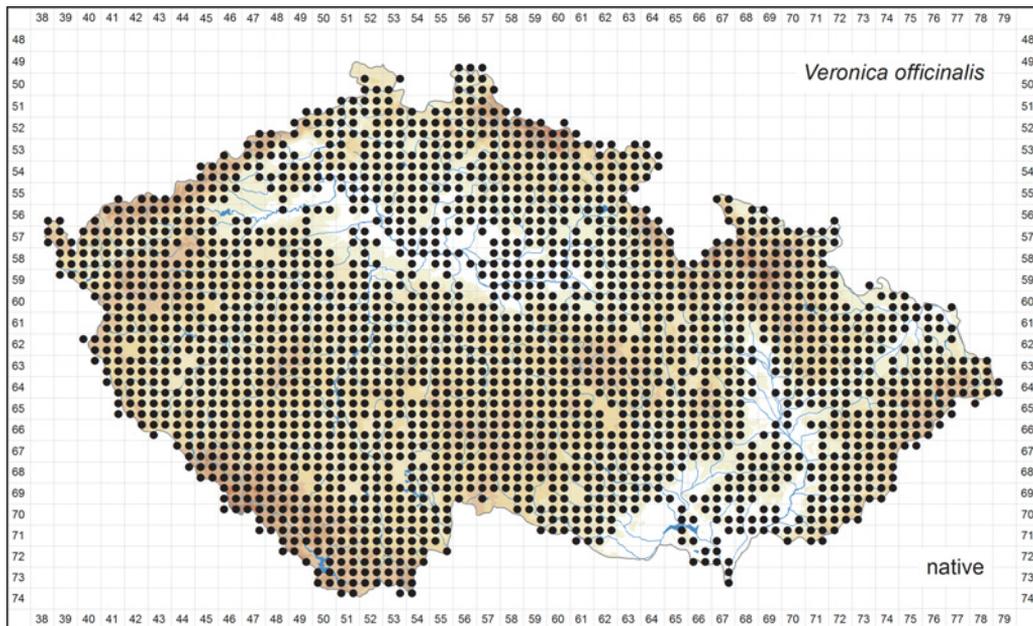
Fig. 83. – Distribution of *Veronica officinalis* in the Czech Republic (2180 occupied quadrants). Prepared by Jiří Danihelka.
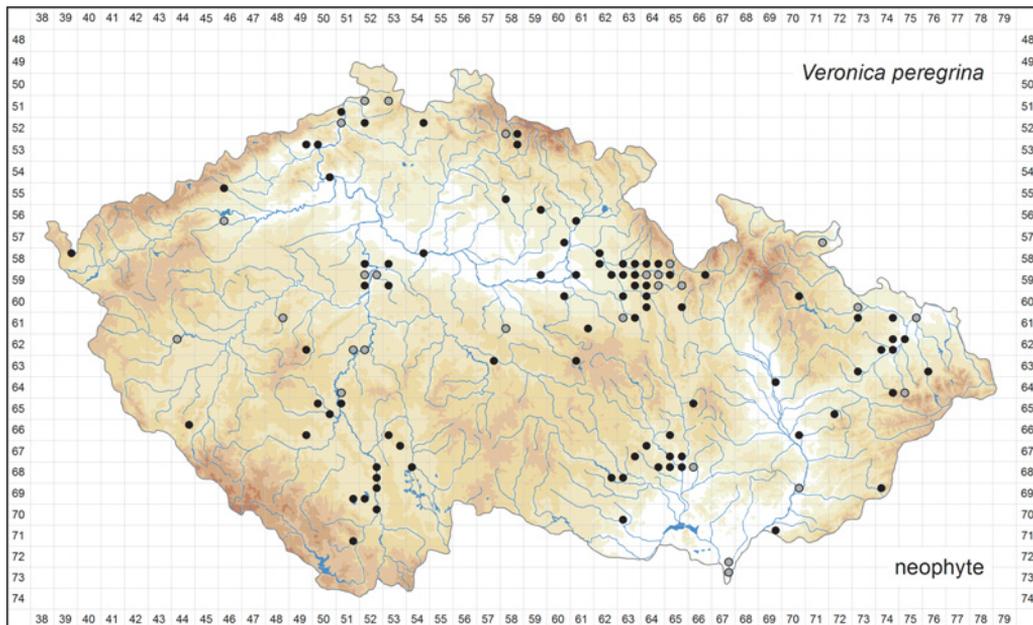
Fig. 84. – Distribution of *Veronica peregrina* in the Czech Republic: ● at least one record in 2000–2016 (86 quadrants), ◉ pre 2000 records only (27 quadrants). Prepared by Jiří Danihelka.

Veronica officinalis (Fig. 83)

Veronica officinalis is a European species with an outpost in the Caucasus Mts, missing only from the northernmost part of the European continent and southern parts of the Mediterranean area (Meusel et al. 1978). It has become naturalized in North America (USDA, NRCS 2016). The species varies in growth habit, indumentum and flower colour but this variation is of little taxonomic value, considerable part of it being attributable to habitat modifications. In the Czech Republic *V. officinalis* grows in broad-leaved and coniferous forests with open canopies, forest clearings, along forest roads and paths, in acidophilous dry grasslands, heathlands, dry and mountain meadows, usually on acidic soils poor in nutrients and developed above siliceous bedrock. If it occurs above carbonate-rich bedrock, then the soil is deep and decalcified in the upper horizons. *Veronica officinalis* is widespread in the Czech Republic, missing only from areas with prevailing arable land, over carbonate-rich bedrock and with very nutrient-rich and wet soils. It occurs from the lowlands up to 1450 m a.s.l. in the Krkonoše Mts.

Veronica peregrina (Fig. 84)

Veronica peregrina is a species native to the mountains of Central and South America (Fischer 2008), in the 17th century introduced to Europe. Nowadays it is naturalized in most countries of western and central Europe (Meusel et al. 1978), and in China, Japan, Korea and Mongolia (Hong & Fischer 1998). The earliest records from central Europe date back to the 19th century, with the very first one in Alsace, France, from 1825. The year of the first record in Bohemia is uncertain. This species was already recorded as occurring in Prague by Schmidt (1789); however, no herbarium specimen is available for this record. Reliable records were published by Čelakovský (1871) and the earliest discovered herbarium specimen was collected in 1882 in the old botanical garden in Prague. Both glabrous and glandular plants have been collected in the Czech Republic, the latter, usually referred to as *V. p.* subsp. *xalapensis*, being far less frequent. The main source of *V. peregrina* in the Czech Republic are plant nurseries and garden centres where it occurs as a weed in irrigated cultivation beds. From there it is transported with plants to private and public gardens, city and chateau parks, cemeteries and flowerbeds in settlements. It occurs also as weed in humid waste places, ruderal vegetation and in field margins. In the 1970s it was for the first time collected on the shores of the Slapy water reservoir, which is the habitat type known from other countries. Since then, the number of records from the shores of water reservoirs, banks of rivers and exposed fishpond bottoms has been steadily growing, and the species became a locally permanent part of their flora. The map suggests that there are some local plant nurseries acting as source of propagules, such as in easternmost Bohemia, but the pattern may be also strongly influenced by varied recording efforts.

*Veronica pumila* (Fig. 85)

Veronica pumila is very closely related to *V. alpina*, and by some authors it is even not recognized at any rank as a separate taxon (Albach et al. 2006; but see Elven 2016). *Veronica alpina* s. l. has a large disjunct distribution range including Iceland, northern Scandinavia, northern European Russia, high mountain ranges of central and southern Europe, mountains of southern Siberia, as well as Labrador and Greenland in North

America (Meusel et al. 1978, Albach et al. 2006, Elven 2016). Based on morphological characters, the central European populations are assigned to *V. pumila* (syn. *V. alpina* subp. *pumila*), described from the Western Alps in northern Italy. In the Czech Republic *V. pumila* with certainty occurred in the Krkonoše Mts near the Luční bouda chalet, as documented by herbarium specimens from the 19th century, probably in subalpine grasslands dominated by *Nardus stricta*. The latest specimen seen was collected by J. v. Sterneck in 1901; he noted on the label that the population, consisting of about 40 plants, is protected from "aliens" by the chalet owner. Only 19th century literature records exist from Mt Sněžka, where another small population may have existed. Not seen for more than a century, the species is now classified as extinct (Grulich 2012), but a small population still survives on the Polish side of the mountains above Mały Staw Lake.

Veronica scutellata (Fig. 86)

Veronica scutellata is a circumboreal species distributed in Europe, western and central Siberia, and western and eastern North America. In southern Europe its distribution is restricted to more humid areas, usually at higher altitudes (Meusel et al. 1978). In the Czech Republic it is usually found in fishpond littorals, on shores of sand pits, in marsh vegetation dominated by tall sedges, fen meadows, along ditches and in reed stands, quite often in somewhat disturbed places. It is a heliophilous species of permanently wet or inundated soils with high groundwater level, usually moderately rich in nutrients and slightly acidic. *Veronica scutellata* is widespread in the Czech Republic, being particularly common at middle altitudes, mainly in fishpond landscapes and other areas harbouring wetland vegetation. In contrast, it is absent from dry and warm areas, such as northwest and east of Prague, or from large parts of central and southern Moravia, where it is more or less confined to river floodplains. It is found at elevations from 151 m up to 950 m in the Šumava Mts but it was recorded as introduced at 1100 m or even higher in the Krkonoše Mts. Having somewhat declined mainly because of drainage, it is currently ranked as lower risk – near threatened (Grulich 2012).

Veronica serpyllifolia (Fig. 87)

Veronica serpyllifolia is an almost cosmopolitan species, native to Eurasia, northern Africa, North and South America. It has become naturalized in South Africa, New Zealand and Australia. It has usually been divided in two subspecies, with the type subspecies originally restricted to Eurasia and *V. s.* subsp. *humifusa*, an arctic-montane subspecies, which is a circumpolar plant with a discontinuous distribution range (Meusel et al. 1978, Hultén & Fries 1986). *Veronica serpyllifolia* is widespread all over Europe, being absent only from some parts of the Mediterranean area. In the Czech Republic only the type subspecies is present. *Veronica serpyllifolia* occurs mainly in mesophilous and floodplain meadows, pastures, along paths, in trampled lawns, gardens and backyards in settlements, on fallow land and in forests with open canopy. It requires humid soils, well supplied with nutrients including nitrogen, usually not above carbonate rock. In sufficiently humid areas it is found in sunny places, whereas in rather dry and warm areas it is confined to semi-shaded habitats. Its altitudinal range spans from 151 m to 1603 m at the summit of Mt Sněžka. In the Czech Republic it is a widespread species with an almost continuous distribution in middle and high altitudes, where the gaps indicate under-recording rather
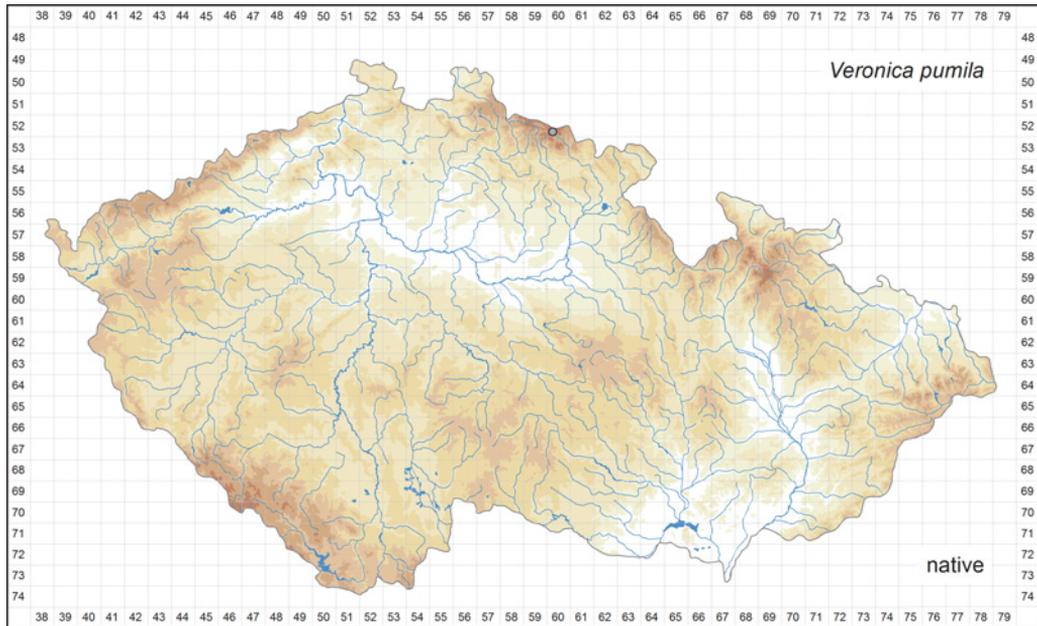
Fig. 85. – Distribution of *Veronica pumila* in the Czech Republic (1 occupied quadrant). Prepared by Jiří Danihelka.
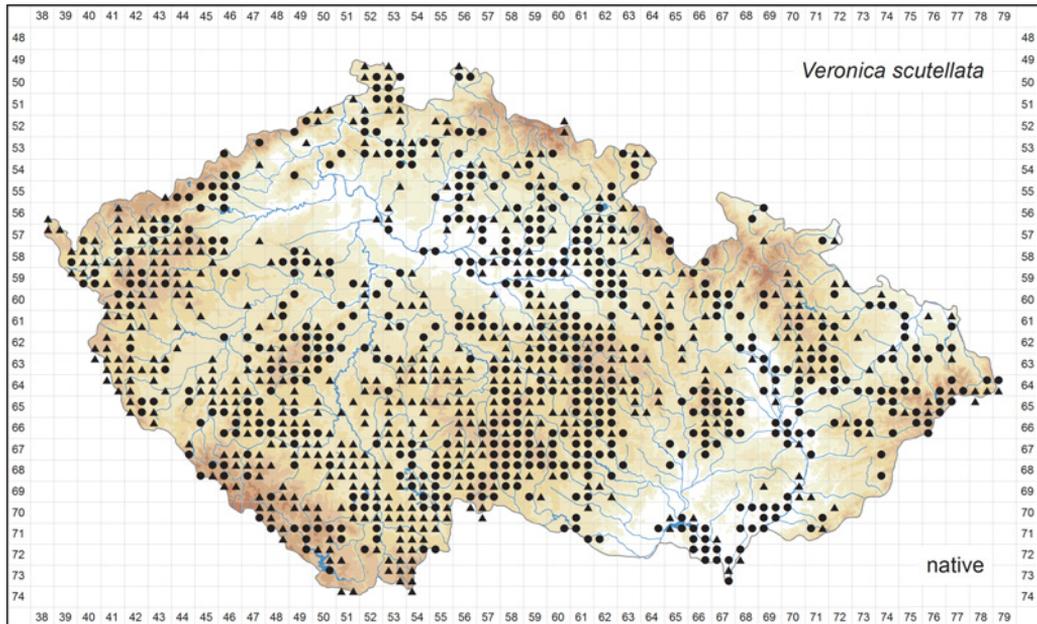
Fig. 86. – Distribution of *Veronica scutellata* in the Czech Republic: ● occurrence documented by herbarium specimens (551 quadrants), ▲ occurrence based on other records (553 quadrants). Prepared by Jiří Danihelka.
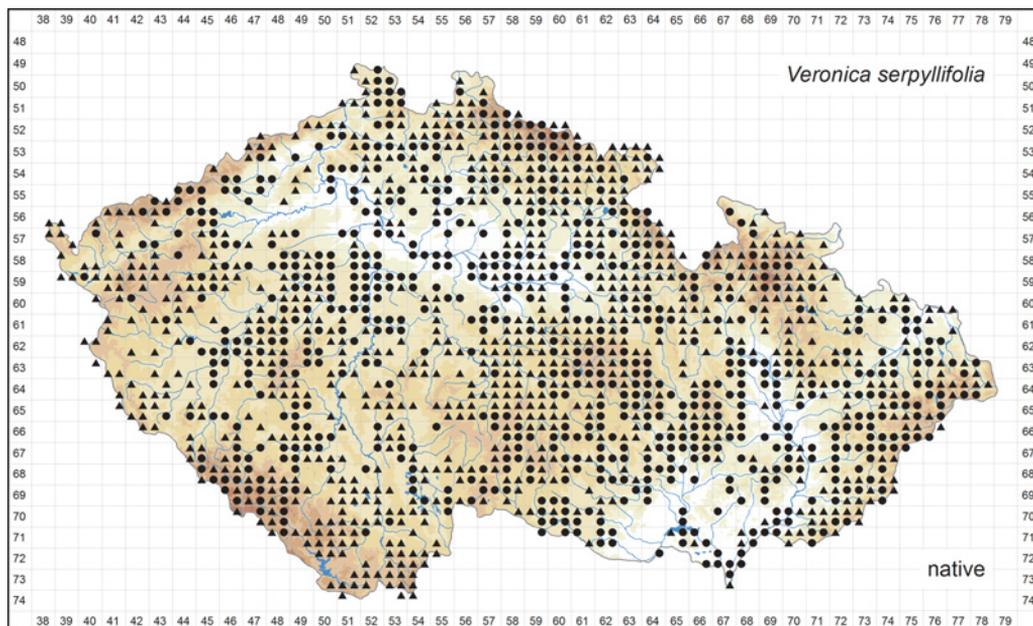
Fig. 87. – Distribution of *Veronica serpyllifolia* in the Czech Republic: ● occurrence documented by herbarium specimens (692 quadrants), ▲ occurrence based on other records (810 quadrants). Prepared by Jiří Danihelka & Michal Ducháček.

than true absences. In contrast, the gaps in warm and dry areas with prevailing arable land in central and north-western Bohemia, and in central and southern Moravia, may be to a large extent true absences.

See www.preslia.cz for Electronic Appendices 1–87

Acknowledgements

Souhrn

Druhá část ze série publikací věnovaných rozšíření cévnatých rostlin v České republice obsahuje síťové mapy a doprovodné komentáře k 87 taxonům z rodů *Antennaria*, *Aposeris*, *Astragalus*, *Avenula*, *Bidens*, *Carex*, *Cenchrus*, *Centunculus*, *Convallaria*, *Crocus*, *Cryptogramma*, *Cyperus*, *Dryopteris*, *Gladiolus*, *Gratiola*, *Helictochloa*, *Hierochloë*, *Lindernia*, *Maianthemum*, *Myriophyllum*, *Notholaena*, *Nymphoides*, *Radiola*, *Schoenoplectus*, *Sisyrinchium*, *Spergularia*, *Tillaea*, *Veratrum* a *Veronica*. Základem jsou údaje získané excerpcí herbářů a literatury, terénní zápisy a nálezy dostupné v databázích, které prověřili taxonomičtí experti. Mnohé taxony patří mezi vzácné nebo ohrožené rostliny a jsou proto zařazeny na Červeném seznamu. Mezi skupiny rostlin zvláště zasažené změnami nebo úplným zničením biotopů patří psamofyty. *Astragalus arenarius*, *Hierochloë odorata* a *H. repens* jsou kriticky ohrožené druhy, které ustoupily zejména v důsledku převodu písčin na ornou půdu, těžbě písku, zalesňování, změnám v obhospodařování krajiny a eutrofizace prostředí následovanou sukcesí. Všechny tři jmenované druhy se dnes vyskytují na malém počtu lokalit a jejich populace jsou většinou velmi chudé. Další skupinou ohroženou kvůli vazbě na specifická stanoviště jsou konkurenčně slabé mokřadní jednoletky, jako jsou *Centunculus minimus*, *Cyperus flavescens*, *C. michelianus*, *Lindernia procumbens*, *Radiola linoides* a *Tillaea aquatica*. Ty se nejčastěji vyskytují na obnažených dnech rybníků nebo řečišti toků, v opuštěných pískovnách a na extenzivně obhospodařovaných vlhkých písčitých polích. Ačkoliv některé byly v minulosti i hojnější, všechny výrazně ustoupily v důsledku intenzifikace hospodaření na rybnících, zejména následkem přihnojování a omezení pravidelného letnění rybníků, a dále v důsledku rozsáhlých změn ve využívání krajiny. Dnes se tyto druhy vyskytují jen na malém počtu posledních lokalit, mnohdy nepravidelně, s delšími periodami absence, a jsou proto řazeny mezi kriticky ohrožené taxony. Článek přináší i první mapu rozšíření středoevropského endemita *Spergularia kurkae*, který byl jako samostatný druh rozlišen teprve nedávno. Dříve vzácné druhy *Astragalus asper*, *Schoenoplectus supinus* a *Veronica pumila* dnes patří mezi taxony na území ČR vyhynulé. Naproti tomu dříve vzácný druh *Spergularia marina*, která se vyskytoval jen na několika přirozených slaniskách, se v důsledku zimního solení silnic rozšířil po většině území ČR. Revize herbářového dokladu ke starému literárnímu údaji o výskytu zavlečeného druhu *Astragalus alopecuroides* ukázala, že se ve skutečnosti jedná o druh, jehož správné jméno je *A. alopecurus*. Mezi další přechodně zavlečené nebo jen lokálně zdomácnělé druhy, jejichž rozšíření je podrobně zpracováno v tomto článku, patří *Bidens pilosus*, *Cenchrus echinatus*, *Gratiola neglecta* a *Lindernia dubia*, které jsou dokumentovány jen z malého počtu lokalit. *Bidens connatus* byl donedávna velmi vzácný, ale v poslední době se začal šířit na nově uvolněná stanoviště po extrémní povodni v roce 2002. Typickým případem zdomácnělých neofytů jsou *Veronica filiformis* a *V. peregrina*, které se již vyskytují na několika až mnoha stovkách lokalit v různých částech ČR. Invazní druhy zastupuje *Bidens frondosus*, který se začal intenzivněji šířit ve 30. letech 20. století a dnes je široce rozšířený a běžný. Celkový obraz rozšíření jednotlivých zpracovávaných taxonů poskytují mapy, konkrétní floristické údaje odrážející odlišné trendy v různých oblastech a v různých obdobích jsou uloženy v databázi Pladias a dostupné v elektronických přílohách. Každou mapu doprovází textový komentář, který obsahuje nástin celkového rozšíření, výčet nejčastějších stanovišť a stručnou charakteristiku rozšíření v České republice, případně i doplňující informace k taxonomii, biologii, změnám v rozšíření a míře ohrožení.

References

Adams L. G., West J. G. & Cowley K. J. (2008): Revision of *Spergularia* (*Caryophyllaceae*) in Australia. – Aust. Syst. Bot. 21: 251–270.

Adler W., Mrkvicka A. Ch. & Fischer M. A. (2008): Nachträge zur „Flora Wiens" (III). – Neilreichia 5: 173–180.

Albach D. C., Schönswetter P. & Tribsch A. (2006): Comparative phylogeography of the *Veronica alpina* complex in Europe and North America. – Mol. Ecol. 15: 3269–3286.

Bayer R. J. (2000): Observations on the morphology and geographic range of *Antennaria dioica* (L.) Gaertn. (*Asteraceae*: *Gnaphalieae*). – Madroño 46: 205–207.

Bílý F. (1931): Druhý příspěvek ku poznání kapradin Moravského krasu [The second contribution to the knowledge of ferns of the Moravian Karst]. – Sborn. Klubu Přírod. Brno 14: 65–77.

Bílý F. (1937): Třetí příspěvek ku poznání kapradin Moravského krasu [The third contribution to the knowledge of ferns of the Moravian Karst]. – Sborn. Klubu Přírod. Brno 19: 22–32.

Bureš L. (2013): Chráněné a ohrožené rostliny chráněné krajinné oblasti Jeseníky [Protected and endangered plants of the Protected Landscape Area Jeseníky]. – Agentura Rubico, Olomouc-Slavonín.

Burkart M. (2001): River corridor plants (Stromtalpflanzen) in Central European lowland: a review of a poorly understood plant distribution pattern. – Global Ecol. Biogeogr. 10: 449–468.

Chater A. O. (1980): *Carex*. – In: Tutin T. G., Heywood V. H., Burges N. A., Moore D. M., Valentine D. H. Walters S. M. & Webb D. A. (eds), Flora Europaea 5: 290–323, Cambridge University Press, Cambridge.

Chen Y.-S. & Hind D. J. N. (2011): Tribe *Heliantheae*. – In: Wu Z. Y., Raven P. H. & Hong D. Y. (eds.), Flora of China 20–21: 852–878, Science Press, Beijing & Missouri Botanical Garden Press, St. Louis.

Chrtek J. & Jirásek V. (1964): Beitrag zur Kenntnis der Veränderlichkeit von *Hierochloë* (L.) Pal.-Beauv. in der Tschechoslowakei. – Preslia 36: 245–250.

Chrtek J., Plačková I., Zahradníková J., Kirschner J., Kirschnerová L., Štěpánek J., Krahulcová A., Krahulec F. & Harčarik J. (2007): Genetická variabilita vybraných horských druhů cévnatých rostlin v Krkonoších [Genetic variation in selected mountain species of vascular plants in the Giant Mountains]. – In: Štursa J. & Knapik R. (eds), Geoekologické problémy Krkonoš, Sborn. Mezin. Věd. Konf., říjen 2006, Svoboda nad Úpou, Opera Corcont. 44: 251–264.

Chytrý M. (2007a): *Euphorbio cyparissiae-Callunetum vulgaris* Schubert 1960. Suchá vřesoviště nížin a pahorkatin [*Euphorbio cyparissiae-Callunetum vulgaris* Schubert 1960. Dry heathlands in lowland and colline landscapes]. – In: Chytrý M. (ed.), Vegetace České republiky 1. Travinná a keříčková vegetace [Vegetation of the Czech Republic 1. Grassland and Heathland Vegetation], p. 308–310, Academia, Praha.

Chytrý M. (2007b): *Festucion valesiacae* Klika 1931. Úzkolisté suché trávníky [*Festucion valesiacae* Klika 1931. Narrow-leaved dry grasslands]. – In: Chytrý M. (ed.), Vegetace České republiky 1. Travinná a keříčková vegetace [Vegetation of the Czech Republic 1. Grassland and Heathland Vegetation], p. 409–425, Academia, Praha.

Chytrý M. & Sádlo J. (2007): *Corynephorion canescentis* Klika 1931. Otevřené trávníky vátých písků s paličkovcem šedavým [*Corynephorion canescentis* Klika 1931. Open vegetation of inland sand dunes dominated by *Corynephorus canescens*]. – In: Chytrý M. (ed.), Vegetace České republiky 1. Travinná a keříčková vegetace. [Vegetation of the Czech Republic. Vol. 1. Grassland and heathland vegetation], p. 323–330, Academia, Praha.

Conert H. J. (ed.) (1998): Gustav Hegi, Illustrierte Flora von Mitteleuropa. Ed. 3. Vol. 1/3. *Spermatophyta*: *Angiospermae*: *Monocotyledones* 1/2. *Poaceae* (Echte Gräser oder Süßgräser). – Parey Buchverlag, Berlin.

Čelakovský L. (1871): Prodromus der Flora von Böhmen. Vol. 2. – Prag.

Čelakovský L. (1875): Prodromus der Flora von Böhmen. Vol. 3. – Prag.

Čížek K. & Král M. (2009): Park v areálu nemocnice v Klatovech [The park of the Klatovy hospital]. – Calluna 14: 7–8.

Danihelka J., Chrtek J. jun. & Kaplan Z. (2012): Checklist of vascular plants of the Czech Republic. – Preslia 84: 647–811.

DeLisle D. G. (1963): Taxonomy and distribution of the genus *Cenchrus*. – Iowa State Coll. J. Sci. 37: 259–351.

Dřevojan P. (2012): Kozinec bezlodyžný (*Astragalus exscapus*) – dosud součástí flóry Znojemska [*Astragalus exscapus* – still a flora element of the Znojmo region, SE Czech Republic]. – Zpr. Čes. Bot. Společ. 47: 291–296.

Dvořák F. (1989): Chromosome counts and chromosome morphology of some selected species. – Scr. Fac. Sci. Natur. Univ. Purkyn. Brun., Biol., 19: 301–322.

Dvořák F. (1990): *Spergularia* (Pers.) J. et C. Presl – kuřinka. – In: Hejný S., Slavík B., Hrouda L. & Skalický V. (eds), Květena České republiky [Flora of the Czech Republic] 2: 81–86, Academia, Praha.

Egorova T. V. (1999): The sedges (*Carex* L.) of Russia and adjacent states (within the limits of the former USSR). – St.-Peterburg State Chemical Pharmaceutical Academy & Missouri Botanical Garden Press, St.-Peterburg & St. Louis.

Ekrt L. (2015): Revize populace podmrvky hadcové (*Notholaena marantae*) na Mohelenské hadcové stepi: rozšíření, početnost, stanovištní preference [Revision of *Notholaena marantae* population on serpentine steppe near Mohelno: distribution, abundance, habitat preferences]. – Acta Rer. Natur. 18: 1–8.

Ekrt L., Lepší M., Boublík K. & Lepší P. (2007): *Dryopteris remota* rediscovered for the flora of the Czech Republic. – Preslia 79: 69–82.

Ekrt L., Linhartová R. & Štech M. (2013): Rozlišování a revize rozšíření kapradě podobné (*Dryopteris expansa*) a jejího křížence *D.* ×*ambroseae* v České republice [Determination and revision of distribution of *Dryopteris expansa* and its hybrid *D.* ×*ambroseae* in the Czech Republic]. – Zpr. Čes. Bot. Společ. 48: 263–291.

Ekrt L., Štech M., Lepší M. & Boublík K. (2010): Rozšíření a taxonomická problematika skupiny *Dryopteris affinis* v České republice [Distribution and taxonomy of the *Dryopteris affinis* group in the Czech Republic]. – Zpr. Čes. Bot. Společ. 45: 25–52.

Ekrt L., Trávníček P., Jarolímová V., Vít P. & Urfus T. (2009): Genome size and morphology of the *Dryopteris affinis* group in Central Europe. – Preslia 81: 261–280.

Elven R. (ed.) (2016): Annotated Checklist of the Panarctic Flora (PAF). Vascular plants. – Natural History Museum, University of Oslo, URL: http://nhm2.uio.no/paf/ (accessed February 2016).

Fischer M. A. (ed.) (2008): Exkursionsflora für Österreich, Liechtenstein und Südtirol. Ed. 3. – OÖ Landesmuseen, Linz.

Formánek E. (1884): Correspondenz. – Oesterr. Bot. Z. 34: 448.

Fraser-Jenkins C. R. (1976): *Dryopteris caucasica* and the cytology of its hybrids. – Fern Gaz. 11: 263–267.

Fraser-Jenkins C. R. (2007): The species and subspecies in the *Dryopteris affinis* group. – Fern Gaz. 18: 1–26.

Friedrich H. C. (1979): Familie *Caryophyllaceae*. – In: Reichinger K. H. (ed.), Gustav Hegi, Illustrierte Flora von Mitteleuropa, ed. 2, vol. 3/2. *Angiospermae*, *Dicotyledones* 1. *Phytolaccaceae*, *Amaranthaceae*, *Chenopodiaceae*, *Illecebraceae*, *Caryophyllaceae*, *Portulacaceae*, p. 763–1182, Verlag Paul Parey, Berlin & Hamburg.

Gibby M. & Walker S. (1977): Further cytogenetic studies and a reappraisal of the diploid ancestry in the *Dryopteris carthusiana* complex. – Brit. Fern Gaz. 11: 313–324.

Grabowski H. (1843): Flora von Ober-Schlesien und dem Gesenke. – Breslau.

Gregor T. (2009): The distribution of *Galeopsis ladanum* in Germany based on an analysis of herbarium material is smaller than that indicated in plant atlases. – Preslia 81: 377–386.

Greuter W. (2006): *Compositae* (pro parte majore). – In: Euro+Med Plantbase – the information resource for Euro-Mediterranean plant diversity. URL: http://ww2.bgbm.org/EuroPlusMed (accessed February 2016).

Grulich V. (1985). Masnice vodní (*Tillaea aquatica* L.) v Československu [*Tillaea aquatica* in Czechoslovakia]. – Sborn. Jihočes. Muz. České Budějovice, Přír. Vědy, 25: 53–56.

Grulich V. (2012): Red List of vascular plants of the Czech Republic: 3rd edition. – Preslia 84: 631–645.

Hadač E. & Hadačová V. (1969): Notes on the ecology and distribution of *Bidens pilosa* L. in Cuba. – Folia Geobot. Phytotax. 4: 165–173.

Hadinec J. & Lustyk P. (eds) (2015): Additamenta ad floram Reipublicae Bohemicae. XIII [Additions to the flora of the Czech Republic. XIII]. – Zpr. Čes. Bot. Společ. 50: 23–129.

Hájková P., Roleček J., Hájek M., Horsák M., Fajmon K., Polák M. & Jamrichová E. (2011): Prehistoric origin of the extremely species-rich semi-dry grasslands in the Bílé Karpaty Mts (Czech Republic and Slovakia). – Preslia 83: 185–204.

Han Y., Cho Y., Kim Y., Lim H., Kwon O. & Nam S-H. (2009): Insect herbivores associated with the introduced weed *Bidens frondosa* L. (*Asteraceae*) in Korea, and their potential role as augmentative biological control agents. – Entomological Research 39: 394–400.

Harpke D., Carta A., Tomović G., Ranđelović V., Ranđelović N., Blattner F. R. & Peruzzi L. (2015): Phylogeny, karyotype evolution and taxonomy of *Crocus* series *Verni* (*Iridaceae*). – Pl. Syst. Evol. 301: 309–325.

Hartl D. (1975): Familie *Scrophulariaceae*. – In: Hartl D. & Wagenitz G. (eds), Gustav Hegi, Illustrierte Flora von Mitteleuropa, ed. 2, vol. 6/1, *Angiospermae*, *Dicotyledones* 4. *Scrophulariaceae*, *Orobanchaceae*, *Lentibulariaceae*, *Globulariaceae*, *Plantaginaceae*, p. 1–469, Verlag Paul Parey, Berlin & Hamburg.

Hartman R. L. & Rabeler K. R. (2005): *Spergularia*. – In: Flora of North America Editorial Committee (eds), Flora of North America North of Mexico 5/2: 16–23, Oxford University Press, New York & Oxford.

Hartvig P. (1987): Chromosome numbers in Nordic populations of the *Carex muricata* group (*Cyperaceae*). – Symb. Bot. Upsal. 27: 127–138.

Hegi G. (1925): Illustrierte Flora von Mittel-Europa. Vol. 6/1. – München.

Hejný S. (1948): Zdomácnění dvouzubce listnatého (*Bidens frondosus* L.) v ČSR [Naturalization of *Bidens frondosus* L. in the Czechoslovak Republic]. – Čs. Bot. Listy 1: 56–63.

Hejný S. (1960): Ökologische Charakteristik der Wasser- und Sumpfpflanzen in den slowakischen Tiefebenen (Donau- und Theissgebiet). – Vydavateľstvo SAV, Bratislava.

Hejný S. (1999): Trojřadka Micheliova (*Dichostylis micheliana*), puštička rozprostřená (*Lindernia procumbens*), šmel okoličnatý (*Butomus umbellatus*) a rybniční soustava Podkrčí u Protivína [*Dichostylis micheliana*, *Lindernia procumbens*, *Butomus umbellatus* and the fishpond system of Podkrčí near Protivín]. – Sborn. Jihočes. Muz. České Budějovice, Přír. Vědy, 39: 47–52.

Hetzel G. (2006): Die Neophyten Oberfrankens. Floristik, Standortcharakteristik, Vergesellschaftung, Verbreitung, Dynamik. – PhD thesis, Universität Würzburg, Fakultät für Biologie, Würzburg.

Hohla M. & Melzer H. (2003): Floristisches von den Autobahnen der Bundesländer Salzburg, Oberösterreich, Niederösterreich und Burgenland. – Linzer Biol. Beitr. 35: 1307–1326.

Holm L. G., Pancho J. V., Herberger J. P. & Plucknett D. L. (1991): A geographic atlas of World weeds. – Krieger Publishing Company, Malabar.

Holub J. (1958): Bemerkungen zur Taxonomie der Gattung *Helictotrichon* Bess. – In: Klášterský I. et al. (eds), Philipp Maxmilian Opiz und seine Bedeutung für die Pflanzentaxonomie, p. 101–133, Praha.

Holub J. (1962): *Helictotrichon desertorum* (Less.) Nevskij – ein bedeutsames Relikt der tschechoslowakischen Flora. – Acta Univ. Carol. – Biol. 2 (1962): 153–188.

Holub J. (1972): A note on the infraspecific classification of *Helictotrichon desertorum* (Less.) Nevskij. – Folia Geobot. Phytotax. 7: 419–421.

Hong D.-Y. & Fischer M. A. (1998): *Veronica*. – In: Wu Z.-Y. & Raven P. H. (eds), Flora of China 18: 65–80, Science Press, Beijing & Missouri Botanical Garden Press, St. Louis.

Howell C. J. & Sawyer J. W. (2006): New Zealand naturalised vascular plant checklist. – New Zealand Plant Conservation Network, Wellington.

Hrivnák R., Kochjarová J., Šumberová K. & Schmotzer A. (2016): *Lindernia dubia* (*Scrophulariaceae*): a new alien species to the Slovak flora. – Biológia, 71: 281–286.

Hsu T.-C., Lin J.-J. & Chung S. W. (2009): Two newly discovered plants in Taiwan. – Taiwania 54: 403–407.

Hultén E. (1958): The amphi-atlantic plants and their phytogeographical connections. – Stockholm.

Hultén E. & Fries M. (1986): Atlas of North European vascular plants north of the Tropic of Cancer. Vols 1–3. – Koeltz Scientific Books, Königstein.

Hylander N. (1966): Nordisk Kärlväxtflora [Scandinavian Flora]. Vol. 2. – Almquist & Wiksell, Stockholm.

Jehlík V. (ed.) (1998): Cizí expanzivní plevele České republiky a Slovenské republiky [Alien expansive weeds of the Czech Republic and Slovak Republic]. – Academia, Praha.

Jessen S., Bujnoch W., Zenner G. & Stark C. (2011): *Dryopteris lacunosa* – eine neue Art des *Dryopteris-affinis*-Aggregats (*Dryopteridaceae*, *Pteridophyta*). – Kochia 5: 9–31.

Jiménez-Mejías P. & Luceño M. (2011): *Cyperaceae*. – In: Euro+Med Plantbase – the information resource for Euro-Mediterranean plant diversity. URL: http://ww2.bgbm.org/EuroPlusMed/ (accessed 11 February 2016).

Juratzka J. (1858): Ueber die Entdeckung von *Notochlaena marantae* R. Br. in Mähren. – Verh. Zool.-Bot. Ges., S.-B., 8: 92.

Juslén A., Väre H. & Wikström N. (2011): Relationships and evolutionary origins of polyploid *Dryopteris* (*Dryopteridaceae*) from Europe inferred using nuclear *pgi*C and plastid *trn*L-F sequence data. – Taxon 60: 1284–1294.

Kaplan Z. (2012): Flora and phytogeography of the Czech Republic. – Preslia 84: 505–573.

Kaplan Z., Danihelka J., Štěpánková J., Bureš P., Zázvorka J., Hroudová Z., Ducháček M., Grulich V., Řepka R., Dančák M., Prančl J., Šumberová K., Wild J. & Trávníček B. (2015): Distributions of vascular plants in the Czech Republic. Part 1. – Preslia 87: 417–500.

Kilián Z. & Krkavec F. (1962): Několik dalších rostlin z rudišť na Ostravsku [Several other plant species from the ore yards in the Ostrava region]. – Zpr. Slezs. Úst. ČSAV Opava, ser. B, 126: 7–10.

Kocián P. (2014): *Linaria pelisseriana* (L.) Mill. – a new alien species in the Czech Republic. – Acta Mus. Siles., Sci. Natur., 63: 283–288.

Kolář F., Kaplan Z., Suda J. & Štech M. (2015): Populations of *Knautia* in ecologically distinct refugia of the Hercynian massif belong to two endemic species. – Preslia 87: 363–386.

Kolbek J. & Boublík K. (2007): Rostlinná společenstva s *Helictotrichon desertorum* v České republice [Plant communities with *Helictotrichon desertorum* in the Czech Republic]. – Severočes. Přír. 38 (2006): 1–10.

Kolenati F. A. (1860): Höhenflora des Altvaters. – Brünn.

Korniak T. (1987): *Carex stenophylla* Wahlenberg – nowy gatunek turzycy dla flory polskiej i inne interesujące rośliny kserotermiczne koło Dąbrowy Białostockiej [*Carex stenophylla* Wahlenberg – a new species of

sedge in the Polish flora and other interesting xerothermic plants near Dąbrowa Białostocka (north-western Poland)] – Fragm. Flor. Geobot. 31–32: 55–63.

Kupriyanova L. A. (1986): Nekotorye voprosy sistematiki i palinomorfologii vidov roda *Convallaria* (*Asparagaceae*) [Some topics of systematics and palynology of *Convallaria* species (*Asparagaceae*)]. – Bot. Zhurn. 71: 185–194.

Küpfer Ph. (1974): Recherches sur les liens de parenté entre la flore orophile des Alpes et celle des Pyrénées. – Boissiera 23: 1–322.

Kurka R. (1990): *Lindernia dubia* – nový zavlečený druh v Československu [*Lindernia dubia* – a new alien species to the flora of the Czech Republic]. – Zpr. Čes. Bot. Společ. 25: 47–48.

Kúr P., Košnar J. & Štech M. (2014): Characterization and cross-species amplification of 16 microsatellite loci in *Spergularia echinosperma* (*Caryophyllales*: *Caryophyllaceae*). – Conserv. Genet. Res. 6: 571–573.

Kúr P., Štech M., Koutecký P. & Trávníček P. (2012): Morphological and cytological variation in *Spergularia echinosperma* and *S. rubra*, and notes on potential hybridization of these two species. – Preslia 84: 905–924.

Lampe M. von (1996): Wuchsform, Wuchsrhythmus und Verbreitung der Arten der Zwergbinsengesellschaften. – Diss. Bot. 266: 1–353.

Lepší M. & Douda J. (2005): *Lindernia dubia* (L.) Pennel – puštička pochybná. – In: Chán V., Lepší M. & Lepší P. (eds), Nálezy zajímavých a nových druhů v květeně jižní části Čech XI [Records of interesting and new plants in the South Bohemian flora XI], Sborn. Jihočes. Muz. České Budějovice, Přír. Vědy, 45: 170–171.

Lepší M., Lepší P., Koutecký P., Bílá J. & Vít P. (2015): Taxonomic revision of *Sorbus* subgenus *Aria* occurring in the Czech Republic. – Preslia 87: 109–162.

Lhotská M. (1966): Der älteste Fund der *Bidens frondosa* L. in Europa. – Folia Geobot. Phytotax. 1: 186–189.

Lhotská M. (1968): Die Gattung *Bidens* L. in der Tschechoslowakei. – Folia Geobot. Phytotax. 3: 65–98.

Liang S.-Y., Tucker G. C. & Koyama T. (2010): *Schoenoplectus*. – In: Wu Z. Y., Raven P. H. & Hong D. Y. (eds), Flora of China 23. *Acoraceae* through *Cyperaceae*, p. 181–188, Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.

Loos G. H. (1996): Zur Identität von *Carex leersiana* Rauschert, *C. chabertii* F. W. Schultz, *C. polyphylla* Kar. et Kir. und *C. guestphalica* (Boenn. ex Rchb.) Boenn. ex O. F. Lang. – Feddes Repert. 107: 61–74.

Lustyk P. (2015): *Gratiola neglecta* Torr. – In: Hadinec J. & Lustyk P. (eds), Additamenta ad floram Reipublicae Bohemicae. XIII [Additions to the flora of the Czech Republic. XIII], Zpr. Čes. Bot. Společ. 50: 66–67.

Meusel H. & Jäger E. J. (eds) (1992): Vergleichende Chorologie der zentraleuropäischen Flora. Vol. 3. – Gustav Fischer, Jena, Stuttgart & New York.

Meusel H., Jäger E., Rauschert S. & Weinert E. (1978): Vergleichende Chorologie der zentraleuropäischen Flora. Vol. 2. – Gustav Fischer, Jena.

Meusel H., Jäger E. & Weinert E. (1965): Vergleichende Chorologie der zentraleuropäischen Flora. Vol. 1. – Gustav Fischer, Jena.

Molina A., Acedo C. & Llamas F. (2008a): Taxonomy and new taxa of the *Carex divulsa* aggregate in Eurasia (section *Phaestoglochin*, *Cyperaceae*). – Bot. J. Linn. Soc. 156: 385–409.

Molina A., Acedo C. & Llamas F. (2008b): Taxonomy and new taxa in Eurasian *Carex* (section *Phaestoglochin*, *Cyperaceae*). – Syst. Botany 33: 237–250.

Monnier P. & Ratter J. A. (1993): *Spergularia* (Pers.) J. & C. Presl. – In: Tutin T. G., Burges N. A., Chater A. O., Edmondson J. R., Heywood V. H., Moore D. M., Valentine D. H., Walters S. M. & Webb D. A. (eds), Flora Europaea, ed. 2, 1: 186–188, Cambridge University Press, Cambridge.

Mouterde P. (1986): Nouvelle flore du Liban et de la Syrie. Vol. 3. Texte. – Librairie orientale, Beyrouth & Secrétariat Optima, Berlin.

Niklfeld H. (1971): Bericht über die Kartierung der Flora Mitteleuropas. – Taxon 20: 545–574.

Niklfeld H. (1999): Mapping the flora of Austria and the Eastern Alps. – Rev. Valdôtaine Hist. Nat. 51, suppl. 51: 53–62.

Nobis M. & Nowak A. (2011): New data on the vascular flora of the central Pamir Alai Mountains (Tajikistan, Central Asia). – Pol. Bot. J. 56: 195–201.

Nobis A., Nobis M., Piotrowicz K., Kącki Z. & Dajdok Z. (2010): *Lindernia procumbens* in Poland: the relationships between weather conditions and the occurrence of the species. – Biodiv. Res. Conserv. 17: 39–46.

Nosova L. M. (1965): Nekotorye vidy s dizjunktivnymi arealami vo flore severnoj (lugovoi) stepi evropejskoi chasti SSSR [Some new species with disjunctive ranges in the flora of northern steppe in the European part of USSR]. – Bjull. Moskov. Obshch. Isp. Prir., sect. biol., 70/6: 116–130.

Novák J. & Chytrý M. (2007): *Cirsio-Brachypodion pinnati* Hadač et Klika ex Klika 1951. Subkontinentální širokolisté suché trávníky [*Cirsio-Brachypodion pinnati* Hadač et Klika ex Klika 1951. Subcontinental

semi-dry broad-leaved grasslands]. – In: Chytrý M. (ed.), Vegetace České republiky 1. Travinná a keříčková vegetace [Vegetation of the Czech Republic 1. Grassland and Heathland Vegetation], p. 425–449, Academia, Praha.

Ohwi J. (1965): Flora of Japan. – Smithsonian Institution, Washington.

Page S. E. & Rieley J. O. (1985): Ecology and distribution of *Carex chordorrhiza* L. fil. – Watsonia 15: 253–259.

Pennell F. W. (1935): The *Scrophulariaceae* of eastern temperate North America. – Acad. Natur. Sci. Phil. Monogr. 1: 1–650.

Peruzzi L., Carta A. & Garbari F. (2013): Lectotypification of the name *Crocus sativus* var. *vernus* L. (*Iridaceae*) and its consequences within *Crocus* ser. *Verni*. – Taxon 65: 1037–1040.

Pichi Sermoli R. E. (1979): A survey of the pteridological flora of the Mediterranean region. – Webbia 34: 175–242.

Plocek A. (1986): Květena Jizerských hor. 4. *Balsaminaceae* až *Cyperaceae* [Flora of the Jizerské hory Mts. 4. *Balsaminaceae* to *Cyperaceae*]. – Sborn. Severočes. Muz. – Přír. Vědy 15: 5–52.

Podlech D. & Zarre Sh. (2013): A taxonomic revision of the genus *Astragalus* L. (*Leguminosae*) in the Old World. Vol. 1–3. – Naturhistorisches Museum Wien, Wien.

Podpěra J. (1930): Květena Moravy ve vztazích systematických a geobotanických. Fasc. 6/3 [Flora of Moravia in taxonomic and geobotanical context]. – Pr. Morav. Přírod. Společ. 5 (1928)/5: 57–415.

Popiela A. (1998): The distribution of character species of the *Isoëto-Nanojuncetea*-class in Poland. Part I. *Centunculus minimus*, *Radiola linoides* and *Illecebrum verticillatum*. – Fragm. Florist. Geobot. 43: 223–230.

Procházka F. (1977). Současné změny východočeské flóry a poznámky k rozšíření chráněných druhů rostlin [Recent changes in the flora of eastern Bohemia and notes on the distribution of protected species]. – Hradec Králové.

Pyke S. B., Michaud H., Ferrer, P., Argagnon O., Virevaire, M. & Montserrat J. M. (2008): Notes on vascular plants in Morocco. – Lagascalia 28: 473–480.

Pyšek P., Chytrý M., Pergl J., Sádlo J. & Wild J. (2012a): Plant invasions in the Czech Republic: current state, introduction dynamics, invasive species and invaded habitats. – Preslia 84: 575–629.

Pyšek P., Danihelka J., Sádlo J., Chrtek J. Jr., Chytrý M., Jarošík V., Kaplan Z., Krahulec F., Moravcová L., Pergl J., Štajerová K. & Tichý L. (2012b): Catalogue of alien plants of the Czech Republic (2nd edition): checklist update, taxonomic diversity and invasion patterns. – Preslia 84: 155–255.

Raabe U. (2007): Ein Vorkommen des Übersehenen Gnadenkrautes (*Gratiola neglecta* Torr.) in Brandenburg. – Flor. Runbr. 40: 111–112.

Randall R. P. (2007): The introduced flora of Australia and its weed status. – CRC for Australian Weed Management, Adelaide.

Rohlena J. (1926): Příspěvky k floristickému výzkumu Čech. VI. [Contributions to the research into the flora of Bohemia]. – Čas. Nár. Mus., sect. sci. natur., 100: 139–158.

Roleček J., Hájek M., Karlík P. & Novák J. (2015): Reliktní vegetace na mezických stanovištích [Relict vegetation on mesic sites]. – Zpr. Čes. Bot. Společ. 50: 201–245.

Romero-Zarco C. (2011): *Helictochloa* Romero Zarco (*Poaceae*), a new genus of oat grass. – Candollea 66: 87–103.

Röser M. (1996): Ecogeography of the grass genus *Helictotrichon* (*Poaceae*: *Aveneae*) in the Mediterranean and adjacent regions. – Plant Syst. Evol. 203: 181–281.

Rünk K., Zobel M. & Zobel K. (2012): Biological flora of the British Isles: *Dryopteris carthusiana*, *D. dilatata* and *D. expansa*. – J. Ecol. 100: 1039–1063.

Řepka R. (1983): Poznámky k výskytu *Carex stenophylla* Wahlenb. na Moravě [Notes on the occurrence of *Carex stenophylla* in Moravia]. – Zpr. Čs. Bot. Společ. 18: 123–128.

Řepka R. (1988): Předběžné sdělení o výskytu *Carex chabertii* F. W. Schultz v Československu [A preliminary report on the occurence of *Carex chabertii* in Czechoslovakia]. – Zpr. Čs. Bot. Společ. 23: 81–90.

Řepka R. (2003): *Carex muricata* aggregate in the Czech Republic: multivariate analysis of quantitative morphological characters. – Preslia 75: 233–248.

Sádlo J. (2011): *Schoenoplectetum tabernaemontani* De Soó 1947. Brakické rákosiny se skřípincem Tabaernemontanovým [*Schoenoplectetum tabernaemontani* De Soó 1947. Brackish marshes dominated by *Schoenoplectus tabernaemontani*]. – In: Chytrý M. (ed.), Vegetace České republiky 3. Vodní a mokřadní vegetace [Vegetation of the Czech Republic 3. Aquatic and wetland vegetation], p. 437–440, Academia, Praha.

Sarika-Hatzinikolaou M., Koupli-Sovantzi L. & Yannitsaros A. (1994): *Myriophyllum alterniflorum* DC. (*Haloragaceae*), a new record for the Greek flora. – Phyton (Horn) 34: 243–246.

Scalone R. & Albach D. (2012): Degradation of sexual reproduction in *Veronica filiformis* after introduction to Europe. – BMC Evolutionary Biology 12: 233.

Schmidt F. W. (1793): Flora Boëmica inchoata, exhibens plantarum regni Boëmiae indigenarum species. Cent. 1. – Pragae.

Schultze-Motel W. (1980): *Cyperus* L. – In: Conert H. J., Hamann U., Schultze-Motel W. & Wagenitz G. (eds), Gustav Hegi, Illustrierte Flora von Mitteleuropa, ed. 3, vol. 2/1. *Angiospermae*. *Monocotyledones* 2. *Cyperaceae*, *Typhaceae*, incl. *Sparganiaceae*, *Araceae*, *Lemnaceae*, *Juncaceae*, p. 80–92, Verlag Paul Parey, Berlin & Hamburg.

Scribailo R. W. & Alix M. S. (2014): *Haloragaceae*. – In: Flora of North America Editorial Committee (eds), Flora of North America, Provisional Publication (May 28, 2014), p. 1–23, Flora of North America Association. URL: http://floranorthamerica.org/files/Haloragaceae.provisional.Gal_.pdf (accessed 10 February 2016).

Scott N. E. & Davison A. W. (1982): De-icing salt and the invasion of road verges by maritime plants. – Watsonia 14: 41–52.

Sessa E. B., Zimmer E. A. & Givnish T. J. (2012): Unraveling reticulate evolution in North American *Dryopteris* (*Dryopteridaceae*). – BMC Evol. Biol. 12: 104.

Simon C. (1960): *Gratiola neglecta* Torr. im Oberelsaß. – Bauhinia 1: 184–188.

Skokan J. (1928): Konitrud lékařský (*Gratiola officinalis* L.). – Věda Přír. 9: 131–144, 212–216, 247–251.

Smith S. G. (2002): *Schoenoplectus*. – In: Flora of North America Editorial Committee (eds), Flora of North America north of Mexico 23: 44–60, Oxford University Press, New York & Oxford.

Soriano I. & Romero T. (2008): Sobre el género *Gratiola* L. (*Scrophulariaceae*) en el norte de Marruecos y Andalucía. – Lagascalia 28: 443–450.

Soukup M., Štech M., Paulič R. & Chán V. (2010): Rozšíření ovsíře lučního *Avenula pratensis* (L.) Dumort. v jižní části Čech [Distribution of *Avenula pratensis* (L.) Dumort. in southern Bohemia]. – Sborn. Jihočes. Muz. České Budějovice, sci. natur., 50: 105–114.

Stace C. (2010): Flora of the British Isles. Ed. 3. – Cambridge University Press, Cambridge.

Stein D. B., Hutton C., Conant D. S., Haufler C. H. & Werth C. R. (2010): Reconstructing *Dryopteris* "*semicristata*" (*Dryopteridaceae*): Molecular profiles of tetraploids verify their undiscovered diploid ancestor. – Am. J. Bot. 97: 998–1004.

Stuckey R. L. (1974): The introduction and distribution of *Nymphoides peltatum* (*Menyanthaceae*) in North America. - Bartonia 42: 14–23.

Suominen J. (1984): *Gratiola neglecta* (*Scrophulariaceae*), Mantsalanjoen rantakasvi [*Gratiola neglecta*, a North American wetland plant naturalized in Finland]. – Memor. Soc. Fauna Fl. Fenn. 60: 5–9.

Šprynar P. (2004): Poznámky k překvapivému výskytu podmrvky jižní (*Notholaena marantae*) a sleziníku hadcového (*Asplenium cuneifolium*) na ultrabazickém pikritu v Českém krasu [Notes on the surprising occurrence of *Notholaena marantae* and *Asplenium cuneifolium* on the ultrabasic picrite in the Bohemian Karst (central Bohemia)]. – Zpr. Čes. Bot. Společ. 30: 321–338.

Šumberová K. (2003): Veränderungen in der Teichwirtschaft und ihr Einfluß auf die Vegetation in der Tschechischen Republik. Mit Beispielen von *Isoëto-Nanojuncetea*-, *Isoëto-Littorelletea*- und *Bidentetea*-Arten im Becken von Třeboň (Wittingauer Becken). – Mitt. Bad. Landesver. Naturkunde u. Naturschutz, N. F., 18: 7–24.

Šumberová K. (2011a): *Nymphoidetum peltatae* Bellot 1951. Vegetace mělkých stojatých vod teplých oblastí s plavínem štítnatým [*Nymphoidetum peltatae* Bellot 1951. Vegetation of shallow water bodies in warm areas dominated by *Nymphoides peltata*]. – In: Chytrý M. (ed.), Vegetace České republiky 3. Vodní a mokřadní vegetace [Vegetation of the Czech Republic 3. Aquatic and wetland vegetation], p. 124–127, Academia, Praha.

Šumberová K. (2011b): *Schoenoplectetum lacustris* Chouard 1924. Rákosiny se skřípincem jezerním [*Schoenoplectetum lacustris* Chouard 1924. Marshes dominated by *Schoenoplectus lacustris*]. – In: Chytrý M. (ed.), Vegetace České republiky 3. Vodní a mokřadní vegetace [Vegetation of the Czech Republic 3. Aquatic and wetland vegetation], p. 394–397, Academia, Praha.

Šumberová K. (2013a): *Cyperus fuscus* L. – šáchor hnědý, š. tmavý. – In: Lepší P., Lepší M., Boublík K., Štech M. & Hans V. (eds), Červená kniha květeny jižní části Čech [Red Data Book of the flora of southern part of Bohemia], p. 151–152, Jihočeské muzeum v Českých Budějovicích, České Budějovice.

Šumberová K. (2013b): *Gratiola neglecta* Torr. – In: Hadinec J. & Lustyk P. (eds), Additamenta ad floram Reipublicae Bohemicae. XI. [Additions to the flora of the Czech Republic. XI], Zpr. Čes. Bot. Společ. 48: 92–90.

Šumberová K. (2013c): *Radiola linoides* Roth – stozrník lnovitý. – In: Lepší P., Lepší M., Boublík K., Štech M. & Hans V. (eds), Červená kniha květeny jižní části Čech [Red Data Book of the flora of southern part of Bohemia], p. 341, Jihočeské muzeum v Českých Budějovicích, České Budějovice.

Šumberová K. & Ducháček M. (2009): *Gratiola neglecta* – nový zavlečený druh pro květenu České republiky [*Gratiola neglecta* – a new alien species to the flora of the Czech Republic]. – Zpr. Čes. Bot. Společ. 44: 151–175.

Šumberová K., Ducháček M. & Lososová Z. (2012a): Life-history traits controlling the survival of *Tillaea aquatica*: a threatened wetland species in intensively managed fishpond landscapes of the Czech Republic. – Hydrobiologia 689: 91–110.

Šumberová K., Lososová Z., Ducháček M., Horáková V. & Fabšičová M. (2012b): Distribution, habitat ecology, soil seed bank and seed dispersal of threatened *Lindernia procumbens* and alien *Lindernia dubia* (*Antirrhinaceae*) in the Czech Republic. – Phyton (Horn) 52: 39–72.

Šumberová K., Štech M. & Rektoris L. (2013): *Lindernia procumbens* (Krock.) Borbás – puštička pouzdernatá, p. rozprostřená. – In: Lepší P., Lepší M., Boublík K., Štech M. & Hans V. (eds), Červená kniha květeny jižní části Čech [Red Data Book of the flora of southern part of Bohemia], p. 248–249, Jihočeské muzeum v Českých Budějovicích, České Budějovice.

Thiers B. (2016): Index Herbariorum: a global directory of public herbaria and associated staff. – New York Botanical Garden's Virtual Herbarium. URL: http://sciweb.nybg.org/science2/IndexHerbariorum.asp (accessed February 2016)

Timohina S. A. (1990): *Scirpus* L. – Kamyš. – In: Peshkova G. A. & Malyshev L. I. (eds), Flora Sibiri [Flora of Siberia] 3: 20–74, Nauka, Novosibirsk.

Toman M. (1973): Rozšíření některých stepních druhů v Čechách [Distribution of some steppe species in Bohemia]. – Sborn. Pedagog. Fak. Ústí nad Labem, ser. sci. natur., 2: 21–102.

Tsvelev N. N. (1980): Nizmyanka *Centunculus* (*Primulaceae*): novyi rod flory Dal'nego Vostoka [*Centunculus* (*Primulaceae*): a new genus of the flora of the Far East]. – Bot. Zhurn. 65: 587–588.

Turland N. J. & Jarvis C. E. (1997): Typification of Linnaean specific and varietal names in the *Leguminosae* (*Fabaceae*). – Taxon 46: 457–485.

USDA, NRCS (2016): The Plants Database. – United States Department of Agriculture.
URL: http://plants.usda.gov (accessed 9 February 2016).

Valdés B., Scholz H., Raab-Straube E. von & Parolly G. (2009): *Poaceae* (pro parte majore). – In: Euro+Med Plantbase – the information resource for Euro-Mediterranean plant diversity.
URL: http://ww2.bgbm.org/EuroPlusMed (accessed February 2016).

van den Berg L. J. L., Dorland E., Vergeer P., Hart M. A. C., Bobbink R. & Roelefs J. G. M. (2005): Decline of acid sensitive plant species in heathland can be attributed to ammonium toxicity in combination with low pH. – New Phytol. 166: 551–564.

von Lampe M. (1996): Wuchsform, Wuchsrhythmus und Verbreitung der Arten der Zwergbinsengesellschaften. – Diss. Bot. 226: 1–353.

Vydrina S. N. (1994): *Astragalus* L. – Astragal. – In: Polozhiy A. V. & Malyshev L. I. (eds), Flora Sibiri [Flora of Siberia] 9: 20–74, Nauka, Novosibirsk.

Walsh N. G. (2003): *Primulaceae*. – In: Walsh N. G. &. Entwisle T. J. (eds), Flora of Victoria 3: 517–522, Inkata Press, Melbourne.

Webb C. J., Sykes W. R. & Garnock-Jones P. J. (1988): Flora of New Zealand. Vol. 4. Naturalised pteridophytes, gymnosperm and dicotyledons. – Christchurch, New Zealand.

Weimarck G. (1971): Variation and taxonomy of *Hierochloë* (*Gramineae*) in the Northern Hemisphere. – Bot. Not. 124: 129–175.

Weyl P. S. R. & Coetzee J. A. (2014): The invasion status of *Myriophyllum spicatum* L. in southern Africa. – Manag. Biol. Invas. 5: 31–37.

Widén C.-J., Fraser-Jenkins C. R., Reichstein T., Gibby M. & Sarvela J. (1996): Phloroglucinol derivatives in *Dryopteris* sect. *Fibrillosae* and related taxa (*Pteridophyta*, *Dryopteridaceae*). – Ann. Bot. Fenn. 33: 69–100.

Yu D., Wang D., Li Z.-Y. & Funston A. M. (2002): The taxonomic revision of the genus *Myriophyllum* (*Haloragaceae*) in China. – Rhodora 104: 396–421.

Zuloaga F. O. & Morrone O. (1999): Catálogo de las plantas vasculares de la República Argentina [Catalogue of vascular plants of Argentina]. Vol. 2. – Missouri Botanical Garden, St. Louis.

Paper 10

Hornych O., Ekrt L., Riedel F., Koutecký P. & Košnar J. (2019): Asymmetric hybridization in the Central European populations of the *Dryopteris carthusiana* group. – American Journal of Botany 106(11): 1477–1486.

Asymmetric hybridization in Central European populations of the *Dryopteris carthusiana* group

Ondřej Hornych[1,4] (iD), Libor Ekrt[1], Felix Riedel[2,3], Petr Koutecký[1], and Jiří Košnar[1]

PREMISE: Hybridization is a key process in plant speciation. Despite its importance, there is no detailed study of hybridization rates in fern populations. A proper estimate of hybridization rates is needed to understand factors regulating hybridization.

METHODS: We studied hybridization in the European *Dryopteris carthusiana* group, represented by one diploid and two tetraploid species and their hybrids. We sampled ~100 individuals per population in 40 mixed populations of the *D. carthusiana* group across Europe. All plants were identified by measuring genome size (DAPI staining) using flow cytometry. To determine the maternal parentage of hybrids, we sequenced the chloroplast region *trn*L–*trn*F of all taxa involved.

RESULTS: We found hybrids in 85% of populations. Triploid *D.* ×*ambroseae* occurred in every population that included both parent species and is most abundant when the parent species are equally abundant. By contrast, tetraploid *D.* ×*deweveri* was rare (15 individuals total) and triploid *D.* ×*sarvelae* was absent. The parentage of hybrid taxa is asymmetric. Despite expectations from previous studies, tetraploid *D. dilatata* is the predominant male parent of its triploid hybrid.

CONCLUSIONS: This is a thorough investigation of hybridization rates in natural populations of ferns. Hybridization rates differ greatly even among closely related fern taxa. In contrast to angiosperms, our data suggest that hybridization rates are highest in balanced parent populations and support the notion that some ferns possess very weak barriers to hybridization. Our results from sequencing cpDNA challenge established notions about the correlation of ploidy level and mating tendencies.

KEY WORDS antheridiogens; Dryopteridaceae; ferns; flow cytometry; hybridization rate; interspecific hybridization; polyploidy; reproductive isolation; speciation; *trn*L–*trn*F.

Polyploidization (whole-genome duplication) plays a major role in plant speciation (Arnold, 1997; Otto and Whitton, 2000; Landis et al., 2018). Many polyploid species originally appear as infertile interspecific hybrids that undergo whole-genome duplication to regain fertility (Barrington et al., 1989; Arnold, 1992; Soltis et al., 2000). The resultant allopolyploids are reproductively isolated from their progenitors and combine their characteristics. Therefore, the study of processes that influence hybridization is essential to understanding plant speciation via allopolyploidization (Twyford and Ennos, 2012), especially in ferns, as more speciation events are correlated with polyploidy in ferns than in angiosperms (Wood et al., 2009).

Hybridization is restricted by two types of barriers: prezygotic (limiting mating and fertilization) and postzygotic (limiting the

viability of hybrids from zygote onward; Rieseberg and Carney, 1998). The combined strength of these barriers affects hybridization rates (i.e., the frequency of hybrids). A wealth of literature exists describing hybridization rates in seed plants from several different perspectives (e.g., Bacilieri et al., 1996; Lepais et al., 2009; Koutecký et al., 2011; Ma et al., 2014), and the frequency of hybrids and recombinants has been studied in mosses (Shaw, 1994; van der Velde and Bijlsma, 2004). Hybridization is considered to be common in ferns (Barrington et al., 1989; Sigel, 2016); however, surprisingly, no accurate estimation of hybridization rates in natural populations has been published for ferns. Many studies have described fern hybrid taxa, including intergeneric ones (e.g., Brownsey, 1977; Reichstein, 1981; Knobloch et al., 1984; Rothfels et al., 2015), or studied the presence of hybrids in natural fern populations (Zhang

et al., 2013; Testo et al., 2015; Ekrt and Koutecký, 2016; Hori et al., 2018; Hanušová et al., 2019). However, hybridization rates have only been described qualitatively in these studies, without any precise evaluation. Various hybridization barriers were described for mosses (Natcheva and Cronberg, 2004) and angiosperms (Baack et al., 2015). However, apart from general genetic incompatibilities (Maheshwari and Barbash, 2011), very few hybridization barriers have been suggested to exist in ferns (Xiang et al., 2000; Testo et al., 2015), and there are almost no data gauging their strength. This missing information is preventing us from better understanding hybridization and allopolyploid speciation in ferns.

A key factor affecting hybridization is the relative abundance of the parent species. Presuming a complete lack of hybridization barriers, a given hybrid should be most abundant in balanced populations in which both parent species have an equally high prevalence of interspecific interactions (Rieseberg et al., 1998). In angiosperms we see the opposite trend: even weak barriers decrease the chance of hybridization in balanced populations (Arnold et al., 1993; Carney et al., 1994; Rieseberg et al., 1995; Emms et al., 1996), and hybridization is frequent only when one species is in the minority and the overabundance of foreign pollen overwhelms the barriers (Prentis et al., 2007; Lepais et al., 2009; Koutecký et al., 2011). Nevertheless, the situation could be different in ferns, in which very few barriers have been described. Therefore, understanding hybridization rates will require considering the parent ratio.

In general, both prezygotic and postzygotic barriers can act differently, based on the direction of the cross. This is commonly termed "asymmetric hybridization," meaning that viable hybrid individuals are more likely to have received one type of gamete from one parent taxon rather than the other (Rieseberg et al., 1998). Among the prezygotic barriers that can lead to asymmetric hybridization are, for example, differences in mating systems and gamete performance (Lewis and Crowe, 1958; Buggs and Pannell, 2006; Testo et al., 2015; Nieto-Lugilde et al., 2018). Various genetic incompatibilities can function as an asymmetric postzygotic barrier and affect the viability and fertility of plant hybrids (Arnold and Bennett, 1993; Peng and Chiang, 2000; Hamzeh et al., 2007). The presence of asymmetric hybridization provides an additional perspective and can help explain results of hybridization studies.

Our study group, the *Dryopteris carthusiana* complex (Fig. 1), is a sexually reproducing fern complex represented in continental Europe by *D. carthusiana* (Vill.) H. P. Fuchs (tetraploid), *D. dilatata* (Hoffm.) A. Gray (tetraploid), and *D. expansa* (C. Presl) Fraser-Jenk. & Jermy (diploid). Because these species are among the most abundant ferns in European forests, considerable effort has been

put into studying their ecology (Rünk et al., 2010, 2012; Bennert et al., 2012), phylogeny and evolution (Stein et al., 2010; Juslén et al., 2011; Sessa et al., 2012a), and cytology (Ekrt et al., 2010; Bennert et al., 2012). Within the group, all three possible hybrid combinations exist: *D.* ×*ambroseae* Fraser-Jenk. & Jermy (triploid) = *D. dilatata* × *D. expansa*; *D.* ×*deweveri* (Jansen) Jansen & Wacht. (tetraploid) = *D. carthusiana* × *D. dilatata*; *D.* ×*sarvelae* Fraser-Jenk. & Jermy (triploid) = *D. carthusiana* × *D. expansa*. *Dryopteris* ×*ambroseae* and *D.* ×*deweveri* are widespread in Europe, whereas *D.* ×*sarvelae* is very rare and has only been reported from northern parts of Europe (reviewed in Ekrt et al., 2010). Hybrid individuals mostly form aborted spores (Wagner and Chen, 1965; Ekrt et al., 2010; Hornych and Ekrt, 2017) and are therefore generally incapable of forming subsequent generations. In Central European forests, this group often forms a mixed population in which hybrids have frequently been found (Ekrt et al., 2010). The availability of mixed populations and the formation of three different hybrid combinations make the *D. carthusiana* group useful for analyzing hybridization patterns in ferns.

To understand the dynamics of hybridization in natural populations of ferns, we ask three main questions. First, what is the rate of formation of the three hybrid taxa within the *D. carthusiana* group in Europe? Second, does the relative abundance of parent species influence hybridization rates in natural populations? Finally, is there asymmetric hybrid formation among any of the three hybrid taxa?

MATERIALS AND METHODS

Field Collection

A total of 40 mixed (i.e., at least two species present) populations of the *Dryopteris carthusiana* group were sampled during 2016 and 2017 in Austria, the Czech Republic, Germany, Slovakia, and Sweden (Appendix S1). All mature (i.e., bearing sporangia) plants in a continuous area containing ~100 individuals were collected from each population. Additional individuals were collected or obtained from the authors' herbaria for molecular analyses. Vouchers (Appendix S2) of all plants used for molecular analyses were deposited in herbarium CBFS (Thiers, 2019).

Flow cytometry

Genome size of all studied individuals was determined using flow cytometry. This method allows for the unambiguous identification
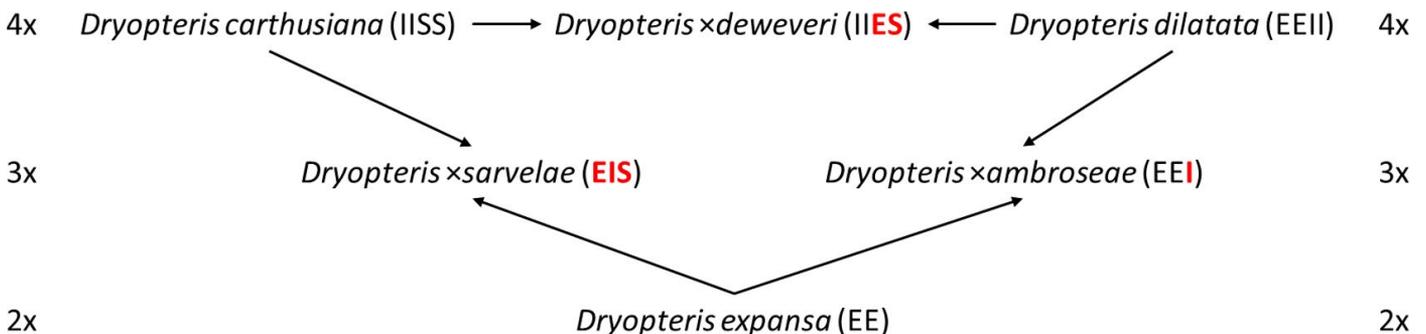
FIGURE 1. Overview of the three species and their hybrids in the *Dryopteris carthusiana* group. Letters in parentheses denote genomic composition of taxa (E = *D. expansa*, I = *D. intermedia*, S = *D.* "semicristata"; Sessa et al., 2012b). Letters in red indicate chromosome sets without homologs present.

of all the studied taxa, including hybrids, because even the two tetraploid species differ by ~21% in their genome size (Ekrt et al., 2010). The samples were measured using a Partec PA II flow cytometer equipped with a mercury arc lamp (Partec, now part of Sysmex, Münster, Germany) employing DAPI as a fluorescent stain (for details on methodology, see Ekrt et al., 2010). Very rarely, individuals had sample-to-standard ratio out of the norm for the taxa involved (based on Ekrt et al., 2010) and were excluded from further analyses. A single clone of *Chlorophytum comosum* (all-green-leaved cultivar; 2C = 24.14 pg) was used as an internal standard because it provides high-quality results and its genome size does not overlap with any of the studied plants. The genome size of *C. comosum* was determined by calibration with *Pisum sativum* 'Ctirad' (2C = 9.09 pg; Doležel et al., 1998) based on 10 measurements on five different days, using the same method of sample preparation described above except that propidium iodide staining was used (for details, see, e.g., Doležel et al., 2007), and the samples were measured using a Partec CyFlow SL flow cytometer equipped with a 100 mW 532 nm (green) solid state laser as a light source (Partec, now part of Sysmex, Münster, Germany).

In total, 3962 individuals of the *Dryopteris carthusiana* group were analyzed, pooling up to five individuals into one analysis (e.g., Ekrt and Koutecký, 2016; Hanušová et al., 2019). The fluorescence histograms were evaluated using FloMax version 2.6 (Partec, now part of Sysmex, Münster, Germany) and FlowJo version 10 (FlowJo, Ashland, Oregon, USA). Mean fluorescence, coefficient of variation, and number of nuclei were recorded for all fluorescent peaks. The relative genome size was then calculated as the ratio between the mean fluorescence of the sample and the internal standard.

Determining chloroplast origin in hybrids

To provide a reference, we sequenced 10 *D. carthusiana*, 13 *D. dilatata*, and 10 *D. expansa* samples. These sequences were then compared with those from 63 individuals of *D. ×ambroseae*, 35 of *D. ×deweveri*, and three of *D. ×sarvelae*. This method can demonstrate which species provided which gamete to the hybrids, because chloroplasts are maternally inherited in ferns (Vogel et al., 1998). The sequences are available in GenBank (accession nos. MK697576–MK697585). Total genomic DNA was extracted, using Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany), from silica-dried plant material and herbaria specimens. The chloroplast region *trn*L–*trn*F was amplified using primers FernL (GGYAATCCTGAGCCAAATC; Li et al., 2009) and TabF (ATTTGAACTGGTGACACGAG; Taberlet et al., 1991). The polymerase chain reaction (PCR) mixture contained 1 µL genomic DNA, 2.5 mM MgCl$_2$, 0.2 mM of each dNTP, 0.3 µM of each primer, 0.5 U Taq polymerase (Top-Bio, Praha,

Czech Republic) in the manufacturer's reaction buffer, and sterile water to make up a final volume of 10 µL. Amplifications were performed with an initial denaturation of 3 min at 94°C; followed by 40 cycles of 1 min at 94°C, 30s at 51°C, and 1 min at 72°C; and a final extension of 10 min at 72°C. The PCR product was sequenced at Eurofins Genomics (Ebersberg, Germany).

Data analysis

The relationship between the abundance of the most common hybrid taxon (*D. ×ambroseae*) and the relative abundance of the parent species (*D. dilatata* and *D. expansa*) in a population was plotted and compared with a null model of completely random mating. Populations of the *D. carthusiana* group are often established after a disturbance in a single colonization event (O. Hornych et al., personal observation). Infrequent disturbances after the initial colonization event enable more individuals to establish. However, turnover is rather small, and the composition of a population remains relatively stable. Therefore, we assume that the current adult individual composition of a population reflects the proportion of colonizing spores and first-generation gametophytes of the parent species that gave rise to the hybrids. The model also presumes that hybridization is bidirectional—that is, each progenitor has an equal chance of providing either gamete to the hybrid. Finally, hybrid individuals are sterile (Hornych and Ekrt, 2017). Therefore, they do not form subsequent generations and their frequency depends solely on the frequencies of the parents. Presuming a complete lack of barriers to hybrid formation, the expected frequency of hybrids under random mating is 2*d*e, where d and e are frequencies of the parent species *D. dilatata* and *D. expansa*; and frequency of intraspecific offspring is d^2 and e^2, respectively. Our model is similar to the Hardy-Weinberg model of allele frequencies, with progeny substituted for alleles. The second-order polynomial model was used because it explained the most variation. The percentage of *D. ×ambroseae* was arcsin transformed for the analysis, and only the populations containing at least one individual of *D. ×ambroseae* were used. This selection also leads to the inclusion of all populations with *D. expansa*. All statistical analyses were performed in R version 3.4.3 (R Core Team, 2017).

RESULTS

Frequency of taxa in wild populations

All 40 studied populations (Table 1) included *D. dilatata*. The other two sexual species, *D. carthusiana* and *D. expansa*, were found in

TABLE 1. Overview of the abundance of all studied taxa in the *Dryopteris carthusiana* group and the number of populations in which the taxon was present. The abundance per parent population expresses the minimum, mean, and maximum percentages of hybrid individuals present in a population in which both parent species are present.

Taxon	Total number of individuals	Total number of populations	Abundance per parent population: (min)-mean-(max)
D. carthusiana	697 (17.59%)	30 (75%)	
D. dilatata	1722 (43.46%)	40 (100%)	
D. expansa	1102 (27.81%)	32 (80%)	
D. ×ambroseae	426 (10.75%)	33 (82.5%)	(0.0)-13.4-(51.2)%
D. ×deweveri	15 (0.38%)	5 (12.5%)	(0.0)-0.5-(5.3)%
D. ×sarvelae	0 (0%)	0 (0%)	0%
Total	3962	40	

75% and 80% of the populations, respectively, and all three species co-occurred in 55% of the populations. The three species were present relatively evenly in northern populations, *D. expansa* dominated the populations of the Alps and the Carpathians, and *D. carthusiana* was most abundant at low elevations (Fig. 2A).

Hybrids were collected in 34 (85%) populations with no geographic pattern (Fig. 2B). The highest hybridization rate was found in population 14 (for details, see Appendix S1), where 51.6% of plants were of hybrid origin. The most frequent hybrid was *D. ×ambroseae* (426 samples, 10.75%), which was found in all populations in which its parents co-occurred (on average, 13.7% individuals per population) and in a single population without *D. expansa* (Fig. 3). By contrast, only 15 (0.38%) individuals of *D. ×deweveri* were sampled from five populations despite a similar number of populations in which both parents were present for *D. ×ambroseae* and *D. ×deweveri* (Fig. 3). No individuals of *D. ×sarvelae* were found during population sampling.

Frequency of *D. ×ambroseae* (the only hybrid for which enough data are available) depended on the frequencies of the parent taxa: this hybrid was more common in populations with a balanced proportion of the parents than in populations in which one of the parents dominated (modeled by the second order polynomial, $F_{2, 37} = 32.68$, $P < 0.001$, $R^2 = 0.619$). However, compared to the null model of the random mating, the hybrid was less frequent than expected in balanced populations and populations in which *D. dilatata* dominated, whereas it was more frequent than expected in *D. expansa*-dominated populations.

Chloroplast DNA analyses

A single unique *trn*L–*trn*F sequence was found in each of the parent species. Among hybrid taxa, 52 of 63 (~83%) of the *D. ×ambroseae* samples received their chloroplast haplotype from *D. expansa* (Fig. 4). For *D. ×deweveri*, hybridization is almost unidirectional, with 33 of 35 (~94%) tested individuals obtaining the chloroplast haplotype from *D. carthusiana* (Fig. 4). The chloroplast haplotype of two of the three individuals of *D. ×sarvelae* match with *D. expansa*.

DISCUSSION

Fern hybridization rates in natural populations

Using flow cytometry, we determined 3962 individuals from 40 mixed populations of the three taxa of the *Dryopteris carthusiana* group, providing us with a perspective on past interactions leading to hybridization. We have found hybrids in 34 of the 40 (85%) sampled populations. However, we revealed a striking difference in abundance among the three hybrid taxa. No hybrids between *D. carthusiana* and *D. expansa* (*D. ×sarvelae*) were found in our sampled populations. Hybrids between *D. carthusiana* and *D. dilatata* (*D. ×deweveri*) occur rarely. Finally, hybrids between *D. expansa* and *D. dilatata* (*D. ×ambroseae*) are frequent. To our knowledge, this is the first quantitative record of fern hybridization rates in natural populations. In a preceding study of the *D. carthusiana* group (Ekrt et al., 2010), the authors sampled all taxa within a population nonrandomly and reported the frequency of populations containing hybrids but provided no information on frequencies of the taxa within populations.

Dryopteris ×ambroseae: the ever-present hybrid

The hybrid between *D. dilatata* and *D. expansa*, *D. ×ambroseae*, was found in all 32 populations with both parents and even in one population without *D. expansa*. These numbers are even higher than those described by Ekrt et al. (2010), who collected the hybrid in only 72% of populations that contained both parents. Averaging 13.4% of the individuals in populations in which it occurred, the barriers to forming this triploid hybrid appear to be extremely weak.

Chloroplast analyses of 63 individuals of the triploid *D. ×ambroseae* from 11 populations demonstrate strong asymmetry in hybridization. Asymmetry in chloroplast origin of heteroploid hybrids has been reported in seed plants (Buggs and Panell, 2006) as well as ferns (Vogel et al., 1998; Xiang et al., 2000; Testo et al., 2015). Based on these studies, the diploid ought to be the predominant paternal parent. Surprisingly, our results present the opposite trend, in that the tetraploid *D. dilatata* provided the sperm to 83% of tested hybrids.

The prevalence of *D. ×ambroseae* allows us to compare its frequency with the relative abundance of its parents (Fig. 5). Hybridization was most pronounced when both parents were equally abundant. In comparison, results from seed plants demonstrate that hybridization rates tend to increase as species frequency in mixed populations becomes more uneven (Arnold et al., 1993; Carney et al., 1994; Jorgensen and Andersen, 1994; Rieseberg et al., 1995; Emms et al., 1996; Prentis et al., 2007; Lepais et al., 2009; Koutecký et al., 2011; Ma et al., 2014). However, the curve estimated from our data is not symmetric and the hybridization rate is generally lower than would be expected under the random-mating null model. Some differences between our data and our model might be attributed to deviations from our assumptions and the presence of hybridization barriers. For example, our model assumes bidirectional hybridization. However, the chloroplast analyses clearly show that hybridization is highly asymmetric, *D. expansa* being the female parent about four times more often than *D. dilatata*. Thus, offspring of a *D. dilatata* female and a *D. expansa* male are much less likely to occur within the populations. Frequency of hybridization might also be influenced by environment-related expression of sexes in ferns. It is known that fern gametophytes in suboptimal conditions produce mainly male structures (antheridia; Korpelainen, 1994; DeSoto et al., 2008). If a species is rare at a site because of suboptimal environmental conditions, it might produce more male and fewer female gametes, and thus more hybrids and fewer of its own offspring, than would be expected on the basis of sporophyte frequency. In case of asymmetric hybridization, this effect might lead to different hybrid frequencies in the two types of uneven populations. If the male parent is in the minority, hybridization can be enhanced by the production of mainly male gametophytes. In the opposite scenario, if the female parent is in the minority, production of mainly male gametophytes may suppress hybridization. Indeed, in the present study, the fitted curve of hybrid frequency (Fig. 5) is asymmetric, with more hybrids in populations in which *D. expansa* (mostly female parent) is dominant and *D. dilatata* (mostly male parent) is rare, compared to the opposite scenario.

Dryopteris ×deweveri: the rare hybrid

The hybrid between *D. carthusiana* and *D. dilatata*, *D. ×deweveri*, is rare. Although both of its parents grew together in 30 populations, the hybrid was found in just five of them (16.6%). Similarly, Ekrt
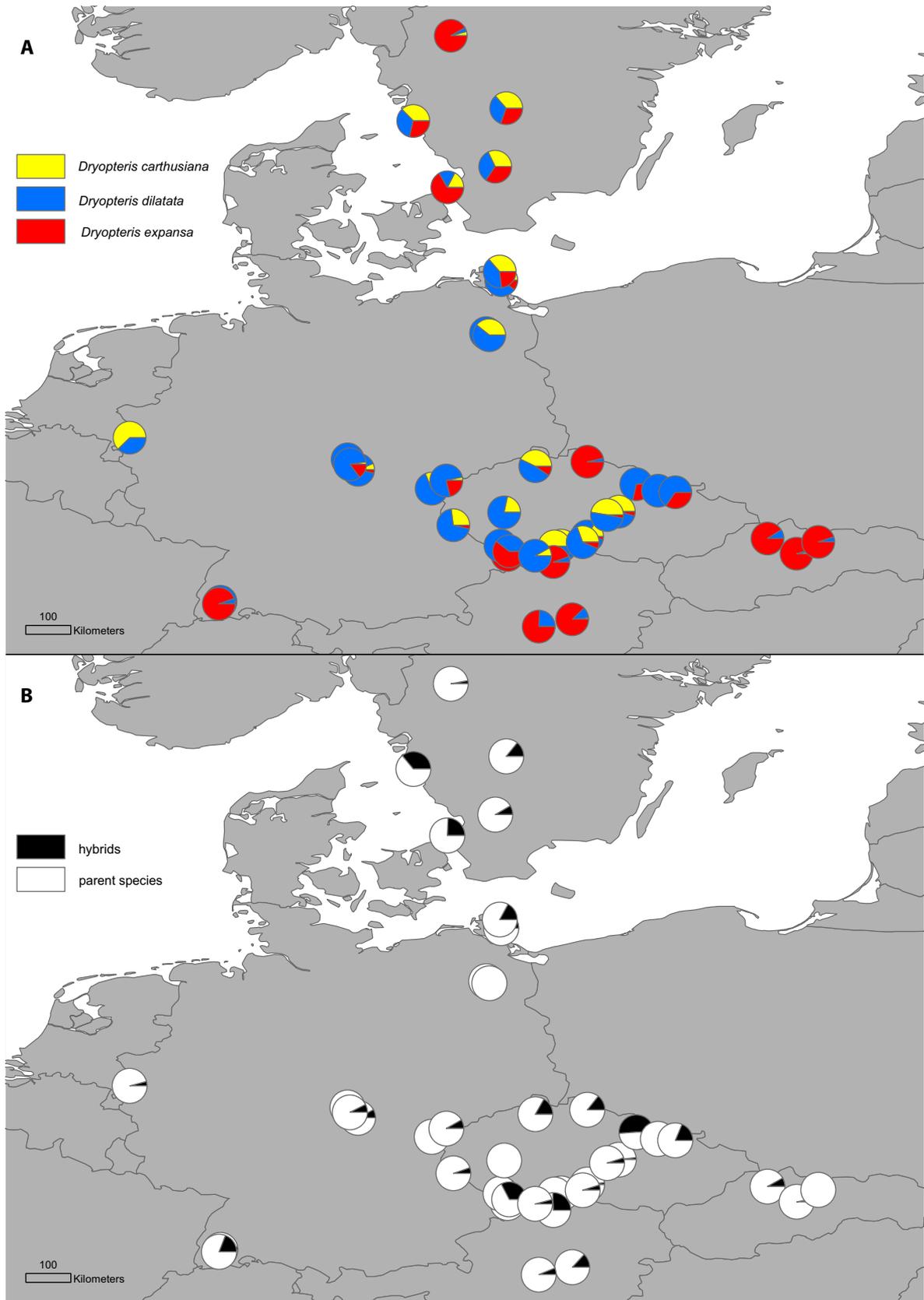
FIGURE 2. Distribution of (A) parent species and (B) hybrids collected in 40 sampled populations of the *Dryopteris carthusiana* group.
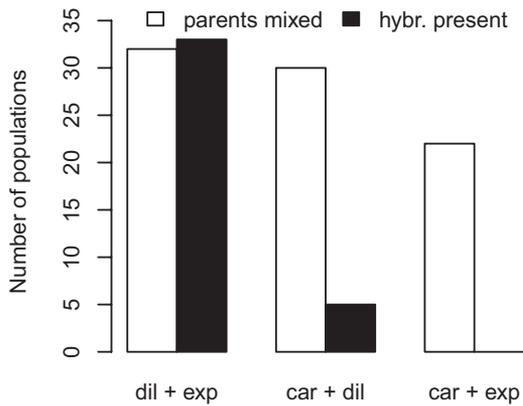
FIGURE 3. A comparison of the numbers of populations in which both parent species were present (mixed) and the numbers in which hybrids were present for all three parent combinations in the *Dryopteris carthusiana* group (car = *D. carthusiana*, dil = *D. dilatata*, exp = *D. expansa*).

et al. (2010) recorded this hybrid taxon in only about 5% of their populations. However, they sampled selectively and substantially fewer individuals per population than the present study. Despite having a similar number of populations in which parent species co-occurred, this hybrid was almost 30 times less abundant than *D. ×ambroseae*.

The vast majority (~94%) of *D. ×deweveri* samples tested for chloroplast origin received their chloroplast from *D. carthusiana*, suggesting an almost unidirectional pattern of hybridization. This striking asymmetry of chloroplast origin suggests that the hybrid combination of *D. dilatata* female and *D. carthusiana* male is either highly unlikely to originate or has low viability. Similar unidirectional hybridization patterns are well established in seed plants (Arnold and Bennett, 1993; Bacilieri, 1996; Peng and Chiang, 2000; Zhou et al., 2008; Beatty et al., 2009; Trucco et al., 2009; Ma et al., 2014) and have also been reported for homoploid hybrid mosses (van der Velde and Bijlsma, 2004) and ferns (Hunt et al., 2011; Zhang et al., 2013). By contrast, homoploid hybrids of lycopods (*Diphasiastrum*) and of some ferns (*Polystichum*) are formed with no preferred direction of hybridization (Kentner and Mesler, 2000; Schnittler et al., 2018)

Dryopteris ×sarvelae: the absent hybrid

Interestingly, the hybrid between *D. carthusiana* and *D. expansa*, *D. ×sarvelae*, was not found in any of the 22 populations in which both of its parent species grew in sympatry. These results are congruent with Ekrt et al. (2010), who were also unable to find this hybrid. The absence of this elusive hybrid is even more surprising because our sampling covered general areas where it has previously been found, northern Germany (Jessen and Rasbach, 1987) and Sweden (L. Ekrt, personal observation).

Apart from the genetic dissimilarity (discussed below), the unusual rarity of *D. ×sarvelae* may be explained by

microhabitat differences. Of the three *D. carthusiana* group species analyzed, *D. carthusiana* and *D. expansa* are the most ecologically distinct (Rünk et al., 2012). Although they can be present together in the same areas (Kaplan et al., 2016), they tend to occupy different microhabitats. Therefore, the two species might have limited opportunities to hybridize. This hypothesis is congruent with the fact that the hybrid has so far been observed only in parts of Northern Europe (Widén et al., 1967; Sorsa and Widén, 1968; Corley and Gibby, 1981; Jessen and Rasbach, 1987) where *D. expansa* grows more commonly together with *D. carthusiana* (Rünk et al., 2012). Nevertheless, even there, the hybrid seems to be extremely rare.

Given the low number of individuals of *D. ×sarvelae* tested for chloroplast origin and the fact that both types of chloroplast haplotypes were found, we can only conclude that hybridization is not unidirectional.

Factors explaining hybridization patterns

Numerous possible barriers may influence fern hybridization. Within our dataset, one of the three hybrids is extremely rare, perhaps because of (micro)habitat differentiation of the parent species. The other two hybrids have one parent species in common, *D. dilatata*. Interestingly, these two hybrids differ in ploidy level (*D. ×ambroseae* is a heteroploid triploid, whereas *D. ×deweveri* is a homoploid tetraploid). Comparing the hybrids gives us an opportunity to examine the role of prezygotic and postzygotic barriers.

Prezygotic barriers—The chance of a hybrid forming in the first place is limited by prezygotic barriers. Among the factors limiting fern hybrid formation are differences in mating strategies, gamete performance, and antheridiogen use.

Mating strategies impact hybridization and are, in turn, influenced by ploidy level. Polyploidization has been associated with a shift between monoecy and dioecy in mosses (Perley and Jesson, 2015) and seed plants (Buggs and Pannell, 2006; Njuguna et al., 2013) altering the ability to form the two types of gametes on a single plant. However, sexual determination of homosporous ferns is environmental, and one gametophyte can form both types of gametes under the right conditions. Nevertheless, ploidy level may influence fern mating strategies. Most ferns studied to date employ a mixed mating system (Soltis and Soltis, 1987;
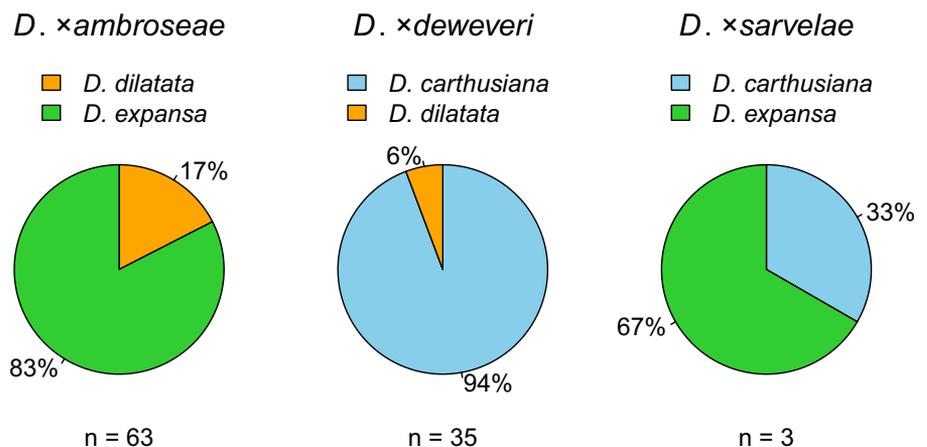
FIGURE 4. Distribution of chloroplast genome (*trn*L–*trn*F region) inheritance in the three hybrid taxa within the *Dryopteris carthusiana* group (*n* = number of individuals analyzed).
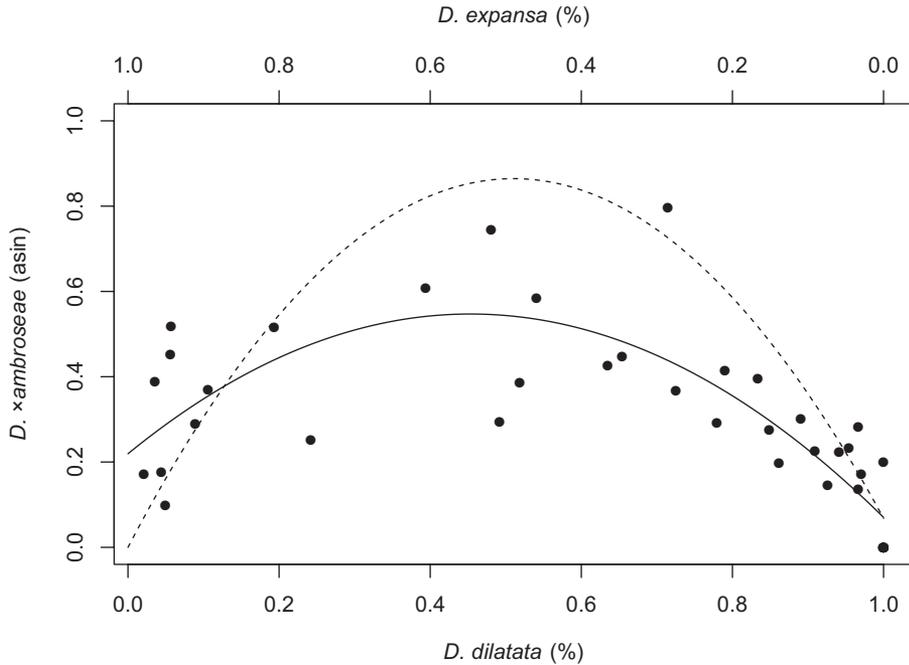
FIGURE 5. Correlation of abundance of *Dryopteris ×ambroseae* and the relative abundance of its parent species, which is expressed as the ratio of the parent to the sum of both parent taxa (bottom axis: *D. dilatata*, top axis: *D. expansa*). Solid line = second-order polynomial regression; dashed line = null model of random mating. Frequency of hybrids (*y*-axis) is arcsine transformed.

Wubs et al., 2010; de Groot et al., 2012; Peredo et al., 2013; Sessa et al., 2016). Under mixed mating, the gametophyte is capable of both selfing and outcrossing. In some cases, polyploid ferns have been demonstrated to better tolerate gametophytic selfing (Masuyama, 1979; Soltis and Soltis, 2000; Pangua et al., 2003; Flinn, 2006; Testo et al., 2015; Sessa et al., 2016). In theory, mating strategies of sexual fern species could influence hybridization in two opposing ways. First, predominant selfers may have fewer eggs available for hybridization. Apart from skewing the ratio of male parentage in favor of polyploids, this effect would also constitute a prezygotic barrier to hybridization. In our case, *D. ×deweveri* would be affected more by the barrier than the triploids, because both of its parents are polyploid and *D. carthusiana* is a known facultative selfer (Testo et al., 2015). Second, predominant outcrossers may have a greater proportion of sperm in the environment to facilitate outcrossing. These outcrossers could then swamp selfers with overabundant sperm. Although there are no data for ferns, an analogous process, pollen swamping, is well known in seed plants (Petit et al., 1997; Buggs and Pannell, 2006; Ouayjan and Hampe, 2018). The diploid would then be the predominant male parent. These two concepts directly contradict each other. Contrary to previously published results (Xiang et al., 2000; Testo et al., 2015), our results indicate that the former process may be influential, depending on the direction of asymmetry in the parentage of *D. ×ambroseae*.

Gamete performance plays a major role in plant reproduction and, consequently, hybridization. While pollen of seed plants use many vectors to move over long distances (Endress, 1994), sperm of mosses and ferns tend to reach archegonia by swimming in a film of water (Sharpe et al., 2010). This limitation greatly reduces the distance at which two gametophytes may interact (Schneller et al., 1990; van der Velde et al., 2001). In ferns, ploidy level may

influence sperm motility. For example, haploid sperm (of diploid species) may swim up to three times farther than diploid sperm (from tetraploid species) in *Dryopteris* (Testo et al., 2015). This increased performance of haploid sperm may increase the likelihood of the diploid species being the paternal parent of hybrids. The formation of *D. ×ambroseae* involves a diploid parent (*D. expansa*) and is more common than that of the tetraploid hybrid. However, contradicting this hypothesized mechanism, the diploid species is less likely to be the paternal parent in our study.

Some ferns also possess a mechanism affecting mating strategies via the use of antheridiogens. Antheridiogens are pheromones that female or bisexual gametophytes release to the environment, inducing development of antheridia in nearby gametophytes (Raghavan, 1989; Schneller, 2008). This system promotes outcrossing by reducing the amount of eggs and increasing the amount of sperm in proximity to female gametophytes. A system promoting outcrossing may also increase hybridization rates if the barriers are weak. However, not all species use antheridiogens and, should only one parent species be antheridiogen-sensitive, we can predict that many of its gametophytes will be male-only and that a relatively higher proportion of sperm will be formed by that parent in mixed populations of gametophytes (Testo et al., 2015). Therefore, the use of antheridiogens predisposes a species to be the paternal parent in this case. Reportedly, both *D. carthusiana* and *D. dilatata* do not react to congeneric antheridiogens (Barker, 1988; Testo et al., 2015). The third species, *D. expansa*, has not been tested yet; we are currently performing these tests. Antheridiogens could partly explain our results. For *D. ×deweveri*, the insensitivity of both parent species reduced the rates of their interactions and may effectively serve as a hybridization barrier. However, in the case of *D. ×ambroseae*, the insensitivity of *D. dilatata* precludes the use of antheridiogens as a viable explanation for the male parentage of the tetraploid. Nevertheless, future studies on dynamics of fern hybridization should take this pheromonal system into consideration.

Postzygotic barriers—Once the hybrid forms, it must overcome various postzygotic barriers. These often take the form of various genetic incompatibilities resulting in reduced viability and/or fertility. Our three studied hybrids differ markedly in abundance in nature. This difference is correlated with their genomic composition (Fig. 1). The most frequent hybrid, *D. ×ambroseae*, has as one of its parents *D. dilatata*, which originated by hybridization between the second parent, *D. expansa*, and *D. intermedia* (Fig. 1; Sessa et al., 2012b). Therefore, the formation of *D. ×ambroseae* involves merging two shared subgenomes with one different subgenome. Two of the four genomes involved in the formation of the intermediately abundant *D. ×deweveri* are probably shared by its parents, namely those of *D. intermedia* (Sessa et al., 2012b). Contrary to Juslén et al. (2011), the chloroplast

haplotype detected in our *D. dilatata* samples differed from *D. expansa* and was identical with *D. intermedia* (AY268821, FR731994) and *D. azorica* (FR731969). Finally, the parents of the very rare *D. ×sarvelae* have no genomes in common: *D. carthusiana* has I and S subgenomes, and *D. expansa* has E genome (Fig. 1; Sessa et al., 2012b).

The disparity in hybrid abundance potentially based on genetic differences may be attributable to various genetic incompatibilities. To our knowledge, these incompatibilities have not been studied explicitly in ferns. Nevertheless, improper epistatic and cytonuclear interactions or the influence of the maternal effect may limit hybrid formation in general or asymmetrically (Turelli and Moyle, 2007; Maheshwari and Barbash, 2011). Rare sex/parent combinations of *D. ×ambroseae* and *D. ×deweveri* may not be less likely to form (i.e., they may not be limited by prezygotic barriers) but simply less likely to survive. Fern sexual hybrids tend to form mostly aborted spores (Wagner and Chen, 1965; Hornych and Ekrt, 2017). So, hybrids may form and be viable, but their contribution to future generations is severely limited. Nevertheless, the presence of many polyploid taxa in ferns (Barrington et al., 1989; Wood et al., 2009; Schneider et al., 2017) indicates that hybrids occasionally regain fertility via polyploidy and form new species. However, some widely known incompatibilities do not affect ferns. For example, contrary to seed plants and some bryophytes, fern sexual determination is environmental, so incompatibilities involving sex chromosomes are inapplicable. Similarly, abnormal formation of triploid endosperm in hybrid angiosperms, limiting seed development (i.e., the "triploid block"; Köhler et al., 2010), has no analogy in ferns.

CONCLUSIONS

As in seed plants, hybridization rates in ferns may vary considerably even between closely related taxa. However, unlike in seed plants (Carney et al., 1994; Jorgensen and Andersen, 1994; Prentis et al., 2007; Lepais et al., 2009; Koutecký et al., 2011), formation of *D. ×ambroseae* is most frequent when both parents are equally abundant. Nevertheless, compared to the predictions of our random mating model, hybridization rate increases when *D. dilatata* is heavily outnumbered. This indicates that there are probably multiple factors affecting fern hybridization. In general, our results support the notion that some ferns may possess very weak barriers to hybridization.

We did not expect asymmetric hybridization in *D. ×deweveri*, and the expected direction of asymmetry in *D. ×ambroseae* was the reverse of our results. There seem to be one or more traits of *D. dilatata* that makes it the paternal parent. Some possibilities include unusual sperm motility, increased propensity toward outcrossing, or specific genetic incompatibilities. Nevertheless, the established correlation between ploidy levels and mating strategies expressed by asymmetric hybridization (e.g., Testo et al., 2015) is not universal. More research is required if we want to fully understand hybridization barriers in ferns and their effect on evolution.

AUTHOR CONTRIBUTIONS

O.H. and L.E. conceived the study. O.H., F.R., and L.E. collected field data. All authors performed laboratory analyses, contributed to writing the manuscript, and gave final approval for publication.

DATA AVAILABILITY

The sequences generated in this study are available in GenBank under accession nos. MK697576–MK697585.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. List of locations of all populations of the *Dryopteris carthusiana* group, including date of sampling, collectors (OH = Ondřej Hornych, LE = Libor Ekrt, FR = Felix Riedel), and numbers of each taxon in a population.

APPENDIX S2. List of all samples of the *Dryopteris carthusiana* group used in chloroplast (*trn*L–*trn*F region) analyses, including haplotype and collection information.

LITERATURE CITED

Arnold, M. L. 1992. Natural hybridization as an evolutionary process. *Annual Review of Ecology, Evolution, and Systematics* 1992: 237–261.

Arnold, M. L. 1997. Natural hybridization and evolution. Oxford University Press, Oxford, UK.

Arnold, M. L., and B. D. Bennett. 1993. Natural hybridization in Louisiana Irises: Genetic variation and ecological determinants. *In* R. G. Harrison [ed.], Hybrid Zones and the Evolutionary Process, 115–139. Oxford University Press, New York, USA.

Arnold, M. L., J. L. Hamrick, and B. D. Bennett. 1993. Interspecific pollen competition and reproductive isolation in *Iris. Journal of Heredity* 84: 13–16.

Baack, E., M. C. Melo, L. H. Rieseberg, and D. Ortiz-Barrientos. 2015. The origins of reproductive isolation in plants. *New Phytologist* 207: 968–984.

Bacilieri, R., A. Ducousso, R. J. Petit, and A. Kremer. 1996. Mating system and asymmetric hybridization in a mixed stand of European oaks. *Evolution* 50: 900–908.

Barker, J. 1988. The sexual expression and development in vitro of gametophytes of Dryopteris filix-mas (L.) Schott and Dryopteris dilatata (Hoffm.) A. Gray. M. Phil Thesis. Derbyshire College of Higher Education, Derby, UK.

Barrington, D. S., C. H. Haufler, and C. R. Werth. 1989. Hybridization, reticulation, and species concepts in the ferns. *American Fern Journal* 79: 55–64.

Beatty, G. E., M. Phillipp, and J. Provan. 2009. Unidirectional hybridization at a species' range boundary: implications for habitat tracking. *Diversity and Distribution* 16: 1–9.

Bennert, H. W., W. Jäger, H. Bäppler, M. Lubienski, R. Theimann, R. Viane, and A. Sarazin. 2012. *Dryopteris expansa* (C. Presl) Frasek-Jenkins & Jermy und *D. ×ambroseae* Fraser-Jenk. & Jermy (Dryopteridaceae) in Nordrhein-Westfalen – Identifizierung, Verbreitung, Ökologie. *Decheniana* 165: 37–53.

Brownsey, P. J. 1977. *Asplenium* hybrids in the New Zealand flora. *New Zealand Journal of Botany* 15: 601–637.

Buggs, R. J. A., and J. R. Pannell. 2006. Rapid displacement of a monoecious plant lineage is due to pollen swamping by a dioecious relative. *Current Biology* 16: 996–1000.

Carney, S. E., M. B. Cruzan, and M. L. Arnold. 1994. Reproductive interactions between hybridizing irises: analyses of pollen-tube growth and fertilization success. *American Journal of Botany* 81: 1169–1175.

Corley, H. V., and M. Gibby. 1981. *Dryopteris × sarvelae* in Scotland: A new hybrid for the British Isles. *Fern Gazette* 12: 178.

DeSoto, L., L. G. Quintanilla, and M. Méndez. 2008. Environmental sex determination in ferns: effects of nutrient availability and individual density in *Woodwardia radicans*. *Journal of Ecology* 96: 1319–1327.

Doležel, J., J. Greilhuber, S. Lucretti, A. Meister, M. A. Lysák, L. Nardi, and R. Obermayer. 1998. Plant genome size estimation by flow cytometry: Interlaboratory comparison. *Annals of Botany* 82: 17–26.

Doležel, J., J. Greilhuber, and J. Suda. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* 2: 2233–2244.

Ekrt, L., and P. Koutecký. 2016. Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (Dryopteridaceae). *Annals of Botany* 117: 97–106.

Ekrt, L., R. Holubová, P. Trávníček, and J. Suda. 2010. Species boundaries and frequency of hybridization in the *Dryopteris carthusiana* (Dryopteridaceae) complex: A taxonomic puzzle resolved using genome size data. *American Journal of Botany* 97: 1208–1219.

Emms, S. K., S. A. Hodges, and M. L. Arnold. 1996. Pollen-tube competition, siring success, and consistent asymmetric hybridization in Louisiana irises. *Evolution* 50: 2201–2206.

Endress, P. K. 1994. Diversity and evolutionary biology of tropical flowers. Cambridge University Press, Cambridge, UK.

Flinn, K. M. 2006. Reproductive biology of three fern species may contribute to differential colonization success in post-agricultural forests. *American Journal of Botany* 93: 1289–1294.

de Groot, G. A., B. Verduyn, E. R. J. Wubs, R. H. J. Erkens, and H. J. During. 2012. Inter- and intraspecific variation in fern mating systems after long-distance colonization: the importance of selfing. *BMC Plant Biology* 12: 3.

Hamzeh, M., C. Sawchyn, P. Périnet, and S. Dayanandan. 2007. Asymmetrical natural hybridization between *Populus deltoides* and *P. balsamifera* (Salicaceae). *Canadian Journal of Botany* 85: 1227–1232.

Hanušová, K., M. Čertner, T. Urfus, P. Koutecký, J. Košnar, C. J. Rothfels, V. Jarolímová, et al. 2019. Widespread co-occurrence of multiple ploidy levels in fragile ferns (*Cystopteris fragilis* complex; Cystopteridaceae) likely stems from similar ecology of cytotypes, their efficient dispersal and inter-ploidy hybridization. *Annals of Botany* 123: 845–855.

Hori, K., Y. Okuyama, Y. Watano, and N. Murakami. 2018. Recurrent hybridization without homoeologous chromosome paring in the *Dryopteris varia* complex (Dryopteridaceae). *Chromosome Botany* 13: 9–24.

Hornych, O., and L. Ekrt. 2017. Spore abortion index (SAI) as a perspective tool of evaluation of spore fitness in ferns: An insight into sexual and apomictic species. *Plant Systematics and Evolution* 303: 497–507.

Hunt, H. V., S. W. Ansell, S. J. Russell, H. Schneider, and J. C. Vogel. 2011. Dynamics of polyploid formation and establishment in the allotetraploid rock fern *Asplenium majoricum*. *Annals of Botany* 108: 143–157.

Jessen, S., and H. Rasbach. 1987. *Dryopteris × sarvelae*, erster Nachweis für Mitteleuropa. Abstammung, verwandte Arten und Hybriden (Pteridophyta). *Gleditschia* 15: 57–72.

Jorgensen, R. B., and B. Andersen. 1994. Spontaneous hybridization between oilseed rape (*Brassica napus*) and weedy *B. campestris* (Brassicaceae): A risk of growing genetically modified oilseed rape. *American Journal of Botany* 81: 1620–1626.

Juslén, A., H. Väre, and N. Wikström. 2011. Relationships and evolutionary origins of polyploid *Dryopteris* (Dryopteridaceae) from Europe inferred using nuclear *pgiC* and plastid *trnL-F* sequence data. *Taxon* 60: 1284–1294.

Kaplan, Z., J. Danihelka, J. Štěpánková, L. Ekrt, J. Chrtek Jr, J. Zázvorka, V. Grulich, et al. 2016. Distributions of vascular plants in the Czech Republic. Part 2. *Preslia* 88: 229–322.

Kentner, E. K., and M. R. Mesler. 2000. Evidence for natural selection in a fern hybrid zone. *American Journal of Botany* 87: 1168–1174.

Knobloch, I. W., M. Gibby, and C. Fraser-Jenkins. 1984. Recent advances in our understanding of pteridophyte hybrids. *Taxon* 33: 256–270.

Köhler, C., O. Mittelsten Scheid, and A. Erilova. 2010. The impact of the triploid block on the origin and evolution of polyploid plants. *Trends in Genetics* 26: 142–148.

Korpelainen, H. 1994. Growth, sex determination and reproduction of *Dryopteris filix-mas* (L.) Schott gametophytes under varying nutritional conditions. *Botanical Journal of the Linnean Society* 114: 357–366.

Koutecký, P., T. Baďurová, M. Štech, J. Košnar, and J. Karásek. 2011. Hybridization between diploid *Centaurea pseudophrygia* and tetraploid *C. jacea* (Asteraceae): the role of mixed pollination, unreduced gametes, and mentor effects. *Biological Journal of the Linnean Society* 104: 93–106.

Landis, J. B., D. E. Soltis, Z. Li, H. E. Marx, M. S. Barker, D. C. Tank, and P. S. Soltis. 2018. Impact of whole-genome duplication events on diversification rates in angiosperms. *American Journal of Botany* 105: 348–363.

Lepais, O., R. J. Petit, E. Guichoux, E. Lavabre, F. Alberto, A. Kremer, and S. Gerber. 2009. Species relative abundance and direction of introgression in oaks. *Molecular Ecology* 18: 2228–2242.

Lewis, D., and L. K. Crowe. 1958. Unilateral interspecific incompatibility in flowering plants. *Heredity* 12: 233–256.

Li, F. W., B. C. Tan, V. Buchbender, R. C. Moran, G. Rouhan, C. N. Wang, and D. Quandt. 2009. Identifying a mysterious aquatic fern gametophyte. *Plant Systematics and Evolution* 281: 77–86.

Ma, Y., W. Xie, X. Tian, W. Sun, Z. Wu, and R. Milne. 2014. Unidirectional hybridization and reproductive barriers between two heterostylous primrose species in north-west Yunnan, China. *Annals of Botany* 113: 763–775.

Maheshwari, S., and D. A. Barbash. 2011. The genetics of hybrid incompatibilities. *Annual Review of Genetics* 45: 331–355.

Masuyama, S. 1979. Reproductive biology of the fern *Phegopteris decursive-pinnata*. I. The dissimilar mating systems of diploids and tetraploids. *Botanical Magazine (Tokyo)* 92: 275–289.

Natcheva, R., and N. Cronberg. 2004. What do we know about hybridization among bryophytes in nature? *Canadian Journal of Botany* 82: 1687–1704.

Nieto-Lugilde, M., O. Werner, S. F. McDaniel, P. Koutecký, J. Kučera, S. M. Rizk, and R. M. Ros. 2018. Peripatric speciation associated with genome expansion and female-biased sex ratios in the moss genus *Ceratodon*. *American Journal of Botany* 105: 1–12.

Njuguna, W., A. Liston, R. Cronn, T.-L. Ashman, and N. Bassil. 2013. Insights into phylogeny, sex function and age of *Fragaria* based on whole chloroplast genome sequencing. *Molecular Phylogenetics and Evolution* 66: 17–29.

Otto, S. P., and J. Whitton. 2000. Polyploid incidence and evolution. *Annual Review of Genetics* 34: 401–437.

Ouayjan, A., and A. Hampe. 2018. Extensive sib-mating in a refugial population of beech (*Fagus sylvatica*) growing along a lowland river. *Forest Ecology and Management* 407: 66–74.

Pangua, E., L. G. Quintanilla, A. Sancho, and S. Pajarón. 2003. A comparative study of the gametophytic generation in the *Polystichum aculeatum* group (Pteridophyta). *International Journal of Plant Sciences* 164: 295–303.

Peng, C.-I., and T.-Y. Chiang. 2000. Molecular confirmation of unidirectional hybridization in *Begonia x taipeiensis* Peng (Begoniaceae) from Taiwan. *Annals of the Missouri Botanical Garden* 87: 273–285.

Peredo, E. L., M. Méndez-Couz, M. A. Revilla, and H. Fernández. 2013. Mating system in *Blechnum spicant* and *Dryopteris affinis* ssp. *affinis* correlates with genetic variability. *American Fern Journal* 103: 27–39.

Perley, D. S., and L. K. Jesson. 2015. Hybridization is associated with changes in sexual system in the bryophyte genus *Atrichum*. *American Journal of Botany* 102: 555–565.

Petit, R. J., E. Pineau, B. Demesure, R. Bacilieri, A. Ducousso, and A. Kremer. 1997. Chloroplast DNA footprints of postglacial recolonization by oaks. *Proceedings of the National Academy of Sciences, USA* 94: 9996–10001.

Prentis, P. J., E. M. White, I. J. Radford, A. J. Lowe, and A. R. Clarke. 2007. Can hybridization cause local extinction: a case for demographic swamping of the Australian native *Senecio pinnatifolius* by the invasive *Senecio madagascariensis*? *New Phytologist* 176: 902–912.

R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Website http://www.R-project.org/ [accessed 19 March 2019].

Raghavan, V. 1989. Developmental biology of fern gametophytes. Cambridge University Press, New York, USA.

Reichstein, T. 1981. Hybrids in European Aspleniaceae (Pteridophyta). *Botanica Helvetica* 91: 89–139.

Rieseberg, L. H., and S. E. Carney. 1998. Plant hybridization. *New Phytologist* 140: 599–624.

Rieseberg, L. H., A. Desrochers, and S. J. Youn. 1995. Interspecific pollen competition as a reproductive barrier between sympatric species of *Helianthus* (Asteraceae). *American Journal of Botany* 82: 515–519.

Rieseberg, L. H., S. J. E. Baird, and A. M. Desrochers. 1998. Patterns of mating in wild sunflower hybrid zones. *Evolution* 52: 713–726.

Rothfels, C. J., A. K. Johnson, P. H. Hovenkamp, D. L. Swofford, H. C. Roskam, C. R. Fraser-Jenkins, M. D. Windham, and K. M. Pryer. 2015. Natural hybridization between parental lineages that diverged approximately 60 million years ago. *American Naturalist* 185: 433–442.

Rünk, K., M. Zobel, and K. Zobel. 2010. Different factors govern the performance of three closely related and ecologically similar *Dryopteris* species with contrastingly different abundance in transplant experiment. *Botany* 88: 961–969.

Rünk, K., Z. Zobel, and K. Zobel. 2012. Biological flora of the British Isles: *Dryopteris carthusiana*, *D. dilatata* and *D. expansa*. *Journal of Ecology* 100: 1039–1063.

Schneider, H., H. M. Liu, Y. F. Chang, D. Ohlsen, L. R. Perrie, L. Shepherd, M. Kessler, et al. 2017. Neo- and paleopolyploidy contribute to the species diversity of *Asplenium* – the most species-rich genus of ferns. *Journal of Systematics and Evolution* 55: 353–364.

Schneller, J. J. 2008. Antheridiogens. *In* T. A. Ranker and C. H. Haufler [eds.], Biology and evolution of ferns and lycophytes. Cambridge University Press, Cambridge, UK.

Schneller, J. J., C. H. Haufler, and T. A. Ranker. 1990. Antheridiogens and natural gametophyte populations. *American Fern Journal* 80: 143–152.

Schnittler, M., K. Horn, R. Kaufmann, R. Rimgailė-Voicik, A. Klahr, M. Bog, J. Fuchs, and H. W. Bennert. 2018. Genetic diversity and hybrid formation in Central European club-mosses (*Diphasiastrum*, Lycopodiaceae) – new insights from cp microsatellites, two nuclear markers and AFLP. *Molecular Phylogenetics and Evolution* 131: 181–192.

Sessa, E. B., E. A. Zimmer, and T. J. Givnish. 2012a. Reticulate evolution on a global scale: A nuclear phylogeny for New World *Dryopteris* (Dryopteridaceae). – *Molecular Phylogenetics and Evolution* 64: 563–581.

Sessa, E. B., E. A. Zimmer, and T. J. Givnish. 2012b. Unraveling reticulate evolution in North American *Dryopteris* (Dryopteridaceae). *BMC Evolutionary Biology* 12: 104.

Sessa, E. B., W. L. Testo, and J. E. Watkins Jr. 2016. On the widespread capacity for, and functional significance of, extreme inbreeding in ferns. *New Phytologist* 211: 1108–1119.

Sharpe, J. M., K. Mehltreter, and L. R. Walker. 2010. Ecological importance of ferns. *In* K. Mehltreter, L. R. Walker and J. M. Sharpe [eds.], Fern ecology, 1–21. Cambridge University Press, Cambridge, UK.

Shaw, J. 1994. Systematics of *Mielichhoferia* (Bryaceae: Musci). III. Hybridization between *M. elongate* and *M. mielichhoferiana*. *American Journal of Botany* 81: 782–790.

Sigel, E. M. 2016. Genetic and genomic aspects of hybridization in ferns. *Journal of Systematics and Evolution* 54: 638–655.

Soltis, D. E., and P. S. Soltis. 1987. Breeding system of the fern *Dryopteris expansa*: Evidence for mixed mating. *American Journal of Botany* 74: 504–509.

Soltis, P. S., and D. E. Soltis. 2000. The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences, USA* 97: 7051–7057.

Soltis, D. E., C. J. Visger, D. B. Marchant, and P. S. Soltis. 2000. Polyploidy: Pitfalls and paths to a paradigm. *American Journal of Botany* 103: 1146–1166.

Sorsa, V., and C. J. Widén. 1968. The *Dryopteris spinulosa* complex in Finland, a cytological and chromatographic study of some hybrids. *Hereditas* 60: 273–293.

Stein, D. B., C. Hutton, D. S. Conant, C. H. Haufler, and C. R. Werth. 2010. Reconstructing *Dryopteris "semicristata"* (Dryopteridaceae): Molecular profiles of tetraploids verify their undiscovered diploid ancestor. *American Journal of Botany* 97: 998–1004.

Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three noncoding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.

Testo, W. L., J. E. Watkins, and D. S. Barrington. 2015. Dynamics of asymmetrical hybridization in North American wood ferns: reconciling patterns of inheritance with gametophyte reproductive biology. *New Phytologist* 206: 785–795.

Thiers, B. 2019 onward (continuously updated). Index Herbariorum: a global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. Available at http://sweetgum.nybg.org/science/ih/ [accessed 28 March 2019].

Trucco, F., T. Tatum, A. L. Rayburn, and P. J. Tranel. 2009. Out of the swamp: unidirectional hybridization with weedy species may explain the prevalence of *Amaranthus tuberculatus* as a weed. *New Phytologist* 184: 819–827.

Turelli, M., and L. C. Moyle. 2007. Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. *Genetics* 176: 1059–1088.

Twyford, A. D., and R. A. Ennos. 2012. Next-generation hybridization and introgression. *Heredity* 108: 179–189.

van der Velde, M., and R. Bijlsma. 2004. Hybridization and asymmetric reproductive isolation between the closely related bryophyte taxa *Polytrichum commune* and *P. uliginosum*. *Molecular Ecology* 13: 1447–1454.

van der Velde, M., H. J. During, L. van de Zande, and R. Bijlsma. 2001. The reproductive biology of Polytrichum formosum: clonal structure and paternity revealed by microsatellites. *Molecular Ecology* 10: 2423–2434.

Vogel, J. C., S. J. Russell, F. J. Rumsey, J. A. Barrett, and M. Gibby. 1998. On hybrid formation in the rock fern *Asplenium ×alternifolium* (Aspleniaceae, Pteridophyta). *Botanica Acta* 111: 241–246.

Wagner, W. H. Jr, and L. C. Chen. 1965. Abortion of spores and sporangia as a tool in the detection of *Dryopteris* hybrids. *American Fern Journal* 55: 9–29.

Widén, C. J., J. Sarvela, and T. Ahti. 1967. The *Dryopteris spinulosa* complex in Finland. *Acta Botanica Fennica* 77: 1–25.

Wood, T. E., N. Takebayashi, M. S. Barker, I. Mayrose, P. B. Greenspoon, and L. H. Rieseberg. 2009. The frequency of polyploid speciation in vascular plants. *Proceedings of the National Academy of Sciences, USA* 106: 13875–13879.

Wubs, E. R. J., G. A. de Groot, H. J. During, J. C. Vogel, M. Grundmann, P. Bremer, and H. Schneider. 2010. Mixed mating system in the fern *Asplenium scolopendrium*: implications for colonization potential. *Annals of Botany* 106: 583–590.

Xiang, L., C. R. Werth, S. N. Emery, and D. E. McCauley. 2000. Population-specific gender-biased hybridization between *Dryopteris intermedia* and *D. carthusiana*: evidence from chloroplast DNA. *American Journal of Botany* 87: 1175–1180.

Zhang, R., T. Liu, W. Wu, Y. Li, L. Chao, L. Huang, Y. Huang, et al. 2013. Molecular evidence for natural hybridization in the mangrove fern genus *Acrostichum*. *BMC Plant Biology* 13: 74.

Zhou, R., X. Gong, D. Boufford, C.-I. Wu, and S. Shi. 2008. Testing a hypothesis of unidirectional hybridization in plants: Observations on *Sonneratia*, *Bruguiera* and *Ligularia*. *BMC Evolutionary Biology* 8: 149.

Paper 11

Ekrt L., Podroužek J., Hornych O., Košnar J. & Koutecký P. (2021): Cytotypes of bracken (*Pteridium aquilinum*) in Europe: widespread diploids and scattered triploids of likely multiple origin. – Flora 274: 151725.

Cytotypes of bracken (*Pteridium aquilinum*) in Europe: widespread diploids and scattered triploids of likely multiple origin

Libor Ekrt [*], Jan Podroužek, Ondřej Hornych, Jiří Košnar, Petr Koutecký

Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, České Budějovice, CZ 37005, Czech Republic

A B S T R A C T

Polyploidization is an important speciation and evolution mechanism in ferns. Initially, new cytotypes face challenges in maintaining themselves within the majority cytotype populations. Unlike in most even-ploidy cytotypes, fern triploids are often apomictic or infertile, due to genetic imbalance. An interesting opportunity to study these phenomena has emerged with the discovery of triploid bracken (*Pteridium aquilinum*), a triploid fern that is fertile but not apomictic. Originally found in one Welsh population, the distribution of this cytotype in Europe is unknown as is its origin and how it maintains itself among the presumed diploid majority. We sampled 135 populations of *P. aquilinum*, focusing on Central Europe. Ploidy level of all samples was analyzed by flow cytometry. We compared the two cytotypes via micromorphological characters (spore and stomata size), fertility characteristics (spore abortion and proportion of populations with sporangia-bearing fronds). Additionally, genetic difference between ploidy levels was tested as well. The diploid cytotype of *P. aquilinum* is dominant in continental Europe with 121 entirely diploid populations found, but we also found 9 mixed and 5 entirely triploid populations. Fertile diploid and triploid plants were found only in 17.7% and 21.4% of populations, respectively. The cytotypes are distinguishable using both tested micromorphological characters, but stomata are more reliable due to overall reduced fertility. Unlike the Welsh specimen, our tested triploid has most spores aborted, ca 97.4%, compared to mean 6.0% of spores aborted in diploids. The triploid cytotype is rare and likely originated multiple times from the diploids and relies on clonal and possibly limited sexual reproduction to maintain itself. However, diploids and triploids are often genetically different within a population, indicating that the triploid may migrate between populations. Due to its vegetative growth and presumed continuous formation, the triploid cytotype is likely to remain established in Central Europe, although in small numbers.

1. Introduction

Polyploidization is an important mechanism of plant evolution (Otto and Whitton, 2000; Soltis and Soltis, 2009; Landis et al., 2018) often resulting in the formation of new species (Rieseberg and Willis, 2007; Wood et al., 2009). Polyploids may be ecologically separated from their parental diploids (Rodriguez, 1996; Brochmann et al., 2004), but diploids and polyploids usually coexist in mixed populations (Kolář et al., 2017; Hanušová et al., 2019). Consequently, newly emergent polyploids face challenges summarized by the minority cytotype exclusion principle (Levin, 1975). According to the minority cytotype exclusion principle (Levin, 1975), newly formed polyploids are initially rare and may have trouble finding appropriate mates of its own cytotype, resulting in a large proportion of ineffective mating with the majority cytotype.

There are several means of escaping minority cytotype exclusion, such as niche differentiation (Baack, 2005, Pangua et al., 2019), self-fertilization (Rodriguez, 1996) or asexual reproduction, including apomixis (Baldwin and Husband, 2013; Chung et al., 2015). The principle was initially described for angiosperms and its application in ferns must consider their specific reproduction features. Most ferns are perennial and polycarpic while also being capable of self-fertilization (Sessa et al., 2016). These characteristics may reduce the potential impact of minority cytotype disadvantage (Rodriguez, 1996). It is possibly due to this combination of characteristics that speciation is more commonly associated with polyploidization in ferns than in angiosperms (Wood et al., 2009). Despite the success of many fern polyploids, established triploid lineages are generally rare in ferns because of additional problems associated with their unbalanced genome.

Like in angiosperms (Popelka et al., 2019, Chrtek et al., 2020), triploid ferns tend to have severely limited reproductive capabilities or may even be completely sterile (Hornych and Ekrt, 2017; Hornych et al., 2019; Hori, 2020), due to their uneven ploidy level. Alternatively, triploids may reproduce via apomixis (Hori et al., 2017; Saggoo and Kaur, 2017), the formation of sporophytes without syngamy (Grusz, 2016). A notable exception is the triploid *Pteridium aquilinum* (L.) Kuhn discovered by Sheffield et al. (1993) in a single population. This cyto-type formed a large proportion of viable spores capable of germination and yet apomixis was deemed unlikely. This set of characteristics in *P. aquilinum* is highly unusual in ferns and requires further study.

In the taxonomic treatment of Tryon (1941), *P. aquilinum* was the sole species in the genus. However, contemporary studies recognize two vicariant diploid species, one of which, *P. aquilinum*, is occurring in the northern hemisphere and Africa (Der et al., 2009; Wolf et al., 2019). Several polyploids were also described, including the aforementioned triploid discovered by Sheffield et al. (1993) and two other allotetra-ploid (of hybrid origin) species (Wolf et al., 2019). The diploid chromosome count is considered to be 2n=104 (summarized by Sheffield et al., 1993). A lower count (2n=52) has been reported from Spain (Löve and Kjellqvist, 1972), but other researchers (Sheffield et al., 1989a) were unable to verify this account. The taxonomy of *P. aquilinum* is complicated and unresolved (Thomson, 2000; Thomson, 2004) but we will follow the simplified two diploid species concept with the diploid chromosome count being 104 (Der et al., 2009; Wolf et al., 2019). As the triploid cytotype was originally described in Wales, Great Britain, the focus of this study will be on European *P. aquilinum*.

In Europe, *P. aquilinum* is considered an aggressive colonizer and a weed, growing in woods, pastures, abandoned fields and various other disturbed habitats (Conway, 1949; Page, 1976). The success of *P. aquilinum* is likely due to a combination of sexual and clonal reproduction. Plants form up to 300 million tiny airborne spores per frond (Conway, 1957) to spread over long distances. Once established, a vigorous rhizome growth enables a quick colonization of the site (Conway, 1949; Oinonen, 1967). Due to the paucity of young sporophytes records in nature (Conway, 1953; Page, 1976) and the presence of many populations without any sporangia-bearing fronds (Conway, 1957; Kaplan et al., 2018), the extent of sexual reproduction in *P. aquilinum* is uncertain.

In previous research using isozyme markers, *P. aquilinum* was deemed to be predominantly outcrossing (Speer et al., 1998; Wolf et al., 1991), despite its homospory enabling gametophytic selfing (Haufler et al., 2016). Analyzing markers such as nuclear microsatellite DNA reveals the patterns of genetic variation (Jiménez et al., 2010) and allows us to study the likely origin of the triploid cytotype as well. However, the triploid first needs to be found and confirmed.

The triploid cytotype of *P. aquilinum* was originally discovered via a peculiar isozyme pattern and its ploidy level was subsequently confirmed by chromosome counting (Sheffield et al., 1993). However, a more expedient method exists today for quickly estimating the ploidy level of plants, flow cytometry (Ekrt and Koutecký, 2016; Hanušová et al., 2019, Liang et al., 2019). Similarly, the size of stomata and spores may be used for the same purpose as these often correlate well with ploidy levels in ferns (Barrington et al., 2020). Furthermore, stomata and spore size measurements of a small number of diploid and triploid plants was provided by Sheffield et al. (1993), so plants from continental Europe can be directly compared with the first discovered population in Wales.

The focus of our study lies in the unusually fertile but not apomictic triploid cytotype of a widespread weed *P. aquilinum*. We ask the following questions: Can triploid *P. aquilinum* be distinguished by molecular markers, ploidy related characteristics (spore, stomata size) and flow cytometry? How prevalent is the triploid cytotype in continental Europe? Are the triploids of a single origin or did they form several times?

2. Material and methods

2.1. Field Collection

A total of 135 populations of *Pteridium aquilinum* were sampled during 2013–2019 across Europe (see Supplement 1). Usually, ten fronds were collected per population. Fronds were collected ca 10 meters apart to limit the chance of sampling of the same clone. Herbarium vouchers are deposited in CBFS.

2.2. Flow cytometry

Genome size of all fronds collected was analyzed using flow cytometry. Fresh tissue was used to determine DNA ploidy levels. Fluorescence intensity of 3000 particles was analyzed using a Partec PA II flow cytometer equipped with a mercury arc lamp (Partec, now part of Sysmex, Münster, Germany), employing DAPI as a fluorescent stain. Samples were prepared using the simplified two step protocol described by Doležel et al. (2007), using *Vicia faba* 'Inovec' (2C=26.90 pg; Doležel et al., 1992) as an internal standard. Up to five fronds were pooled together and reanalyzed individually if multiple DNA ploidy levels occurred.

The resultant histograms were evaluated using FloMax 2.6 (Partec, now part of Sysmex, Münster, Germany), recording mean fluorescence, coefficient of variation and number of nuclei for all fluorescent peaks. Relative genome size was calculated as the ratio between the mean fluorescence of the sample and the internal standard.

2.3. Fertility

Fertility of *Pteridium aquilinum* was studied by determining the fertility of fronds and by calculating the spore abortion index (SAI), the percentage of aborted spores (Hornych and Ekrt, 2017). A frond was considered fertile, if it possessed developed sporangia. The presence of indusia was not enough to consider the plant fertile. All samples collected for this study had ploidy level determined, so a comparison of fertile frond formation between ploidy levels was possible. Spore abortion was evaluated for five diploid plants (locations L11, L19, L21, L52 and L93) and one triploid plant (location L128) using the methodology of Hornych and Ekrt (2017) except that only 500 spores were used per plant due to the lack of spores. Only a single triploid plant was used as fertile triploids from other populations did not have enough spores left for counting SAI.

2.4. Stomatal and spore sizes

Stomatal (guard cell length) and spore (exospore length) sizes were measured as additional ploidy-related factors under a light microscope (Olympus BX50) at 1000x magnification with QuickPHOTO MICRO 3.1 (Promicra). Ten diploid plants (locations L26 and L100) and ten triploid plants (locations L11, L23, L30, L32, L69, L79, L97 and L99) were studied, 20 stomata per plant. Spore size was measured for ten diploid plants (locations L11, L19, L21, L52, L55, L93 and L104) and six triploid plants (locations L26, L99 and L128) using 20 spores per plant. Most spores produced by the triploids were aborted and were not included in this measurement. Due to the presence of very large spores with irregular shapes, spores larger than 50 μm were not included within the 20 spores per plant measured and were excluded from statistical analyses. Measurement was taken as distance between the two most distant points of the spore. The differences in stomata and spore sizes between cytotypes were compared by one-factor ANOVA using R version 3.4.3 (R Core Team, 2017).

2.5. Genetic variation

Microsatellite markers were used to analyze genetic variation of

P. aquilinum. A total of 237 fronds (118 diploid and 119 triploid) from 17 populations in nine regions were analyzed (Table 1). Of the 17 populations, six contained only diploid fronds, three only triploids and eight populations were mixed. Within this study, two loci (Pter12 and Pter04) published by Chen et al. (2008) were used, others were not sufficiently variable. To complement them, five new microsatellite loci (Table 2) were developed based on transcriptome data by Der et al. (2011) using Primer3 (Koressaar et al., 2007; Untergasser et al., 2012).

Total genomic DNA was extracted using the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany). Isolates were diluted 1:20 with sterile water. PCR amplification was performed as described by Schuelke (2000). The PCR mixture contained 1 µl of template DNA, 0.3 µM fluorescent labelled M13 forward primer, 0.075 µM of specific forward-tailed primer, 0.3 µM specific reverse primer, 2.5 µl of 2x Plain PP Master Mix (Top-Bio, Czech Republic), and 0.15 µl of sterile water to make up a final volume of 5 µL. The equimolar pools of resultant PCR products from each frond were sent for a fragment analysis at SEQme company (Dobříš, Czech Republic).

The results of the fragment analysis were evaluated using Gene-Marker 1.8 (SoftGenetics, LLC.). Due to the presence of polyploid samples and inconsistent numbers of alleles per locus for both ploidy levels (up to four and five alleles for diploid and triploid samples, respectively) the data were scored as dominant (i.e., presence/absence of each allele). Relationship among individual sampled fronds and structure of genetic variation were evaluated using neighbor joining (NJ) tree, Principal Coordinate Analysis (PCoA) and Analysis of Molecular Variance (AMOVA) performed in FAMD 1.2 (Schlüter and Harris, 2006) using Standard Similarity distances. The main AMOVA groups were the two ploidy levels and subgroups were populations (for mixed populations, each ploidy level considered separately). Private alleles for both cytotypes were detected in FAMD 1.2.

Table 1

General information about the populations of *Pteridium aquilinum* used in microsatellite analysis. Locations CS-2, CS-4b and CS-4c were sampled in 2015 and 2016 and the fronds were pooled.

Pop. ID (code in Supplement 1)	Location	Region	Ploidy level	No. of fronds (diploids + triploids for mixed-ploidy populations)
AUS (L97)	Walcherbauer	Austria	2x+3x	10 (8+2)
CN (L21)	Chřibská	N Bohemia	2x	10
CS-1 (L11)	Mříč	S Bohemia	2x+3x	10 (9+1)
CS-2 (L26)	Pernek	S Bohemia	2x+3x	35 (16+19)
CS-3 (L99)	Loučovice	S Bohemia	2x+3x	22 (10+12)
CS-4a (L7)	Třeboňsko basin Mladošovice	S Bohemia	2x+3x	10 (9+1)
CS-4b (L10)	Třeboňsko basin, rybník Kukla	S Bohemia	2x+3x	40 (10+30)
CS-4c (L9)	Třeboňsko basin, Holičky	S Bohemia	2x+3x	40 (15+25)
CW-1 (L68)	Rokycansko, Kokotský ryb.	W Bohemia	2x	10
CW-2 (L69)	Rokycansko, Čilina	W Bohemia	3x	10
CW-3 (L79)	Rokycansko, Dobřív	W Bohemia	3x	10
FRA1 (L44)	St. Guilhem	France 1	2x	5
FRA2 (L48)	Laveissière	France 2	2x	5
GER1 (L30)	Settichen	Germany 1	3x	5
GER2 (L31)	Lietzow	Germany 2	2x	5
NOR (L34)	Hemsjoen	Norway	2x	5
SWE (L32)	Skövde	Sweden	2x+3x	5 (1+4)

3. Results

3.1. DNA ploidy level

A total of 1456 fronds of *Pteridium aquilinum* among 135 populations were determined as diploid or triploid via flow cytometry. The majority, 1293 (88.8%), of the samples were diploid, only 163 (11.2%) were triploid. Solely diploid populations were the most prevalent, numbering 121, followed by 9 mixed and 5 triploid populations. During our study, diploids were found commonly throughout the studied area and triploids were revealed rarely in Norway, Sweden, Germany, Switzerland, the Czech Republic and Austria. There seems to be no obvious geographical patterns in distribution of cytotypes (Fig. 1.).

Using *Vicia faba* 'Inovec' as the internal standard, the ratio of relative fluorescence averages 0.418 (SEM = 0.001, min 0.376, max 0.440) for diploid and 0.616 (SEM = 0.003, min 0.539, max 0.650) for triploid DNA ploidy levels, allowing for a clear DNA ploidy level determination (Fig. 2). The relative monoploid fluorescence (the ratio of relative fluorescence divided by ploidy level) averages are 0.209 and 0.200 for diploids and triploids, respectively.

3.2. Fertility

The proportion of populations containing at least one fertile diploid (23 of 130 – 17.7%) and one fertile triploid frond (3 of 14 – 21.4%) is similar. For both cytotypes, the number of fertile fronds per population ranged from one to all fronds. Among the three populations sampled in two consecutive years, two changed from fertile to sterile from 2015 to 2016, while one remained sterile.

Spores of diploid plants were mostly well-developed, the mean SAI of the five diploid plants studied was 6.0% (min. 3.0%, max. 9.6%). In contrast, spores of the triploid were predominantly aborted (SAI 97.4%).

3.3. Stomatal and spore sizes

Stomatal measurements reveal a significant difference between diploid and triploid fronds (F=166.05; df=1, 18; p<0.001). Stomatal size averaged 36.6 µm in diploids and 46.9 µm in triploids, there is no overlap in stomatal size between cytotypes. (Fig. 3). Similarly, spore size differs significantly (F=19.47; DF=1, 14; p<0.001) between ploidy levels (Fig. 3). Spores of diploids average 30.1 µm in length. Comparably, triploids produce two classes of well-developed spores: regular tetrahedral spores (presumably reduced) with average length of 33.8 µm and larger spherical spores (presumably unreduced diplospores) 50–70 µm in length. These large spores were found in all analyzed triploid samples.

3.4. Genetic variation

A total of 237 samples were analyzed from 17 populations, including diploid, triploid and mixed populations. Four private alleles were found in each cytotypes. No genotype shared by the two ploidy levels was observed. Except for a single population (L69), all populations were genetically variable. The results from AMOVA (Table 3) indicate that most variation occurred among populations within ploidy level (88%), followed by variation within populations (22%), whereas no variation was found between ploidy levels (-10%).

Similarly, both PCoA and NJ tree of analyzed populations (Fig. 4, 5A) also indicate no separation of ploidy levels. Distinct clustering of plants from the same population was always found in ploidy-uniform populations. In mixed-ploidy populations, the two ploidy levels formed distinct separate clusters in case of two populations (SWE, AUS), or showed various clustering in Czech populations.

In the two Czech areas with more dense populations sampling (CW region - W Bohemia, CS region - S Bohemia), samples from individual populations formed separate clusters (CW region – three ploidy-uniform

Table 2
Basic characteristics of loci used in microsatellite analysis of Pteridium aquilinum. Loci Pt12cp and Pt04 were taken from Chen et al. (2008). Pt12cp is a plastid locus, all others are nuclear.

Marker name	Primer sequence (5′ → 3′)	T_a – primer annealing temperature (°C)	Fluorescent labeling	size (bp)	No. of markers	motif
AC1	F: GCTCAAAACACGGACACACA	57	VIC	307-321	7	$(CA)_{10-18}$
	R: GGGTGAAGGTATGGAAGAGG					
AG3	F: ATTGACGGCAGTAGCGGTAT	57	6-FAM	374-392	8	$(AG)_{10-19}$
	R: ATCGAAGACCCCATCTACCC					
TGA7	F: GATGAGGACGGTCTCTTTGC	57	PET	167-191	9	$(TGA)_{14-22}$
	R: CTGATCATCGGAGCAGCTTA					
TTC8	F: CGGCTTGAACACCTCCATAA	57	PET	297-312	6	$(TTC)_{12-17}$
	R: CAGTGCCCATACCTTACCAA					
TG12	F: ATGAGCGAGCAGAAGCTAGG	57	VIC	191-212	12	$(TG)_{13-24}$
	R: TGCTGCAGAGTGTGTCTAGAGTG					
Pt12cp	F: TGGTGAAGTTGTGATGCCTAC	57	NED	379-389	3	$(AT)_{15-21}$
	R: TATCGGTGGAAAGAAAGAGTG					
Pt04	F: ATCAAGCCAAGGTCAC	48	6-FAM	231-233	2	$(CA)_{14-15}$
	R: AACCCATGATTGCTAAT					
Total					**47**	
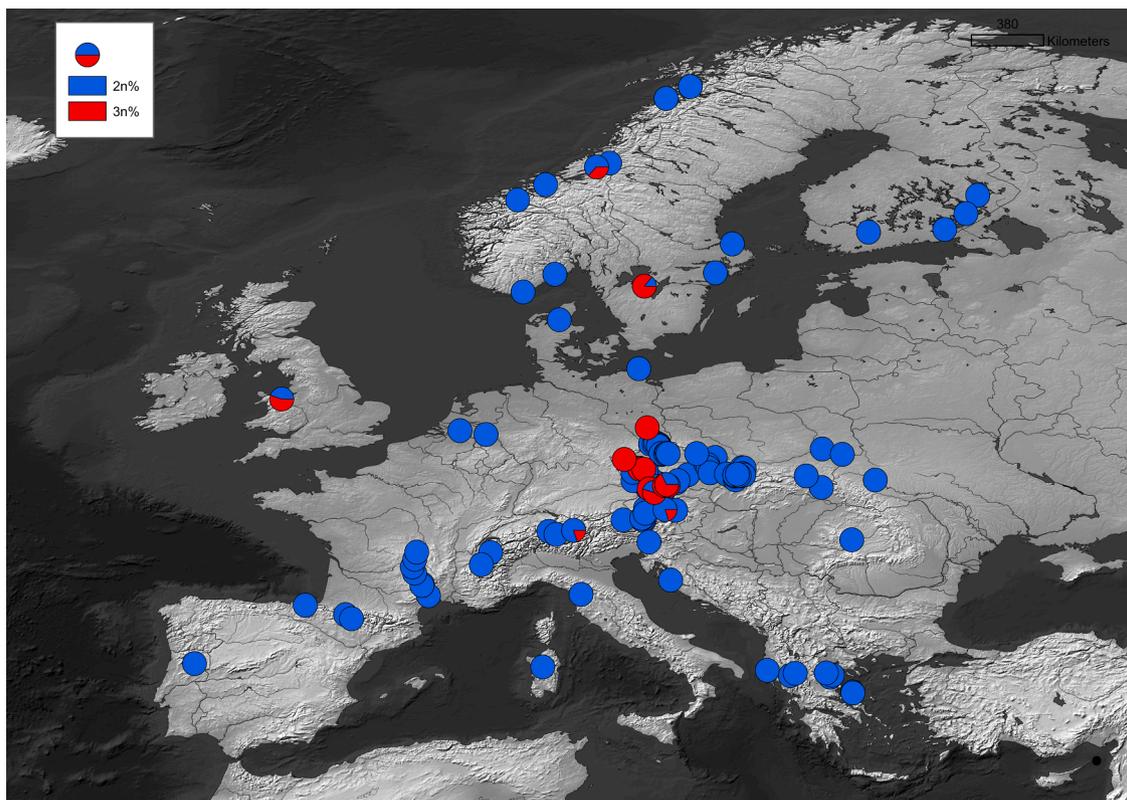
Fig. 1. Spatial distribution of cytotypes in *Pteridium aquilinum* in Europe. Mixed-ploidy populations are depicted as pie charts showing the local frequency of DNA ploidy levels. Triploids and mixed-ploidy populations are situated in the front of diploids for improved clarity. The map consists of our data (localities in Supplement 1) except occurrence in Great Britain, which is taken from Sheffield et al. (1993).

populations) or were admixed, usually with plants from other populations of the same region only (CS region – six mixed-ploidy populations, Fig. 5B). Among mixed-ploidy populations of CS region, triploids were related to lineages involving diploids from the same population in two cases (populations CS-2 and CS-3), or to diploids from other populations within the CS region in four cases (populations CS-1, CS-4a, CS-4b, CS-4c). However, in case of three adjacent populations of Třeboň basin (CS-4a, CS-4b, CS-4c), triploids from two populations were closely related to diploids from other populations of the basin (CS-4a, CS-4c), and only triploids of CS-4b were related to other CS region populations outside the basin.

4. Discussion

4.1. Distinguishing the cytotypes

Using flow cytometry, we identified two different cytotypes in *Pteridium aquilinum* in continental Europe. The difference in relative fluorescence (ca 50%) support the concept of a diploid-triploid complex, matching the findings of Sheffield et al. (1993), who provided chromosome counts for both ploidy levels, basing the triploid level calculation on a single specimen from Wales. Although there were no obvious macromorphological differences, the two cytotypes can also be distinguished by microscopic characteristics.

Spore and stomata size correlate well with ploidy level in ferns
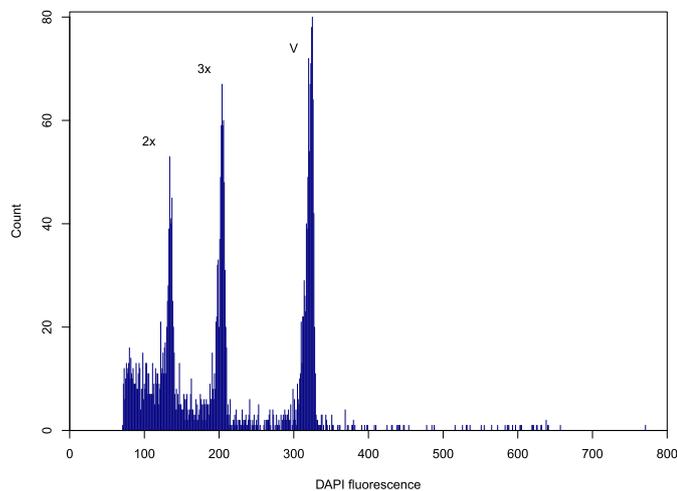
Fig. 2. A flow cytometry histogram estimating the relative genome sizes of diploid and triploid *Pteridium aquilinum* via DAPI staining. *Vicia faba* 'Inovec' is used as an internal standard at ca 300 relative fluorescence.

(Barrington et al., 2020) and can be used as identification characters (Dyer et al., 2012). Our results show significant differences in stomata and spore sizes between diploid and triploid *P. aquilinum*. The average stomata sizes in our study were 36.6 µm in diploids and 46.9 µm in triploids. While the triploid size corresponds with the average 47.3 µm found by Sheffield et al. (1993), they reported a somewhat larger size for diploids, on average 42.3 µm; this difference may have been caused by small methodological differences or geographical variation. Indeed, Thomson (2000) found variable stomata sizes for several *Pteridium* diploids and tetraploids from around the world, but the diploids generally had stomata smaller than 45 µm. The results of spore size calculations are more consistent. The spore size of diploids averaged 30.1 µm. This number corresponds well with those provided for diploid *P. aquilinum* by other researchers (25–34 µm, McVaugh, 1935; Conway, 1949; Sheffield et al., 1993; Thomson and Alonso-Amelot, 2002). The average size of spores produced by triploid fronds was 33.8 µm, similar to the 34.1 µm reported by Sheffield et al. (1993) and intermediate

between the size of aforementioned diploids and tetraploid *P. caudatum* (37.9 µm, Thomson and Alonso-Amelot, 2002). Interestingly, a small amount of much larger spores, 50–70 µm, in length were produced by triploids. They were reminiscent of the unusual diplospores of triploid *Cystopteris protrusa* formed in dyads (Haufler et al., 1985). The unreduced *C. protrusa* spores were deemed to be viable by Haufler et al. (1985) and the presumed unreduced spores of *P. aquilinum* found in our study were viable too. Upon cultivation, spores of diploid *P. aquilinum* produced only reduced (1x) gametophytes, while triploids produced reduced (1x) as well as unreduced (3x) gametophytes (Podroužek, unpublished data, obtained via flow cytometry). Sheffield et al. (1993) were also able to successfully cultivate spores from triploid plants. Overall, proper measurement of spore and stomata size allows for an unambiguous identification of the two *P. aquilinum* cytotypes; the use of stomata is recommended because many populations contain only infertile fronds producing no spores.

4.2. Cytotype fertility

Sexual reproduction of ferns requires the production of sporangia containing viable spores. Many factors affect the production of *P. aquilinum* sporangia and spores, including plant age, phenology, weather and environmental factors (Conway, 1957; Wynn et al., 2000). Under optimal conditions, a single bracken frond may produce up to 300 milion spores, although the real numbers are lower (Conway, 1957).

Table 3
The distribution of genetic variation in *Pteridium aquilinum* based on Analysis of Molecular Variance (AMOVA); d.f. – degrees of freedom, SSD – sum of squared deviations.

Source of variation	d.f.	SSD	Variance %	Fixation index
Between diploid and triploid groups of populations	1	0.02	-10.0	$Phi_{RT} = -0.100$
Among populations within ploidy level	24	41.79	88.1	$Phi_{SR} = 0.801$
Within populations	211	9.41	21.9	$Phi_{ST} = 0.781$
Total	**236**	**51.21**		
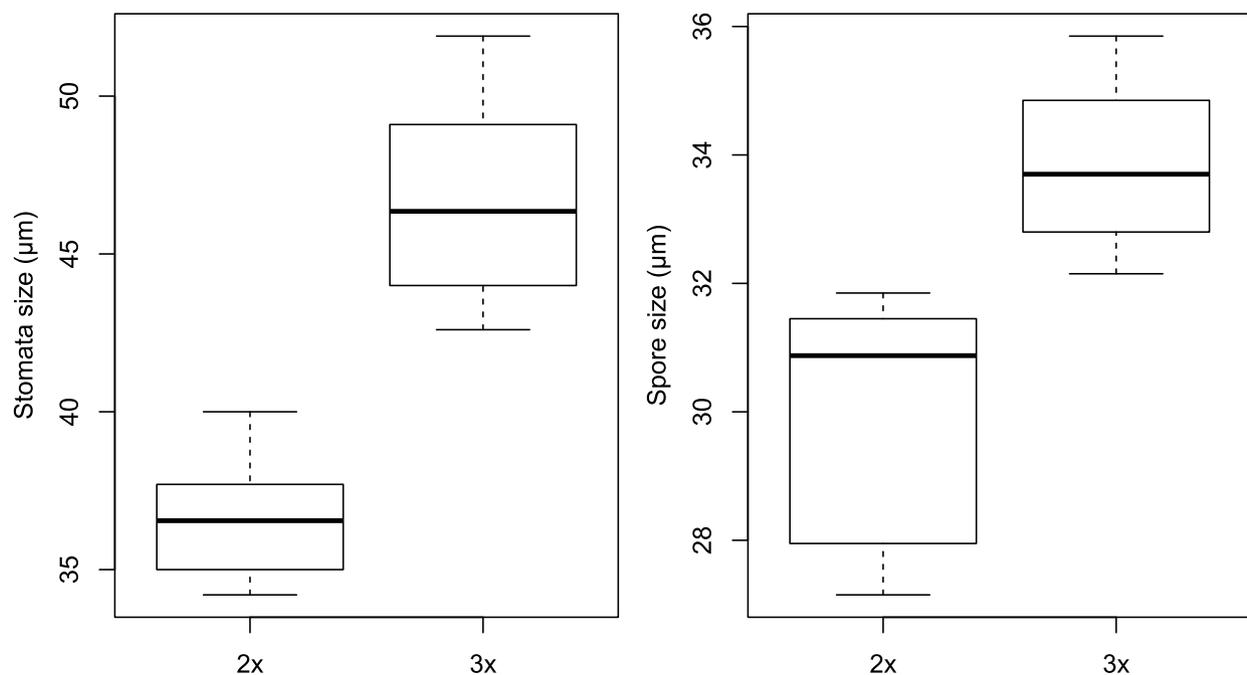
Fig. 3. Stomata and spore size of diploid and triploid *Pteridium aquilinum*. (Diplo)spores larger than 50 µm (formed by triploids) are not included.
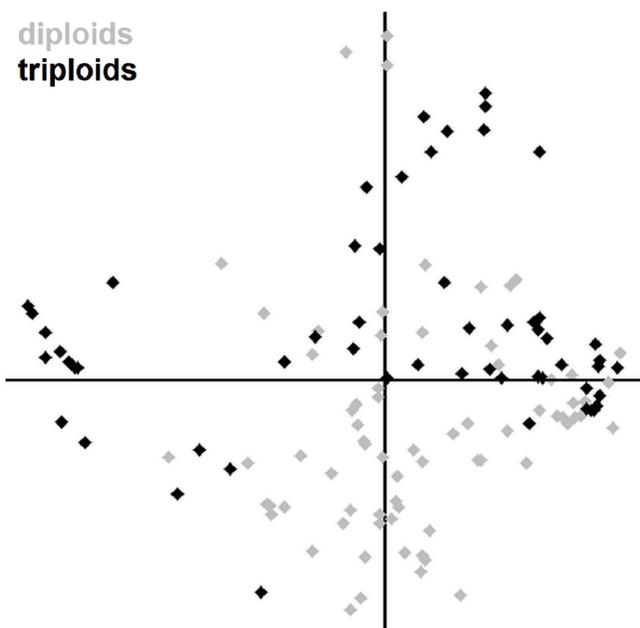
Fig. 4. PCoA of diploid and triploid *Pteridium aquilinum* samples analyzed for seven microsatellite loci. The first (horizontal) and the second (vertical) axes explained 18,6 % and 10,6 %, respectively, of total variation.

Despite the reports of massive spore production (Conway, 1957; Page, 1976), many bracken populations have no fertile (sporangia-bearing) fronds. In our study, diploid and triploid populations were fertile in ca 17.7 and 21.4% of the cases, respectively. The differences in the proportion of fertile populations might be caused by the overall low number of sampled triploid populations, reducing the accuracy of estimate. Often only a few fronds in a population were fertile. Similarly, 15.3% (187 of 1001), voucher specimens deposited in Czech public herbaria bore sporangia (Kaplan et al., 2018), but fertile fronds may have been preferentially collected as a rarity. The generally low proportions of fertile fronds might affect the dispersal and distribution of *P. aquilinum*. Interestingly, the two fertile populations sampled in 2015 and resampled in 2016, became sterile. This might be caused by seasonal weather differences, but this finding highlights the possibility of potential false negatives in our sampling as populations deemed sterile could be fertile in a different year.

Another important metric of fertility is the proportion of aborted spores formed. While the diploids in our study formed <10% of aborted spores, the triploid plant formed mostly aborted spores (97.4%). Still, some of the seemingly viable spores were occasionally much larger and could be unreduced. In stark contrast, Sheffield et al. (1993) reported 23–53% spore germination rate in triploid, similar to the 51–75% germination rate in their diploid specimen. But, results from other fern triploids also found spores to be mostly aborted (Bennert et al., 2005; Hornych and Ekrt, 2017), with occasionally large, presumably unreduced spores formed (Morzenti, 1962; Haufler et al., 1985; Schuettpelz et al., 2015). The reduced ability to form viable spores likely limits the dispersal capabilities of triploids.

4.3. Cytotype distribution

Our study confirms the presence of triploid *P. aquilinum* in continental Europe. While diploids are widespread and often act as noxious weeds (Page, 1976), the triploid cytotype is rare. Triploids were originally discovered by Sheffield et al. (1993) in Wales, Great Britain in just one population together with diploids. In our sampling, triploids comprised 11.2% of fronds sampled and often were found in mixed populations with diploids (9 populations mixed, 5 solely triploid). Our

sampling covered 19 European countries, but most of the sampled triploids were found in Central Europe and rarely in Scandinavia. This may be due to only extensive sampling in other regions or due to particularly favorable conditions for the triploid in certain areas. Still, a more thorough sampling across other regions would likely discover triploid or mixed populations and the overall pattern of distribution could be random. Especially western continental Europe is a good candidate for more research as it lies between the confirmed samples from Central Europe and Great Britain.

4.4. The origin of the triploid cytotype

Our data indicate an autopolyploid origin of the triploid cytotype as both PCoA and AMOVA showed no distinguishable difference in the microsatellite makeup of the two cytotypes. All triploids fall well within the diversity of the diploids, and both cytotypes showed the same number of private alleles. Similar outcomes were also found in some triploid seed plants (Chung et al., 2015), ferns (Haufler et al. 1985) and lycophytes (Rumsey et al., 1993). In contrast, tetraploids within the genus *Pteridium* are of allopolyploid origin (Der et al., 2009). As the means of origin, Sheffield et al. (1993) hypothesized a merger of gametes from a regular haploid gametophyte and a diploid aposporous (vegetatively generated from sporophyte tissue) gametophyte. This is due to the ease with which *P. aquilinum* forms aposporous gametophytes (Sheffield and Bell, 1987) and the rarity of unreduced diploid spores produced by diploid plants (Wolf et al., 1987). Our spore measurements also found no disproportionately large spores in the diploid cytotype indicating that unreduced spores are probably rare. However, the origin via unreduced spores is possible, despite their rarity, especially if the triploid cytotype was formed only once.

Multiple origins of polyploids are often considered the norm in plants (Soltis and Soltis, 1999, Mandáková et al., 2019), including ferns (Perrie et al., 2010; Beck et al., 2012). Our data show that the triploid cytotype is comprised of several genetically distinct groups, so a single origin would have to be followed by dispersal and the subsequent differentiation by mating with various diploids. The original triploid would have to be vigorous and fertile (discussed below), to overcome the odds of establishing within a diploid-dominated region. Moreover, triploids were in several cases closely related to diploids of the same population or to diploids of adjacent populations, implying multiple origins of the triploid as a more parsimonious explanation.

We outline several possible reasons why triploid *P. aquilinum* has avoided the minority cytotype exclusion. First, new triploid lineages could be continuously formed from the diploids. However, the formation and establishment of triploids is likely a rare event, as triploids are much less frequent than diploids. Second, triploids likely have some means of dispersal. For example, rhizome fragments are typical for *P. aquilinum* (Conway, 1949; Oinonen, 1967) and could be dispersed between localities (e.g. by forestry). Moreover, vegetative reproduction via rhizome growth ensures persistence in time, as exemplified by the Welsh triploid clone estimated to be 250–500 years old (Sheffield et al., 1993). Spores could also be a rare but possible means of dispersal for triploid *P. aquilinum*, although the ratio of viable spores is much lower than in diploids and gametophytes from triploids were not yet observed to form apogamous sporophytes under laboratory conditions. Sexual reproduction may also be highly restricted as archegonia were only formed extremely rarely by triploids (Sheffield et al., 1993).

Based on our data, within population variation was considerably lower but not negligible as all but one population was variable. Low within and high among population variation is generally associated with selfing, mating among related genotypes or clonality. However, *P. aquilinum* produces antheridiogens, pheromones estimated to be used by the majority of fern species (Hornych et al., 2020) to promote outcrossing (Schneller 2008), and previous isozyme studies revealed low levels of selfing (Wolf et al., 1987, 1988). Reduced within population variation in *P. aquilinum* may be due to its potential for extensive clonal
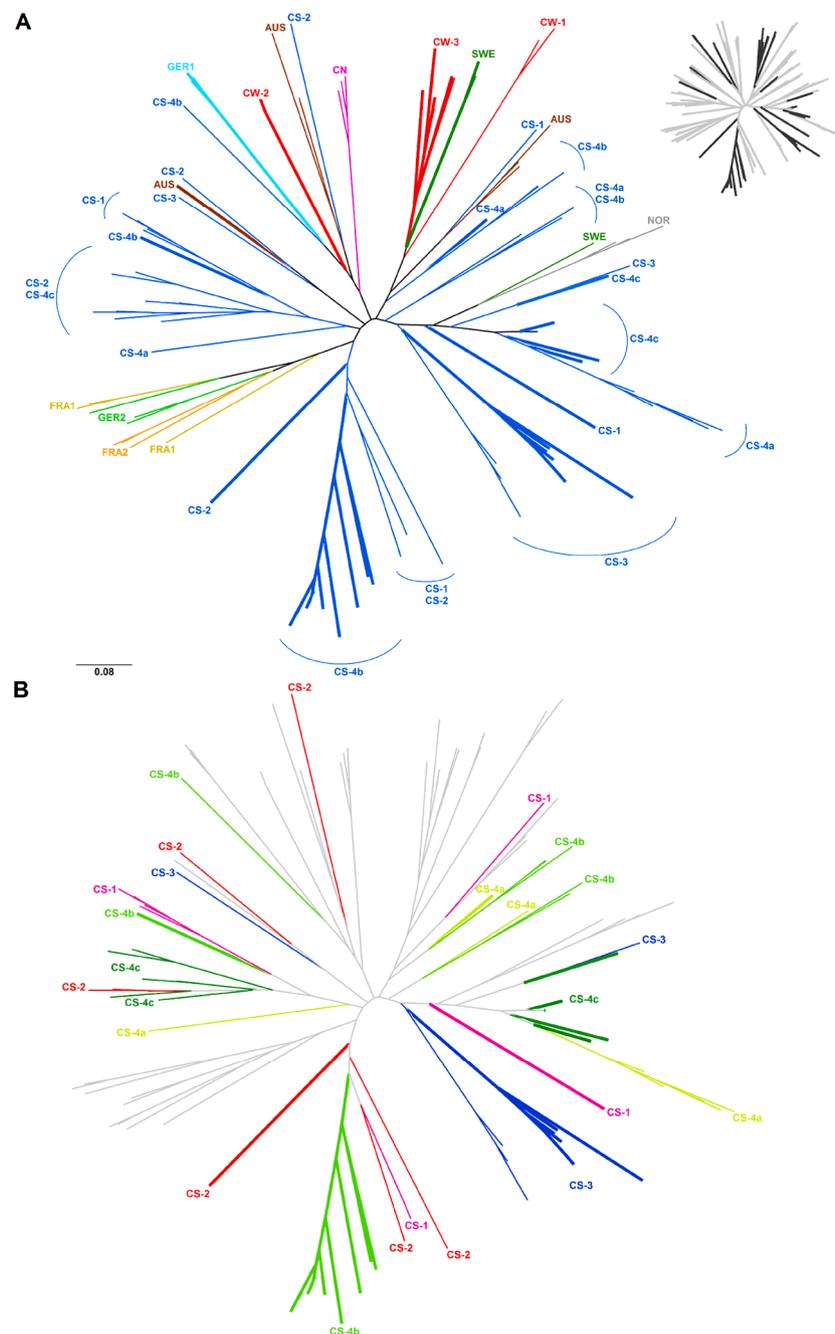
Fig. 5. Neighbor-joining tree of diploid and triploid *Pteridium aquilinum* samples analyzed for seven microsatellite loci. Bold lines indicate triploid accessions. A – populations are indicated by colors, Czech populations are grouped into three regions (CS, CW and CN); top-right greyscale image indicates triploid (dark gray) and diploid (light gray) populations. B – detailed view of Czech populations of the CS region with populations indicated by colors.

growth via rhizomes. Several different spores may arrive to colonize an open habitat, and the resultant gametophytes outcross to form variable sporophytes. However, the extensive growth and perennial nature of mature sporophytes likely limits establishment of genotypes arriving later. This is congruent with the limited number of young *P. aquilinum* sporophytes found in nature (Conway, 1949; 1953; Page, 1976). Furthermore, clones of *P. aquilinum* can be considerably larger (up to 1, 015 m across, Sheffield et al., 1989b; Parks and Werth, 1993) than our chosen 10 meter distance for collecting fronds, but as we found some within population variation, we expect to have generally collected multiple individuals in each population.

In summary, the triploid cytotype of *P. aquilinum* is rare but distinguishable by micromorphology and flow cytometry. Relying on its

extensive vegetative growth, the triploid can maintain itself within the diploid majority practically indefinitely, despite being limited in sexual reproduction. The likely continuous formation of new triploid lineages from the diploids guarantees the long-term establishment of the triploid cytotype and enables further potential evolution.

Supplement 1: General information of populations of *Pteridium aquilinum* collected for this study. Molecular label refers to the labelling of populations in Fig. 5.

CRediT authorship contribution statement

Libor Ekrt: Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing, Visualization, Supervision,

Project administration, Funding acquisition. **Jan Podroužek:** Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Ondřej Hornych:** Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Jiří Košnar:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing, Visualization. **Petr Koutecký:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.flora.2020.151725.

References

Baack, E.J., 2005. Ecological factors influencing tetraploid establishment in snow buttercups (*Ranunculus adoneus*, Ranunculaceae): Minority cytotype exclusion and barriers to triploid formation. Amer. J. Bot- 92, 1827–1835.

Baldwin, S.J., Husband, B.C., 2013. The association between polyploidy and clonal reproduction in diploid and tetraploid *Chamerion angustifolium*. Mol. Ecol 22, 1806–1819.

Barrington, D.S., Patel, N.R., Southgate, M.W., 2020. Inferring the impacts of evolutionary history and ecological constraints on spore size and shape in the ferns. Appl. Plant Sci. 8, e11339.

Beck, J.B., Allison, J.R., Pryer, K.M., Windham, M.D., 2012. Identifying multiple origins of polyploid taxa: A multilocus study of the hybrid cloak fern (*Astrolepis integerrima*; Pteridaceae). Amer. J. Bot 99, 1857–1865.

Bennert, W., Lubienski, M., Kröner, S., Steinberg, M., 2005. Triploidy in *Equisetum* subgenus *Hippochaete* (Equisetaceae, Pteridophyta). Ann. Bot. 95, 807–815.

Brochmann, C., Brysting, A.K., Alsos, I.G., Borgen, L., Gtundt, H.H., Scheen, A.-C., Elven, R., 2004. Polyploidy in arctic plants. Biol. J. Linnean Soc. 82, 521–536.

Chen, X., Wang, J., Tian, H., Zhang, X., Wen, J., Zhou, S., 2008. Development of microsatellite markers for the bracken fern, *Pteridium aquilinum*. Mol. Ecol. Resour. 8, 1491–1493.

Chrtek, J., Mráz, P., Belyayev, A., Paštová, L., Mrázová, V., Caklová, P., Josefiová, J., Zagorski, D., Hartmann, M., Jandová, M., Pinc, J., Fehrer, J., 2020. Evolutionary history and genetic diversity of apomictic allopolyploids in *Hieracium* s. str.: morphological versus genomic features. Amer. J. Bot. 107, 66–90.

Chung, M.Y., López-Pujol, J., Chung, J.M., Kim, K.-J., Park, S.J., Chung, M.G., 2015. Polyploidy in *Lilium lancifolium*: Evidence of autotriploidy and no niche divergence between diploid and triploid cytotypes in their native ranges. Flora 213, 57–68.

Conway, E., 1949. XXII. – The autecology of bracken (*Pteridium aquilinum* (L.) Kuhn): The germination of the spore, and the development of the prothallus and the young sporophyte. Proc. R. Soc. Edinb., Section B: Biol. Sci. 63, 325–343.

Conway, E., 1953. Spore and sporeling survival in bracken (*Pteridium aquilinum* (L.) Kuhn). J. Ecol 41, 289–294.

Conway, E., 1957. Spore production in bracken (*Pteridium aquilinum* (L.) Kuhn). J. Ecol 45, 273–284.

Der, J.P., Thomson, J.A., Stratford, J.K., Wolf, P.G., 2009. Global chloroplast phylogeny and biogeography of bracken (*Pteridium*; Dennstaedtiaceae). Amer. J. Bot 96, 1041–1049.

Der, J.P., Barker, M.S., Wickett, N.J., de Pamphills, C.W., Wolf, P.G., 2011. De novo characterization of the gametophyte transcriptome in bracken fern, *Pteridium aquilinum*. BMC Genomics. 12, 99.

Doležel, J., Greilhuber, J., Suda, J., 2007. Estimation of nuclear DNA content in plants using flow cytometry. Nat. Protoc. 2, 2233–2244.

Doležel, J., Sgorbati, S., Lucretti, S., 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. Physiol. Plant. 85, 625–631.

Dyer, R.J., Savolainen, V., Schneider, H., 2012. Apomixis and reticulate evolution in the *Asplenium monanthes* fern complex. Ann. Bot 110, 1515–1529.

Ekrt, L., Koutecký, P., 2016. Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (Dryopteridaceae). Ann. Bot. 117, 97–106.

Grusz, A.L., 2016. A current perspective on apomixis in ferns. J. Syst. Evol. 54, 656–665.

Hanušová, K., Čertner, M., Urfus, T., Koutecký, P., Košnar, J., Rothfels, C.J., Jarolímová, V., Ptáček, J., Ekrt, L., 2019. Widespread co-occurrence of multiple ploidy levels in fragile ferns (*Cystopteris fragilis* complex; Cystopteridaceae) likely stems from similar ecology of cytotypes, their efficient dispersal and inter-ploidy hybridization. Ann. Bot. 123, 845–855.

Haufler, C.H., Windham, D.M., Britton, D.M., Robinson, S.J., 1985. Triploidy and its evolutionary significance in *Cystopteris protrusa*. Can. J. Bot. 63, 1855–1863.

Haufler, C.H., Pryer, K.M., Schuettpelz, E., Sessa, E.B., Farrar, D.R., Moran, R., Schneller, J.J., Watkins Jr., J.E., Windham, M.D., 2016. Sex and the single gametophyte: Revising the homosporous vascular plant life cycle in light of contemporary research. BioSci 66, 928–937.

Hornych, O., Ekrt, L., 2017. Spore abortion index (SAI) as a perspective tool of evaluation of spore fitness in ferns: An insight into sexual and apomictic species. Plant Sys. Evol. 303, 497–507.

Hornych, O., Ekrt, L., Riedel, F., Koutecký, P., Košnar, J., 2019. Asymmetric hybridization in Central European populations of the *Dryopteris carthusiana* group. Amer. J. Bot. 106, 1477–1486.

Hornych, O., Testo, W.L., Sessa, E.B., Watkins Jr., J.E., Campany, C.E., Pittermann, J., Ekrt, L., 2020. Insights into the evolutionary history and widespread occurrence of antheridiogen systems in ferns. New Phytol. https://doi.org/10.1111/nph.16836. In press.

Hori, K., 2020. Allelic variation of the *Athyrium christensenianum* complex (Athyriaceae). Cytologia 85, 9–14.

Hori, K., Kuo, L.Y., Chiou, W.L., Ebihara, A., Murakami, N., 2017. Geographical distribution of sexual and apogamous types of *Dryopteris formosana* and *Dryopteris varia* (Dryopteridaceae) in Taiwan. Acta Phytotax. Geobot. 68, 23–32.

Jiménez, A., Holderegger, R., Csencsics, D., Quintanilla, L.G., 2010. Microsatellites reveal substantial among-population genetic differentiation and strong inbreeding in the relict fern *Dryopteris aemula*. Ann. Bot. 106, 149–155.

Kaplan, Z., Koutecký, P., Danihelka, J., Šumberová, K., Ducháček, M., Štěpánková, J., Ekrt, L., Grulich, V., Řepka, R., Kubát, K., Mráz, P., Wild, J., Brůna, J., 2018. Distributions of vascular plants in the Czech Republic. Part 6. Preslia 90, 235–346.

Kolář, F., Čertner, M., Suda, J., Schönswetter, P., Husband, B.C., 2017. Mixed-ploidy species: Progress and opportunities in polyploid research. Trends Plant Sci 22, 1041–1055.

Koressaar, T., Jöers, K., Remm, M., 2007. Enhancements and modifications of primer design program Primer3. Bioinform 10, 1289–1291.

Landis, J.B., Soltis, D.E., Li, Z., Marx, H.E., Barker, M.S., Tank, D.C., Soltis, P.S., 2018. Impact of whole-genome duplication events on diversification rates in angiosperms. Amer. J. Bot. 105, 348–363.

Levin, D.A., 1975. Minority cytotype exclusion in local plant populations. Taxon 24, 35–43.

Liang, S.-Q., Viane, R.L.L., Zhang, X.-C., Wei, R., 2019. Exploring the reticulate evolution in the *Asplenium pekinense* complex and the *A. varians* complex (Aspleniaceae). J. Syst. Evol. https://doi.org/10.1111/jse.12530.

Löve, A., Kjellquist, E., 1972. Cytotaxonomy of Spanish plants. 1. Introduction: Pteridophytes and Gymnosperms. Lagascalia 2, 23–25.

McVaugh, R., 1935. Studies on the spores of some northeastern ferns. Amer. Fern J. 25, 73–85.

Mandáková, T., Pouch, M., Brock, J.R., Al-Shehbaz, I.A., Lysak, M.A., 2019. Origin and evolution of diploid and allopolyploid *Camelina* genomes was accompanied by chromosome shattering. Plant Cell 31, 2596–2612.

Morzenti, V.M., 1962. A first report on pseudomeiotic sporogenesis, a type of spore reproduction by which "sterile" ferns produce gametophytes. Amer. Fern J. 52, 69–78.

Oinonen, E., 1967. The correlation between the size of Finnish bracken (*Pteridium aquilinum* (L.) Kuhn) clones and certain periods of site history. Acta For. Fenn. 83, 1–51.

Otto, S.P., Whitton, J., 2000. Polyploid incidence and evolution. Annu. Rev. Genet. 34, 401–437.

Page, C.N., 1976. The taxonomy and phytogeography of bracken – a review. Bot. J. Linn. Soc. 73, 1–34.

Pangua, E., Pajarón, S., Quintanilla, L.G., 2019. Fitness of an allopolyploid rupicolous fern compared with its diploid progenitors: from sporogenesis to sporophyte formation. Amer. J. Bot. 106, 984–995.

Parks, J.C., Werth, C.R., 1993. A study of spatial features of clones in a population of bracken fern, *Pteridium aquilinum* (Dennstaedtiaceae). Amer. J. Bot. 80, 537–544.

Perrie, L., Shepherd, L., De Lange, P., Brownsey, P., 2010. Parallel polyploid speciation: distinct sympatric gene-pools of recurrently derived allooctoploid *Asplenium* ferns. Molec. Ecol. 19, 2916–2932.

Popelka, O., Sochor, M., Duchoslav, M., 2019. Reciprocal hybridization between diploid *Ficaria calthifolia* and tetraploid *Ficaria verna* subsp. *verna*: evidence from experimental crossing, genome size and molecular markers. Bot. J. Linn. Soc. 189, 293–310.

Core Team, R, 2017. R: A language and environment for statistical computing. In: R Foundation for Statistical Computing. Vienna, Austria. Website: http://www.R-project.org/ [accessed 19 March 2019].

Rieseberg, L.H., Willis, J.H., 2007. Plant speciation. Sci 317, 910.

Rodriguez, J.D., 1996. A model for the establishment of polyploidy in plants. Am. Nat. 147, 33–46.

Rumsey, F.J., Thompson, P., Sheffield, E., 1993. Triploid *Isoetes echinospora* (Isoetaceae: Pteridophyta) in Northern England. Fern Gaz 14, 215–221.

Saggoo, M.I.S., Kaur, M., 2017. *Adiantum lanulatum* Burm. – Naturalized triploid apomicts from Kanga valley of Himachal Pradesh. Cytologia 82, 507–512.

Schlüter, P.M., Harris, S.A., 2006. Analysis of multilocus fingerprinting data sets containing missing data. Mol. Ecol. Notes 6, 569–572.

Schneller, J.J., 2008. Antheridiogens. In: Ranker, T.A., Haufler, C.H. (Eds.), Biology and evolution of ferns and lycophytes. Cambridge University Press, Cambridge, pp. 134–158.

Schuettpelz, E., Pryer, K.M., Windham, M.D., 2015. A unified approach to taxonomic delimitation in the fern genus *Pentagramma* (Pteridaceae). Syst. Bot. 40, 629–644.

Sessa, E.B., Testo, W.L., Watkins Jr., J.E., 2016. On the widespread capacity for, and functional significance of, extreme inbreeding in ferns. New Phytol 211, 1108–1119.

Sheffield, E., Bell, P.R., 1987. Current studies of the pteridophyte life cycle. Bot. Rew. 53, 442–490.

Sheffield, E., Wolf, P.G., Haufler, C.H., Ranker, T., Jermy, A.C., 1989a. A re-evaluation of plants referred to as *Pteridium herediae* (Colmerio) Löve & Kjellqvist. Bot. J. Linn. Soc 99, 377–386.

Sheffield, E., Wolf, P.G., Haufler, C.H., 1989b. How big is a bracken plant? Weed Res. 29, 455–460.

Sheffield, E., Wolf, P.G., Rumsey, F.J., Robson, D.J., Ranker, T.A., Challinor, S.M., 1993. Spatial distribution and reproductive behavior of a triploid bracken (*Pteridium aquilinum*) clone in Britain. Ann. Bot. 72, 231–237.

Soltis, D.E., Soltis, P.S., 1999. Polyploidy: Recurrent formation and genome evolution. Trends Ecol. Evol. 14, 348–352.

Soltis, P.S., Soltis, D.E., 2009. The role of hybridization in plant speciation. Annu. Rev. Plant Biol. 60, 561–588.

Speer, W.D., Werth, C.R., Hilu, K.W., 1998. Relationships between two infraspecific taxa of *Pteridium aquilinum* (Dennstaedtiaceae). II. Isozyme evidence. Syst. Bot. 23, 313–325.

Thomson, J.A., 2000. Morphological and genomic variation in the genus *Pteridium* (Dennstaedtiaceae). Ann. Bot. 85, 77–99.

Thomson, J.A., 2004. Towards a taxonomic revision of *Pteridium* (Dennstaedtiaceae). Telopea 10, 793–803.

Thomson, J.A., Alonso-Amelot, M.E., 2002. Clarification of the taxonomic status and relationships of *Pteridium caudatum* (Dennstaedtiaceae) in Central and South America. Bot. J. Linn. Soc. 140, 237–248.

Tryon Jr., R.M., 1941. A revision of the genus *Pteridium*. Contrib. Gray Herb, 134. Harvard Univ., pp. 1–31, 37-67.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S. G., 2012. Primer3 – new capabilities and interfaces. Nucleic Acids Res 40, e115.

Wolf, P.G., Haufler, C.H., Sheffield, E., 1987. Electrophoretic evidence for genetic diploidy in the bracken fern *Pteridium aquilinum*. Sci 236, 947–949.

Wolf, P.G., Haufler, C.H., Sheffield, E., 1988. Electrophoretic variation and mating system of the clonal weed *Pteridium aquilinum*. (L.) Kuhn (Bracken). Evol 42, 1350–1355.

Wolf, P.G., Sheffield, E., Haufler, C.H., 1991. Estimates of gene flow, genetic substructure and population heterogeneity in bracken (*Pteridium aquilinum*). Biol. J. Linn. Soc. 42, 407–423.

Wolf, P.G., Rowe, C.A., Kinosian, S.P., Der, J.P., Lockhart, P.J., Shepherd, L.D., McLenachan, P.A., Thomson, J.A., 2019. Worldwide relationships in the fern genus *Pteridium* (bracken) based on nuclear genome markers. Amer. J. Bot. 106, 1–12.

Wood, T.E., Takebayashi, N., Barker, M.S., Mayrose, I., Greenspoon, P.B., Rieseberg, L. H., 2009. The frequency of polyploid speciation in vascular plants. Proc. Natl. Acad. Sci. USA. 106, 13875–13879.

Wynn, J.M., Small, J.L., Pakeman, R.J., Sheffield, E., 2000. An assessment of genetic and environmental effects on sporangial development in bracken (*Pteridium aquilinum* (L.) Kuhnl) using a novel quantitative method. Ann. Bot. 85, 113–115.

Paper 12

Hanušová K., Čertner M., Urfus T., Koutecký P., Košnar J., Rothfels C. J, Jarolímová V., Ptáček J., Ekrt L. (2019): Widespread co-occurrence of multiple ploidy levels in fragile ferns (*Cystopteris fragilis* complex; Cystopteridaceae) likely stems from similar ecology of cytotypes, their efficient dispersal and inter-ploidy hybridization. – Annals of Botany 123: 845–855.

ANNALS OF
BOTANY
Founded 1887

Widespread co-occurrence of multiple ploidy levels in fragile ferns (*Cystopteris fragilis* complex; Cystopteridaceae) probably stems from similar ecology of cytotypes, their efficient dispersal and inter-ploidy hybridization

Kristýna Hanušová[1], Martin Čertner[1,2], Tomáš Urfus[1], Petr Koutecký[3], Jiří Košnar[3], Carl J. Rothfels[4], Vlasta Jarolímová[2], Jan Ptáček[1] and Libor Ekrt[3*]

[1]*Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 00 Praha, Czech Republic,* [2]*Institute of Botany, The Czech Academy of Sciences, Zámek 1, CZ-252 43 Průhonice, Czech Republic,* [3]*Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, CZ-370 05 České Budějovice, Czech Republic, and* [4]*University Herbarium and Department of Integrative Biology, University of California, Berkeley, CA 94720, USA*
* *For correspondence. E-mail* libor.ekrt@gmail.com

- **Background and Aims** Polyploidy has played an important role in the evolution of ferns. However, the dearth of data on cytotype diversity, cytotype distribution patterns and ecology in ferns is striking in comparison with angiosperms and prevents an assessment of whether cytotype coexistence and its mechanisms show similar patterns in both plant groups. Here, an attempt to fill this gap was made using the ploidy-variable and widely distributed *Cystopteris fragilis* complex.
- **Methods** Flow cytometry was used to assess DNA ploidy level and monoploid genome size (Cx value) of 5518 *C. fragilis* individuals from 449 populations collected over most of the species' global distributional range, supplemented with data from 405 individuals representing other related species from the complex. Ecological preferences of *C. fragilis* tetraploids and hexaploids were compared using field-recorded parameters and database-extracted climate data.
- **Key Results** Altogether, five different ploidy levels ($2x$, $4x$, $5x$, $6x$, $8x$) were detected and three species exhibited intraspecific ploidy-level variation: *C. fragilis*, *C. alpina* and *C. diaphana*. Two predominant *C. fragilis* cytotypes, tetraploids and hexaploids, co-occur over most of Europe in a diffuse, mosaic-like pattern. Within this contact zone, 40 % of populations were mixed-ploidy and most also contained pentaploid hybrids. Environmental conditions had only a limited effect on the distribution of cytotypes. Differences were found in the Cx value of tetraploids and hexaploids: between-cytotype divergence was higher in uniform-ploidy than in mixed-ploidy populations.
- **Conclusions** High ploidy-level diversity and widespread cytotype coexistence in the *C. fragilis* complex match the well-documented patterns in some angiosperms. While ploidy coexistence in *C. fragilis* is not driven by environmental factors, it could be facilitated by the perennial life-form of the species, its reproductive modes and efficient wind dispersal of spores. Independent origins of hexaploids and/or inter-ploidy gene flow may be expected in mixed-ploidy populations according to Cx value comparisons.

Keywords: Bladder ferns, contact zone, Cx value, *Cystopteris fragilis*, cytotype coexistence, ecological preferences, flow cytometry, genome size, ploidy distribution, pteridophytes.

INTRODUCTION

Polyploidization (whole-genome duplication) is widely considered one of the major forces contributing to the evolutionary diversification of land plants (Soltis *et al.*, 2016; Landis *et al.*, 2018). This is especially so in ferns, where approx. 30 % of speciation events are presumably linked to changes in ploidy, twice the rate predicted for angiosperms (Wood *et al.*, 2009). Specifically, by providing immediate postzygotic reproductive isolation between newly arisen polyploids and their progenitors, polyploidization is an efficient mechanism of sympatric speciation (Ramsey and Schemske, 1998; Coyne and Orr, 2004). Polyploidization was suggested as the predominant mechanism of genome size expansion in ferns, and chromosome number and genome size are tightly correlated in this plant group, unlike in angiosperms (Barker, 2013; Clark *et al.*, 2016). High accumulation of polyploidy in some fern lineages (Clark *et al.*, 2016; Schneider *et al.*, 2017) makes them suitable models for studying polyploid evolution.

The prevailing mode of polyploid origin is via unreduced gametes (i.e. gametes with a somatic chromosome number), produced as a consequence of rare meiotic errors (Ramsey, 2007; Kreiner *et al.*, 2017). The rarity of polyploid formation is compounded by demographic challenges: new polyploids generally suffer from a lack of compatible mating partners, and crosses with their progenitors, which produce sterile odd-ploidy offspring, may lead to their extirpation ('minority cytotype exclusion'; Levin, 1975). The frequency-dependent

selection driving this process may similarly affect otherwise well-established cytotypes if they meet in contact zones, which makes minority cytotype exclusion a main constraint to ploidy coexistence in general (Husband, 2000). In recent decades, several mechanisms facilitating successful polyploid establishment and/or cytotype coexistence have been proposed (reviewed by Kolář *et al.*, 2017). For example, the minority status of one of the coexisting cytotypes may be overcome by its recurrent origin (Ramsey, 2007), efficient vegetative spread (Chrtek *et al.*, 2017), autogamy (Petit *et al.*, 1997), non-random mating (Husband *et al.*, 2008) or a substantial competitive advantage (Felber, 1991). Prominent among these mechanisms is the (fine-scale) spatial segregation of cytotypes, which can increase the rate of compatible, within-ploidy mating (Baack, 2005; Kolář *et al.*, 2017). The most frequently reported cause of such spatial segregation of cytotypes is their different ecological preferences (e.g. Levin, 2002; Laport *et al.*, 2016).

Cytogeography, the study of cytotype diversity and its distribution patterns, is usually the first step towards understanding the mechanisms of polyploid evolution (Soltis *et al.*, 2003). Cytotype distribution patterns may point to differences in habitat preferences among cytotypes and enable the detection of zones of cytotype contact. Such information may provide insights into the temporal stability of ploidy coexistence and the origin of cytogenetic novelty, and possibly demonstrate the potential of contact zones in promoting inter-ploidy gene flow (Kolář *et al.*, 2017). Of importance in this respect is local coexistence of different cytotypes, arising either after an *in situ* polyploidization event ('primary contact') or through cytotype immigration into populations of another cytotype ('secondary contact'; Petit *et al.*, 1999). Cytotype coexistence provides the opportunity for inter-ploidy crosses involving either reduced or unreduced gametes and thus may generate cytogenetic novelty (Kolář *et al.*, 2017).

Despite the importance of polyploidy in ferns, only a handful of detailed cytogeographical studies are available for this plant group (e.g. Moran, 1982; Nakato and Kato, 2005; Chang *et al.*, 2013; Grusz *et al.*, 2014; Dauphin *et al.*, 2018). The situation is further complicated by the limited geographical extent of these studies and their relatively small sample size, which can be largely attributed to using laborious methods of ploidy-level estimation (i.e. chromosome counts or measurements of spore diameter). The dearth of data on cytotype distribution and ecology in ferns, which is especially striking in comparison with the number of studies of angiosperms (reviewed by Kolář *et al.*, 2017), prevents us from assessing whether cytotype coexistence and the mechanisms of polyploid evolution show similar patterns in the two plant groups. Nonetheless, fast and reliable ploidy assessment of high numbers of samples is now available via flow cytometry (Ekrt *et al.*, 2010; Shinohara *et al.*, 2010; Clark *et al.*, 2016). Here, we use this technique to get better insight into fern polyploid evolution. We selected the *Cystopteris fragilis* complex (*sensu* Rothfels *et al.*, 2013) as a suitable model because, firstly, considerable ploidy-level diversity has been reported from natural populations of this group (six distinct cytotypes: 2*x*, 3*x*, 4*x*, 5*x*, 6*x* and 8*x*; Manton, 1950; Vida, 1974; Haufler *et al.*, 1985; Haufler and Windham, 1991; Kawakami *et al.*, 2010). Secondly, the complex (and especially *C. fragilis*) has

an extremely wide geographical distribution (Rothfels *et al.*, 2013), but whether spatial isolation of cytotypes or their sorting along ecological gradients contributes to cytotype coexistence remains unknown. Lastly, our preliminary ploidy screening in Central Europe revealed a high frequency of mixed-ploidy populations in *C. fragilis*. We used a wide array of complementary approaches, consisting of extensive field sampling, flow cytometric analysis of ploidy level and relative genome size, and ecological niche comparisons, to investigate the following questions: (1) What is the cytotype diversity in the *C. fragilis* complex and how is it geographically distributed (with a focus on Europe and North America)? (2) Could monoploid genome size comparisons provide additional clues to the evolution of cytotypes? (3) How common and widespread is cytotype coexistence in *C. fragilis*? (4) Is cytotype coexistence in *C. fragilis* driven by the underlying environmental heterogeneity?

MATERIALS AND METHODS

Model system

Fragile ferns (*Cystopteris fragilis*) are a complex of species within the eupolypod II family Cystopteridaceae (PPG I, 2016). Comprising approximately ten commonly recognized species, two of which are thought to be exclusively diploid (except rare incidence of autotriploidy) and the remainder predominantly polyploid, this complex is highly polymorphic and the species limits within it are uncertain, largely due to extensive patterns of allopolyploidy (Rothfels *et al.*, 2013, 2014, 2017). The complex has a nearly global distribution (Rothfels *et al.*, 2013), but individual taxa may be geographically restricted: the two named diploids (*C. protrusa* and *C. reevesiana*) are restricted to the Americas, as are the Mexican endemics *C. millefolia* and *C. membranifolia* and the north-eastern North American *C. tenuis*; *C. alpina* is European; *C. douglasii* is limited to Hawaii; and *C. tasmanica* occurs in Australia and New Zealand. The remaining diversity tends to be lumped into a heterogeneous north-temperate '*C. fragilis*' or a sub-tropical '*C. diaphana*' – both of which, as typically circumscribed, occur over wide areas of multiple continents and comprise multiple ploidy levels. An additional putative taxon, *C. dickieana*, distinguished based on spore characters, is recognized by some authors (e.g. Vida, 1974; Fraser-Jenkins, 2008) but we here follow the recent tendency (e.g. Haufler and Windham, 1991; Parks *et al.*, 2000; Rothfels, 2012) in lumping *dickieana* into *C. fragilis*. Members of the *C. fragilis* complex occasionally hybridize with the non-complex member *C. bulbifera*, forming the allopolyploids *C. utahensis*, *C. tennesseensis* and *C. laurentiana* (Haufler *et al.*, 1993; Rothfels *et al.*, 2017); these taxa are not included in this study.

The *C. fragilis* complex is a powerful system for studying polyploid evolution because of its broad geographical and habitat range, extensive ploidy variation – four cytotypes have been documented in natural populations of *C. fragilis* (4*x*, 5*x*, 6*x*, 8*x*) each of which probably contains multiple independent evolutionary units (Rothfels *et al.*, 2014, 2017) – and because polyploids in *Cystopteris* reproduce sexually, avoiding the potentially confounding factor of apomixis (Rothfels, 2012).

However, vegetative reproduction and a high frequency of inbreeding have also occasionally been reported in *Cystopteris* (Haufler *et al.*, 1993; Gämperle and Schneller, 2002).

Field sampling

Cystopteris populations were sampled during 2012–2016. The initial strategy was to cover representatively all habitats occupied by *C. fragilis* across Europe with particular emphasis on Central Europe, where a pilot survey documented frequent cytotype coexistence. To put our results into a broader context, we additionally sampled *C. fragilis* populations in western Asia, and North and South America, and also included other species from the *C. fragilis* complex. While the locality selection was generally random, in North America, we deliberately targeted much rarer hexaploid populations of *C. fragilis*. Depending on the plant's abundance at a given locality, we sampled 5–30 randomly selected plants with a minimum distance of 10 cm between sampled individuals. Each site was characterized by geographical coordinates, elevation, type of substrate (i.e. siliceous, alkaline and neutral), and general habitat description (including natural vs. anthropogenic character), and herbarium vouchers were collected (see Supplementary Data Table S1). One leaf per plant was collected for ploidy determination. The leaves were either kept fresh until flow-cytometric analysis or immediately desiccated using silica gel; the method of tissue preservation did not affect the reliability of ploidy-level estimation (data not shown).

Flow cytometry and karyology

DNA ploidy levels and relative genome size of *Cystopteris* individuals were assessed from fluorescence intensities of DAPI-stained nuclei using flow cytometry. Sample preparation followed Čertner *et al.* (2017); *Vicia faba* 'Inovec' (2C DNA = 26.90 pg; Doležel *et al.*, 1992) was used as an internal standard. Fluorescence intensity of 3000 particles was analysed using either Partec PA II or Partec CyFlow ML flow cytometers (Partec GmbH, Münster, Germany) equipped with a mercury arc lamp or a 365-nm UV LED, respectively. Up to five individuals were processed together during the ploidy screening, as our pilot analyses proved reliable detection of minority cytotypes in such pooled samples. We used DAPI-stained analyses for both ploidy-level screening and relative genome size estimation because these provide histograms with high resolution and do not require RNase treatment (Doležel *et al.*, 2007). We are well aware that DAPI preferentially binds AT-rich regions of DNA, which may lead to seeming genome size differences among samples differing strongly in their genomic GC content. To complement and calibrate DAPI-stained analyses, genome size was determined for 51 selected samples using propidium iodide (PI) staining. Sample preparation was identical but only fresh material was used; PI was used as a fluorochrome and RNase IIA was added to the staining solution, both at a final concentration of 50 µg mL^{-1}. Fluorescence intensity of 5000 particles was analysed using a CyFlow SL instrument (Partec GmbH) equipped with a green solid-state laser (Cobolt Samba,

532 nm, 100 mW). In PI analyses, samples were always processed individually and a mean value of three measurements on different days was used for genome size calculation.

The relationship between sample relative fluorescence and ploidy level was calibrated using chromosome counting. Spore mother cells of a single tetraploid *C. fragilis* individual (locality No. 126, see Supplementary Data Table S1) were pretreated with a saturated solution of *p*-dichlorobenzene (3 h, room temperature), fixed in a mixture of ethanol and acetic acid (3: 1), and stained using lacto-propionic orceine. The number of chromosomes was counted using a Carl-Zeiss Jena NU microscope (total magnification 1000×).

Genome size comparisons

Reported genome sizes, unless otherwise stated, are relative genome sizes based on DAPI staining (i.e. the sample to standard fluorescence ratio). We also calculated monoploid genome sizes (Cx values), by dividing the relative genome size by the ploidy level. This trait allows for comparisons of genome size independent of ploidy level; sufficient Cx value differences may in some cases be used to reconstruct modes of cytotype origin, cytotype relationships and, possibly, to detect newly originated polyploids (Čertner *et al.*, 2017).

All Cx value comparisons were conducted on a subset of *C. fragilis* data with high-quality genome size estimates [coefficient of variation (CV) of both the sample and the standard peak <3 %; see Supplementary Data Table S2]; populations from Argentina with a distinct genome size were excluded. For an overall comparison of Cx values of *C. fragilis* tetraploids and hexaploids, and for a test for a latitudinal gradient in Cx values, the dataset was reduced by randomly selecting one sample per ploidy per population. The Cx value differences between the cytotypes (overall and between samples from uniform-ploidy and mixed-ploidy populations) were tested using a Kruskal–Wallis rank sum test. Latitudinal gradients of Cx values were tested separately for tetraploids and hexaploids in linear regression models with latitude as an explanatory variable. To corroborate the recurrent, hybrid origin of pentaploids in mixed-ploidy populations of *C. fragilis*, mean Cx values of cytotypes within populations were extracted. Only mixed-ploidy populations for which high-quality genome size estimates were available for all three coexisting cytotypes (i.e. 4*x*, 5*x* and 6*x*) were retained, resulting in a final dataset of 23 populations. The Cx value of pentaploids was used as a response variable in a linear regression model and the mean of Cx values of co-occurring tetra- and hexaploids served as a predictor. All statistical analyses were conducted in R ver. 3.4.3 (R Core Team, 2016).

Ecology of cytotypes

Three field-recorded parameters, type of substrate, habitat origin (i.e. natural vs. anthropogenic) and elevation, were combined with database-extracted climate data and used in comparisons of abiotic and climatic niches of cytotypes for the representatively sampled *C. fragilis* populations (see Supplementary Data Table S2). We compared ecological niches

among uniformly tetraploid, uniformly hexaploid and mixed-ploidy populations; the last were identified by either co-occurrence of tetraploids and hexaploids at a site or by the presence of pentaploid individuals (resulting from inter-ploidy crosses). While climatic data were available on the global scale, the abiotic niche comparisons of cytotypes had to be restricted to the Eurasian range of *C. fragilis* as we lacked information on local abiotic parameters for the American samples.

The relative incidence of tetraploid, hexaploid and mixed-ploidy populations on different substrates and at habitats of natural or anthropogenic origin were compared using chi-square tests for homogeneity. Where there were significant differences, we conducted pairwise comparisons of populations with different cytotype composition and applied the Bonferroni correction. Only localities with siliceous or alkaline substrates were retained in the dataset because neutral substrates and other, intermediate or unclear assignments constituted a minority of sites (6.8 % of the data). Similarly, localities where natural or anthropogenic habitat status could not be unambiguously determined were excluded from the relevant analyses (1.9 %). The natural vs. anthropogenic origin was intended as a proxy of habitat history (e.g. higher frequency of one of the cytotypes at anthropogenic sites may indicate its relatively recent spread). Differences in mean elevation of tetraploid, hexaploid and mixed-ploidy populations were tested using one-way ANOVA. Elevation was square-root transformed prior to the analysis to meet the model assumptions. The final datasets consisted of 421, 400 and 429 population records for the tests of habitat origin, substrate type and elevation, respectively. All statistical analyses were conducted in R ver. 3.4.3 (R Core Team, 2016).

Georeferenced occurrences of tetraploid, hexaploid and mixed-ploidy populations were used to extract 19 Bioclim climate variables from the WorldClim database (http://www.worldclim.org/bioclim; Hijmans *et al.*, 2005) downloaded in the highest available resolution (30 arc seconds ≈ 1 km^2). To account for heterogeneity in sampling intensity, we used ArcGIS 10.0 (ESRI, Redlands, CA, USA) to divide the global range of *C. fragilis* into a grid of 0.5° × 0.5° cells and when multiple populations of the same cytotype composition were located within a cell, one was randomly selected for the analysis. A principal component analysis (PCA) was used to visualize climatic niches of the resulting 94 tetraploid, 98 hexaploid and 79 mixed-ploidy populations. Differences in climatic niches of the cytotypes were tested using a redundancy analysis (RDA) by applying a Monte Carlo test with 999 permutations. Cytotype composition of populations (tetraploid, hexaploid, mixed-ploidy) was used as an explanatory variable in RDA. Multivariate analyses were conducted in Canoco 5 (ter Braak and Šmilauer, 2012).

RESULTS

Cytotype diversity and its distribution patterns

During our detailed examination of cytotype diversity in *C. fragilis*, we sampled 5518 individuals from 449 localities across four continents (Fig. 1). Four ploidy levels were detected (4*x*, 5*x*, 6*x* and 8*x*; Table 1, Supplementary Data Fig. S1) and chromosome counts confirmed *n* = 84 in tetraploids (Supplementary Data

Fig. S2), consistent with the *Cystopteris* base number of *x* = 42. The two most common cytotypes in *C. fragilis*, tetraploids and hexaploids, occurred at similar frequencies (51 % and 46 % of samples, respectively). However, the relative frequency of tetraploids and hexaploids differed between Eurasia and the Americas (50 % and 47 % in Eurasia vs. 80 % and 15 % in the Americas, respectively), despite the fact that hexaploid populations were deliberately targeted in North America. Pentaploid individuals were quite scarce (3 % of samples) and only a single octoploid individual (0.02 % of samples) was sampled (it grew at a site of anthropogenic origin; see Supplementary Data Table S1).

In the European range of *C. fragilis*, the area with the most intensive sampling, the distribution of tetraploid and hexaploid cytotypes largely overlaps (Fig. 1C, Supplementary Data Fig. S3). The two cytotypes co-occur over most of Europe in a diffuse, mosaic-like pattern with only a few regions seemingly dominated by one cytotype: tetraploids dominate in Iceland and the Iberian Peninsula whereas hexaploids dominate in the Dinaric and French Alps (Fig. 1C). Ploidy coexistence within populations is very common and accounts for 40 % of the localities where more than ten plants were sampled, whereas uniformly tetraploid and uniformly hexaploid populations constitute 35 % and 25 % of such sites, respectively. Moreover, 26.7 % of populations include tetraploids, pentaploids and hexaploids. Pentaploid individuals were only found in ploidy mixtures, and never formed uniformly pentaploid populations.

To put our results into a broader context, we also sampled 405 individuals of other species from the *C. fragilis* complex (Fig. 1). In the exclusively European species *C. alpina*, the vast majority of analysed individuals were hexaploids (87.4 %). However, tetraploids (9.1 %), pentaploids (2.6 %) and octoploids (0.9 %) also occurred, albeit rarely (Fig. 1B, Supplementary Data Table S1). These are the first reports for ploidy levels other than hexaploid in *C. alpina*. The rarest cytotype, octoploid, was discovered in two mixed-ploidy (4*x* + 6*x* and 5*x* + 6*x*) populations in Macedonia. The American species *C. protrusa* and *C. reevesiana* were uniformly diploid, whereas *C. tenuis* was tetraploid. Populations of *C. diaphana* were consistently tetraploid in America but hexaploid in Europe (including Macaronesia). Interestingly, both cytotypes of *C. diaphana* show conspicuously larger genome sizes in comparison with other taxa of the same ploidy (see Table 1).

Monoploid genome size comparisons

Significant differences in monoploid genome size (i.e. the Cx value) were detected between *C. fragilis* tetraploids and hexaploids (Kruskal–Wallis test; χ^2 = 175.0, d.f. = 1, *P* < 0.001): that of tetraploids is on average 2.6 % larger than that of hexaploids (mean = 0.1091 ± 0.0001 s.e. and mean = 0.1063 ± 0.0001 s.e., respectively; Fig. 3A). The lowest Cx values for both tetraploids and hexaploids were found in populations from the arctic island of Svalbard, whereas the highest originated in the Mediterranean region (southern Europe and Turkey). However, linear regression models with latitude as predictor failed to explain much of the overall variation in Cx values of either tetraploids ($F_{1,228}$ = 0.014, *P* = 0.905, R^2 = 0.000) or hexaploids ($F_{1,226}$ = 3.440, *P* = 0.065, R^2 = 0.015). The populations from Argentina, distinct by both their
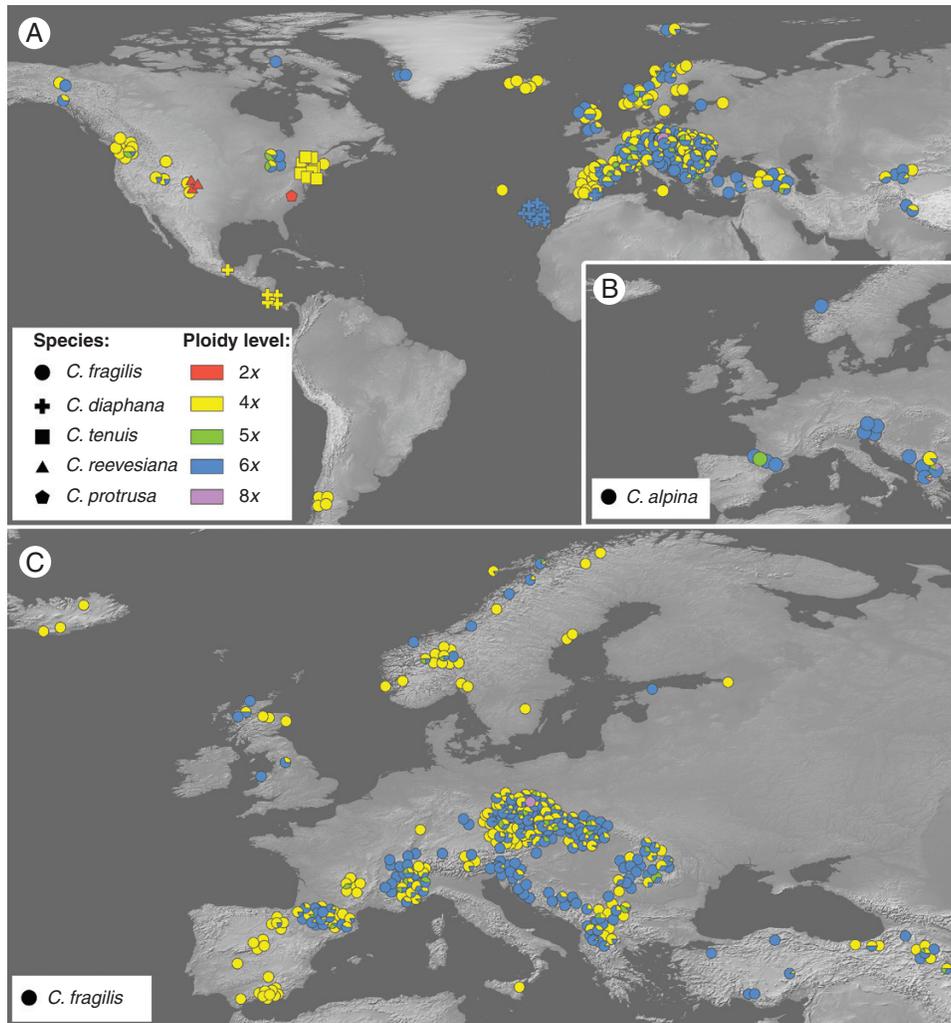
Fig. 1. Spatial distribution of cytotype diversity in *Cystopteris fragilis* and related species. Mixed-ploidy populations are depicted as pie charts showing the local frequency of cytotypes. Sampling at a world-wide scale (A), with inset displaying the sampled populations of *C. alpina* (B). Detail of cytotype distribution in the European *Cystopteris fragilis* populations (C) subjected to more intensive field sampling. For the more detailed distribution of central European populations of *C. fragilis* see Supplementary Data Fig. S3.

isolated geographical position (Fig. 1A) and slightly higher Cx values (Table 1), were excluded from all genome-size-related analyses as these could represent a special type.

Taking into account the Cx value differences between tetra- and hexaploids, we further attempted to use this trait as a clue for reconstructing evolutionary relationships among locally co-occurring cytotypes. Interestingly, while there were no differences in monoploid genome size of tetraploids from mixed-ploidy and uniform-ploidy populations (Kruskal–Wallis test; $\chi^2 = 1.9$, d.f. = 1, $P = 0.163$), hexaploids from mixed-ploidy populations had slightly but significantly higher Cx values than their counterparts from uniform-ploidy populations (mean = 0.1067 ± 0.0002 s.e., mean = 0.1059 ± 0.0002 s.e., respectively; Kruskal–Wallis test: $\chi^2 = 6.6$, d.f. = 1, $P = 0.010$). Mean Cx value of co-occurring tetra- and hexaploids is a very good predictor of Cx value of pentaploids residing in these mixed-ploidy populations ($F_{1,21} = 172.0$, $P < 0.001$, $R^2 = 0.891$; Fig. 3B), consistent with the recurrent origin of pentaploids from inter-ploidy crosses between coexisting tetraploids and hexaploids.

Ecological preferences of C. fragilis *cytotypes*

The sampled *C. fragilis* populations exhibited a substantial elevational range, from sea level up to 4670 m a.s.l. in the Himalayas (Supplementary Data Fig. S4), although no difference in mean elevation was found among the cytotypes ($F_{2,426} = 1.67$, $P = 0.189$). Similarly, tetraploid, hexaploid and mixed-ploidy populations were not differentiated by preferential occurrence at habitats of natural (e.g. rock crevices) or anthropogenic (e.g. walls) origin ($\chi^2 = 1.38$, d.f. = 2, $P = 0.501$; Fig. 2A). However, significant differences were observed for substrate preference ($\chi^2 = 16.56$, d.f. = 2, $P < 0.001$; Fig. 2B), with hexaploid populations being more common on alkaline than siliceous substrates compared to both tetraploid ($\chi^2 = 15.32$, d.f. = 1, $P_{adj.} < 0.001$) and mixed-ploidy populations ($\chi^2 = 6.64$, d.f. = 1, $P_{adj.} = 0.030$). Tetraploid and mixed-ploidy populations do not differ significantly in substrate preferences ($\chi^2 = 1.09$, d.f. = 1, $P_{adj.} = 0.890$).

Climatic niches of tetraploid, hexaploid and mixed-ploidy *C. fragilis* populations, as reconstructed using 19 Bioclim

TABLE 1. *Overview of cytotype diversity and genome size parameters for commonly recognized members of the* Cystopteris fragilis *complex. Using flow cytometry, we estimated the relative genome size (the ratio of sample to standard fluorescence), monoploid genome size (relative genome size divided by ploidy level) and absolute genome size (propidium iodide staining, in pg)*

Taxon	Ploidy	Number of sampled plants	Relative GS* (mean ± s.d.)	Monoploid GS* (Cx value) (mean ± s.d.)	Mean CV	Absolute GS* (pg) (mean ± s.d.)	Mean CV
C. fragilis	4x	2775 (28)[†]	0.436 ± 0.009	0.109 ± 0.002	2.20 ± 0.47	14.26 ± 0.070	2.48 ± 0.31
	5x	176 (7)[†]	0.542 ± 0.010	0.108 ± 0.002	2.18 ± 0.45	17.59 ± 0.059	2.27 ± 0.14
	6x	2524 (12)[†]	0.638 ± 0.013	0.106 ± 0.002	2.09 ± 0.45	20.80 ± 0.424	2.64 ± 0.23
	8x	1	0.863	0.108	1.02		
C. f. from Argentina	4x	42	0.459 ± 0.005	0.115 ± 0.001	2.20 ± 0.37		
C. alpina	4x	21	0.441 ± 0.001	0.111 ± 0.001	2.81 ± 0.22		
	5x	6	0.536 ± 0.004	0.107 ± 0.001	2.09 ± 0.54		
	6x	202 (4)[†]	0.633 ± 0.010	0.106 ± 0.002	2.31 ± 0.36	20.91 ± 0.130	2.75 ± 0.06
	8x	2	0.850 ± 0.034	0.106 ± 0.004	1.77 ± 0.33		
C. diaphana	4x	36	0.577 ± 0.006	0.144 ± 0.001	2.49 ± 0.29		
	6x	55	0.790 ±0.014	0.132 ± 0.002	1.99 ± 0.67		
C. protrusa	2x	9	0.260 ± 0.001	0.130 ± 0.000	2.61 ± 0.54		
C. reevesiana	2x	54	0.214 ± 0.005	0.107 ± 0.002	2.74 ± 0.22		
C. tenuis	4x	20	0.464 ± 0.008	0.116 ± 0.002	2.54 ± 0.26		

*GS = genome size; only flow-cytometric analyses with CV of sample peak <3 % were used for computing genome size statistics.

[†]The number in parentheses indicates for how many samples absolute genome size was estimated using PI flow cytometry.
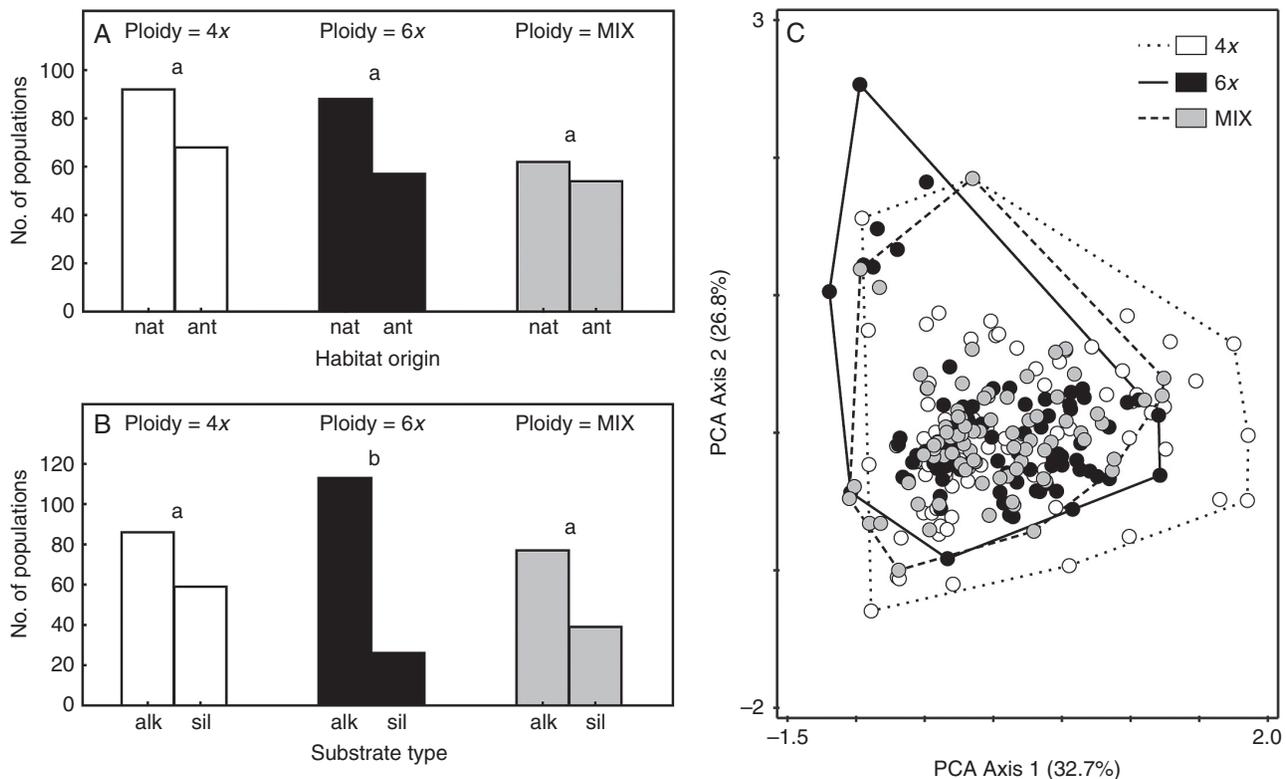
FIG. 2. Comparisons of climatic and abiotic niches among uniformly tetraploid, uniformly hexaploid and mixed-ploidy populations of *Cystopteris fragilis*. Differences in habitat origin, natural vs. anthropogenic (A), and in the type of substrate, alkaline vs. siliceous (B). Lower-case letters indicate significantly different groups in pairwise comparisons using chi-square tests for homogeneity (i.e. differences in relative incidence at particular habitat types are compared between cytotypes). A principal component analysis (C) shows climatic niches of tetraploid, hexaploid and mixed-ploidy populations reconstructed from 19 Bioclim variables. Lines connect the most divergent populations in each group.

variables, showed substantial overlap in a PCA (Fig. 2C). The effect of cytotype composition of populations was not significantly different in a redundancy analysis (*P* = 0.611, 999 permutations) and both constrained axes explained together only 0.6 % of the variation, whereas the first unconstrained axis in the analysis explained 33.2 % of the variation.
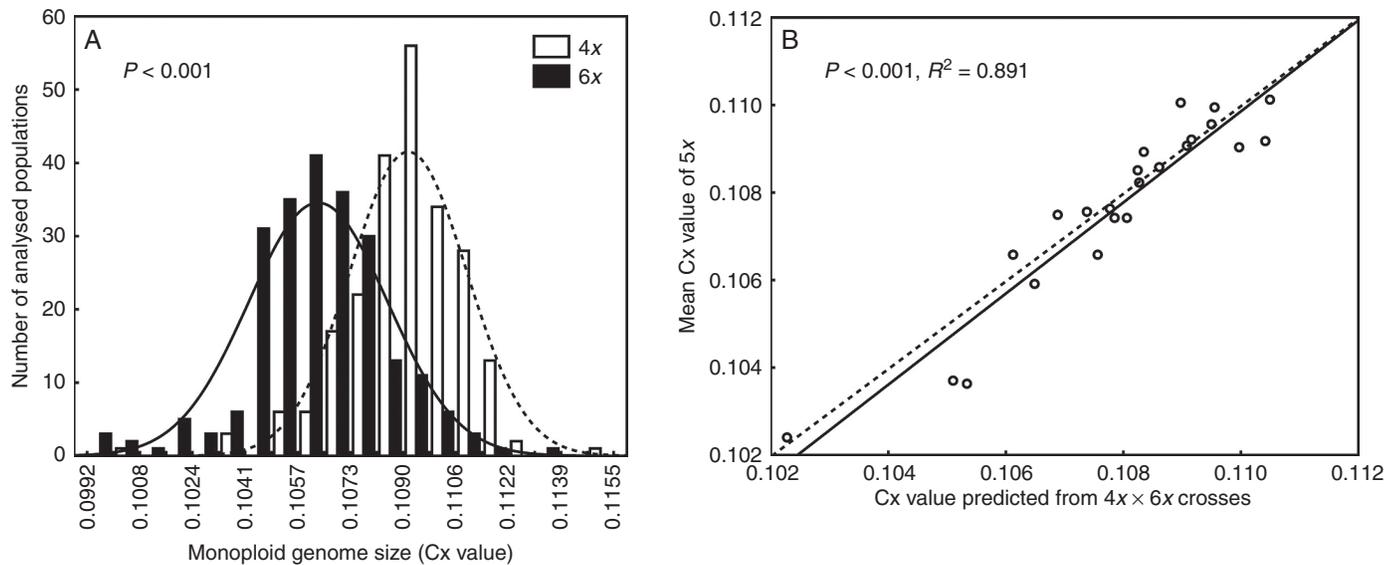
Fig. 3. Differences in monoploid genome size (i.e. relative genome size divided by ploidy level; Cx value) between *C. fragilis* tetraploids and hexaploids (A). Utilization of monoploid genome size divergence to compare two competing scenarios explaining the origin of pentaploids in mixed-ploidy populations of *C. fragilis* (B). Using a linear regression model (solid line), we related the Cx value of pentaploids to the mean Cx value of co-occurring tetraploids and hexaploids. In the case of a local independent origin of pentaploids in each mixed-ploidy population, the Cx values of response and explanatory variables should be identical (as represented by the dashed line), whereas in the case of immigration of established pentaploids from a distinct population, the Cx values should differ.

DISCUSSION

Cytotype diversity

By taking advantage of fast and reliable ploidy estimation using flow cytometry, we examined the ploidy level of 5518 individuals from 449 populations across the geographical range of *C. fragilis*, with particular emphasis on distribution in Europe (Fig. 1). Undoubtedly, this is the most comprehensive cytotype screening ever conducted in ferns (and one of the widest among vascular plants) with respect to both the area covered and the number of sampled individuals. All cytotypes previously reported in natural populations of *C. fragilis* were discovered (i.e. 4*x*, 5*x*, 6*x* and 8*x*; Manton, 1950; Vida, 1974; Kawakami *et al.*, 2010), and these have contrasting frequencies of occurrence. Whereas the high frequencies of tetraploids and hexaploids (51 % and 46 % of sampled individuals, respectively) suggest these are well-established cytotypes, pentaploids and octoploids are rare (3 % and 0.02 %, respectively). Our data indicate that the pentaploids originate within mixed-ploidy populations from inter-ploidy crosses between tetraploids and hexaploids. Pentaploids are only found co-occurring with their putative parental cytotypes, and their recurrent hybrid origin is supported by monoploid genome size, as the Cx value of pentaploids can be accurately predicted from the mean of Cx values of the locally co-occurring tetra- and hexaploids (linear model explaining 89 % of the variation, Fig. 3B). In addition, the pentaploids have greatly reduced spore fertility (69–100 % of spores aborted compared to 0–1 % in tetra- and hexaploids, data not shown), which is generally common in odd-level polyploids (Ramsey and Schemske, 1998; Ekrt and Koutecký, 2016). The single octoploid individual could have originated via three possible scenarios involving different combinations of reduced and unreduced gametes of the other cytotypes (i.e. 4*x* + 4*x* gametes, 6*x* + 2*x*, or 3*x* + 5*x*). However, due to only subtle differences in Cx values of the co-occurring putative parental cytotypes, the three pathways cannot be reliably distinguished based on genome size, and the presence of both tetraploids and hexaploids in nearby populations prevents any inference from the regional frequency of parental cytotypes.

Apart from *C. fragilis*, several other related species were included in our sampling to put our results into context. In *C. alpina*, a European species which has previously been considered uniformly hexaploid (Manton, 1950; Blasdell, 1963; Vida, 1974), four different ploidy levels were detected (4*x*, 5*x*, 6*x* and 8*x*). Nonetheless, hexaploids are clearly the dominant cytotype – they constitute 86 % of our *C. alpina* samples and were found in all but one *C. alpina* population investigated. The remaining three cytotypes were found only in Macedonia and usually occurred alongside hexaploids in mixed-ploidy populations. Populations of American species *C. protrusa* and *C. reevesiana* were uniformly diploid, and populations of *C. tenuis* were uniformly tetraploid, in line with previous studies (Haufler and Windham, 1991). Populations of *C. diaphana* were uniformly tetraploid in America but uniformly hexaploid in Europe (including Macaronesia). While the situation in Europe is consistent with Vida (1974), three ploidy levels (2*x*, 4*x* and 6*x*) were previously reported from American populations by Blasdell (1963), and our results might have been influenced by our low number of *C. diaphana* samples. With the exception of triploids, all cytotypes previously reported in natural populations of the *C. fragilis* complex were discovered (i.e. 2*x*, 4*x*, 5*x*, 6*x* and 8*x*; Manton, 1950; Vida, 1974; Kawakami *et al.*, 2010; Rothfels *et al.*, 2013), although we have not specifically targeted the diploid populations where such triploids would be expected (Haufler *et al.*, 1985). Interestingly, our thorough sampling corroborates the complete lack of diploids in European populations of the *C. fragilis* complex, as suggested by Blasdell (1963).

Ploidy distribution patterns

The distribution of *C. fragilis* tetra- and hexaploids shows distinct patterns in North America and Eurasia. Firstly, whereas these two cytotypes are almost equally represented in Eurasia, a striking predominance of tetraploids is apparent in North America, where they are five times more common than their hexaploid counterparts. Also, in North America, hexaploids seem to occur more often in the north and tetraploids predominate in the south, while the distribution of the two cytotypes in Eurasia is less structured (Fig. 1). However, the low number of populations (27 in total) and non-random sampling pattern in North America precluded comprehensive comparison of the spatial structure of the tetraploid–hexaploid co-occurrence and frequency of mixed-ploidy populations between the two continents. In Europe, the tetraploid–hexaploid contact zone of *C. fragilis* (i.e. the area of ploidy coexistence) is extremely wide and covers most of the continent (Fig. 1C). The contact zone has a diffuse, mosaic-like structure with common incidence of mixed-ploidy populations. This strongly contrasts with substantial spatial isolation of cytotypes documented in many fern species (e.g. Nakato and Kato, 2005; Chang *et al.*, 2013), and when local ploidy coexistence is reported – e.g. in the *Asplenium trichomanes* complex (Moran, 1982; Ekrt and Štech, 2008; Liu *et al.*, 2018) or the *A. ceterach* complex (Trewick *et al.*, 2002) – it is usually restricted to certain regions. Moreover, such a cytotype distribution pattern is quite rare even in well-documented mixed-ploidy angiosperms (but see, e.g. Duchoslav *et al.*, 2010; McAllister *et al.*, 2015; Čertner *et al.*, 2017). The frequency of mixed-ploidy populations in *C. fragilis* is very high (40 % for populations with >10 sampled individuals) and 67 % of $4x + 6x$ populations also include pentaploids. Comparable frequencies of ploidy mixtures have been documented in only a few angiosperm species (e.g. *Gymnadenia conopsea* and *Andropogon gerardii*; Trávníček *et al.*, 2011; McAllister *et al.*, 2015; Kolář *et al.*, 2017). Although multiple cytotypes were previously reported from natural populations of *C. fragilis* (Vida, 1974; Kawakami *et al.*, 2010), our results demonstrate the frequency and scope of ploidy coexistence across the species' entire distributional range. The widespread ploidy coexistence makes *C. fragilis* a convenient model system for studying the microevolutionary mechanisms of polyploid speciation in ferns.

Monoploid genome size divergence

Flow-cytometric analysis has revealed subtle but highly significant differences in monoploid genome size (i.e. Cx value) between *C. fragilis* tetra- and hexaploids. Specifically, the monoploid genome of hexaploids is on average 2.6 % smaller than that of tetraploids (Fig. 3A). This might be a consequence of 'genome downsizing', a process of systematic DNA loss, which is known to commonly accompany polyploidy (Leitch and Bennett, 2004; Tayalé and Parisod, 2013). Some support for this explanation may be provided by two other ploidy-heterogeneous species from the *C. fragilis* complex, *C. alpina* and *C. diaphana*, in which a similar trend of decreasing Cx value with increasing ploidy seems to be present (Table 1). Alternatively, the *C. fragilis* hexaploids could have originated from unsampled ancestors with lower Cx value. Given that our data were based

on flow-cytometric analysis using AT-specific DAPI staining, the observed differences could theoretically be attributed to different base composition (GC content) and not genome size of the two cytotypes. However, when we used base-unspecific PI staining on a selected subset of *C. fragilis* individuals (Table 1), the Cx value differences between tetra- and hexaploids were retained ($F_{1,38} = 11.5$, $P = 0.002$).

The Cx value, allowing genome size comparisons independent of ploidy level, may in some cases be used to reconstruct cytotype relationships and modes of cytotype origin, and, possibly, to detect recurrent origin of polyploids (Čertner *et al.*, 2017). In this study, we used the Cx value differences as a clue to reconstruct evolutionary relationships among locally co-occurring cytotypes in *C. fragilis*, although the potential utility of this approach is substantially limited by the very small between-ploidy difference in this species. Nevertheless, we detected significantly higher Cx values of hexaploids from mixed-ploidy populations (i.e. those with values more similar to the Cx value of tetraploids) when compared with their counterparts from uniformly hexaploid populations. The fact that Cx values of tetra- and hexaploids were more similar in sympatry (mixed-ploidy populations) than in allopatry (uniform-ploidy populations) could be explained by recurrent polyploidization, the presence of locally originated hexaploids in some mixed-ploidy populations (i.e. primary cytotype contact; Petit *et al.*, 1999). Alternatively, such a pattern could result from frequent inter-ploidy hybridization and gene flow, possibly involving pentaploids as intermediates. Were this the case, the gene flow would have to be strongly asymmetric to explain significant Cx value changes only in hexaploids and not tetraploids. Interestingly, such strongly asymmetric gene flow from tetra- to hexaploids but not vice versa was previously reported, e.g. in *Senecio carniolicus* (Hülber *et al.*, 2015).

We also used the Cx value differences between locally co-occurring tetra- and hexaploids to corroborate a local hybrid origin of *C. fragilis* pentaploids in mixed-ploidy populations (Fig. 3B). Our premise was that with prevailing immigration of pentaploids from different populations (in which they are established and spread out), we would not observe a tight correlation between the Cx value of pentaploids and mean Cx value of residing tetra- and hexaploids in $4x + 5x + 6x$ populations. Moreover, the Cx value divergence between tetraploid samples from Argentina and the other *C. fragilis* tetraploids (Table 1) may be a sign that these represent a different, cryptic species. Collectively, our results suggest that Cx value differences may provide interesting insight into microevolutionary processes in ploidy-heterogeneous species. Note, however, that intraspecific genome size variation may occasionally be an artefact caused by technical difficulties (e.g. error of measurement, presence of staining inhibitors in the material, low material quality or improper material storage; Greilhuber, 2005). Here, we are convinced that our results are sufficiently robust, as repeated measurements of selected samples provided highly comparable estimates, fresh leaf material was preferred for *C. fragilis* (only approx. 30 % of samples were silica-gel dried, pilot tests confirmed estimates highly comparable to analysing fresh material), and we only used high-quality analyses (CV <3 %) for statistical comparisons. Nonetheless, caution is needed when interpreting such data and it should be ideally combined with other approaches (e.g. molecular-genetic analyses) before firm conclusions are reached.

The drivers of ploidy coexistence

Due to the reduced fitness (sterility) of offspring from between-ploidy crosses, plant fitness in mixed-ploidy populations depends strongly on cytotype frequency. According to theoretical models, the less common (minority) cytotypes should spend more of their reproductive effort on ineffective between-ploidy mating, and thus decline in frequency and ultimately become excluded (Levin, 1975; Husband, 2000). However, several factors and ecological processes may facilitate ploidy coexistence in natural populations (reviewed by Kolář *et al.*, 2017), one of the most important being spatial clustering of cytotypes (favouring within-ploidy mating; Baack, 2005), which is commonly driven by their sorting along ecological gradients (Manzaneda *et al.*, 2012; Laport *et al.*, 2016).

However, our results imply a very limited effect of environmental heterogeneity on cytotype distribution patterns in *C. fragilis*. Tetraploids and hexaploids occupy the same climatic niches and elevational ranges, and show similar preferences for habitat origin (rocks vs. man-made walls). The only sign of ecological differentiation was found in substrate preference, as the relative incidence of uniformly hexaploid populations at alkaline sites was significantly greater than observed in both uniformly tetraploid and mixed-ploidy populations (Fig. 2B). Given that hexaploids from mixed-ploidy populations showed the same substrate preferences as tetraploids, it seems unlikely these differences were caused by inherently distinct ecophysiology of the two cytotypes. Alternatively, the observed association could stem from an interplay between the founder effect, allowing hexaploids to dominate in some regions with alkaline bedrock (e.g. Dinaric Alps, French Alps), and a sampling bias (e.g. unintentionally high sampling intensity in such regions). When statistical comparisons of substrate preferences were restricted to the Central European *C. fragilis* populations to exclude all tetraploid/hexaploidy-dominated regions, the overall effect of cytotype composition was only marginally significant ($\chi^2 = 6.38$, d.f. = 2, $P = 0.041$) and none of the pairwise comparisons differed significantly after applying the Bonferroni correction. While we cannot rule out some effect of substrate quality on cytotype distribution patterns in *C. fragilis*, our data do not suggest that it has an important role in maintaining tetraploid–hexaploid coexistence.

We are well aware that our survey of environmental parameters may not have been comprehensive enough to reveal other signs of cytotype ecological differentiation. For example, local environmental conditions in the sampled populations could be substantially better described if we employed chemical soil analysis and direct measurements of microclimatic conditions using temperature and moisture probes. Unfortunately, the applied methodology was largely a trade-off caused by the scope of our study (nearly global-scale sampling) and the very high number of sampled populations. Nonetheless, the lack of cytotype ecological differentiation is also indirectly supported by the ploidy distribution patterns (e.g. range-wide ploidy coexistence, high incidence of mixed-ploidy populations). While we cannot rule out a fine-scale ecological segregation of cytotypes within mixed-ploidy populations, during our field sampling, we did not notice any signs of greater microhabitat diversity in mixed-ploidy compared to uniform-ploidy populations. No ecological differences among cytotypes were also documented

in other ploidy-variable plant species (Buggs and Pannell, 2007; Glennon *et al.*, 2014; Hanzl *et al.*, 2014), suggesting that ecological segregation of cytotypes may be a common scenario rather than the rule.

The widespread and frequent ploidy coexistence, however, can be explained by other mechanisms. In general, plant longevity and the ability to reproduce clonally may partially mitigate the effect of minority cytotype exclusion (Yamauchi *et al.*, 2004). In a recent review of cytotype diversity among angiosperms, asexual reproduction resulted in a nearly two-fold increase in the frequency of mixed-ploidy populations and also contributed to the abundance of odd-ploidy cytotypes (Kolář *et al.*, 2017). As with most ferns, *C. fragilis* is a long-lived rhizomatous perennial, and even though the clonal spread of the species is limited and probably locally restricted (Hovenkamp, 1990), these traits could facilitate ploidy coexistence within populations and also favour the persistence of largely sterile pentaploid hybrids suffering from meiotic irregularities. Additionally, several other mechanisms specific to the reproductive biology of ferns could promote local cytotype coexistence. First, the movement of spermatozoids among gametophytes is more spatially restricted than pollen transfer in angiosperms, especially in *Cystopteris*, where suitable micro-habitats (e.g. crevices in walls and rock faces) are often patchily distributed. Secondly, gametophytic selfing documented in the genus *Cystopteris* is likely to occur and may serve as an important mechanism of reproductive assurance for minority cytotypes (Sessa *et al.*, 2016). Lastly, and probably most importantly, efficient wind dispersal of spores might often bring the two cytotypes together and facilitate ploidy coexistence, either by founding new mixed-ploidy populations or by supplying immigrants to the existing ones.

CONCLUSIONS

Our study has provided the first detailed insight into ploidy distribution patterns in populations of a fern species. Contrary to the substantial spatial isolation of cytotypes documented in most ferns, mosaic-like structure of the 4*x*–6*x* contact zone in *C. fragilis* favours ploidy coexistence even within populations and makes it a common and widespread phenomenon across the entire distributional range of the species. Both ploidy-level diversity and the frequency of mixed-ploidy populations observed here suggest that in this respect ferns can match the well-documented patterns in angiosperms (Kolář *et al.*, 2017).

We also focused on possible evolutionary drivers of common coexistence of cytotypes in this species. Because no ecological constraints to ploidy coexistence were detected, the local co-occurrence of tetra- and hexaploids seems to be possible across the entire range of environmental conditions suitable for *C. fragilis*. Persistence of local ploidy mixtures could be facilitated by the perennial life-form of *C. fragilis*, its reproductive modes (occasional clonal spread and gametophytic selfing) and efficient wind dispersal of spores (founding new mixed-ploidy populations or supplying immigrants to existing ones). Moreover, independent origins of hexaploids and/or inter-ploidy gene flow may be expected in mixed-ploidy populations of *C. fragilis* as suggested by Cx value comparisons.

SUPPLEMENTARY DATA

Supplementary data are available online at https://aca-demic.oup.com/aob and consist of the following. Table S1: Complete list of sampled localities. Table S2: Datasets used for ecological niche and genome size comparisons. Fig. S1: Flow-cytometric histogram of the relative DNA content in simultaneous analysis of DAPI-stained nuclei isolated from tetraploid and hexaploid *C. fragilis* plants, with the internal standard *Vicia faba* 'Inovec'. Fig. S2: Microphotograph of chromosomes at meiosis of tetraploid *C. fragilis*. Fig. S3: Detail of cytotype distribution patterns in Central European populations of *C. fragilis*. Fig. S4: Overlapping elevational ranges of uniformly tetraploid, uniformly hexaploid and mixed-ploidy populations of *C. fragilis*.

ACKNOWLEDGEMENTS

LITERATURE CITED

Baack EJ. 2005. To succeed globally, disperse locally: effects of local pollen and seed dispersal on tetraploid establishment. *Heredity* **94:** 538–546.

Barker M. 2013. Karyotype and genome evolution in pteridophytes. In: Greilhuber J, Doležel J, Wendel JF eds. *Plant genome diversity*, vol. 2. Vienna: Springer-Verlag, 245–353.

Blasdell RF. 1963. A monographic study of the fern genus *Cystopteris*. *Memoirs of the Torrey Botanical Club* **21:** 1–102.

Buggs RJA, Pannell JR. 2007. Ecological differentiation and diploid superiority across a moving ploidy contact zone. *Evolution* **61:** 125–140.

Čertner M, Fenclová E, Kúr P, et al. 2017. Evolutionary dynamics of mixed-ploidy populations in an annual herb: dispersal, local persistence and recurrent origins of polyploids. *Annals of Botany* **120:** 303–315.

Chang Y, Li J, Lu S, Schneider H. 2013. Species diversity and reticulate evolution in the *Asplenium normale* complex (Aspleniaceae) in China and adjacent areas. *Taxon* **62:** 673–687.

Chrtek J, Herben T, Rosenbaumová R, et al. 2017. Cytotype coexistence in the field cannot be explained by inter-cytotype hybridization alone: linking experiments and computer simulations in the sexual species *Pilosella echioides* (Asteraceae). *BMC Evolutionary Biology* **17:** 87.

Clark J, Hidalgo O, Pellicer J, et al. 2016. Genome evolution of ferns: evidence for relative stasis of genome size across the fern phylogeny. *New Phytologist* **210:** 1072–1082.

Coyne JA, Orr HA. 2004. *Speciation*. Sunderland, MA: Sinauer Associates, Inc.

Dauphin B, Grant JR, Farrar DR, Rothfels CJ. 2018. Rapid allopolyploid radiation of moonwort ferns (*Botrychium*; Ophioglossaceae) revealed by PacBio sequencing of homologous and homeologous nuclear regions. *Molecular Phylogenetics and Evolution* **120:** 342–353.

Doležel J, Sgorbati S, Lucretti S. 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Physiologia Plantarum* **85:** 625–631.

Doležel J, Geilhuber J, Suda J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* **2:** 2233–2244.

Duchoslav M, Šafářová L, Krahulec F. 2010. Complex distribution patterns, ecology and coexistence of ploidy levels of *Allium oleraceum* (Alliaceae) in the Czech Republic. *Annals of Botany* **105:** 719–735.

Ekrt L, Koutecký P. 2016. Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (Dryopteridaceae). *Annals of Botany* **117:** 97–106.

Ekrt L, Štech M. 2008. A morphometric study and revision of the *Asplenium trichomanes* group in the Czech Republic. *Preslia* **80:** 325–347.

Ekrt L, Holubová R, Trávníček P, Suda J. 2010. Species boundaries and frequency of hybridization in the *Dryopteris carthusiana* (Dryopteridaceae) complex: a taxonomic puzzle resolved using genome size data. *American Journal of Botany* **97:** 1208–1219.

Felber F. 1991. Establishment of a tetraploid cytotype in a diploid population: effect of relative fitness of the cytotypes. *Journal of Evolutionary Biology* **4:** 195–207.

Fraser-Jenkins CR. 2008. *Taxonomic revision of three hundred Indian subcontinental pteridophytes: with a revised census list; a new picture of fern-taxonomy and nomenclature in the Indian subcontinent*. Bishen Singh Mahendra Pal Singh.

Gämperle E, Schneller JJ. 2002. Phenotypic and isozyme variation in *Cystopteris fragilis* (Pteridophyta) along an altitudinal gradient in Switzerland. *Flora* **197:** 203–213.

Glennon KL, Ritchie ME, Segraves KA. 2014. Evidence for shared broad-scale climatic niches of diploid and polyploid plants. *Ecology Letters* **17:** 574–582.

Greilhuber J. 2005. Intraspecific variation in genome size in angiosperms: identifying its existence. *Annals of Botany* **95:** 91–98.

Grusz AL, Windham MD, Yatskievych G, Huiet L, Gastony GJ, Pryer KM. 2014. Patterns of diversification in the xeric-adapted fern genus *Myriopteris* (Pteridaceae). *Systematic Botany* **39:** 698–714.

Hanzl M, Kolář F, Nováková D, Suda J. 2014. Nonadaptive processes governing early stages of polyploid evolution: insights from a primary contact zone of relict serpentine *Knautia arvensis* (Caprifoliaceae). *American Journal of Botany* **101:** 935–945.

Haufler CH, Windham MD. 1991. New species of North American *Cystopteris* and *Polypodium*, with comments on their reticulate relationships. *American Fern Journal* **81:** 7–23.

Haufler CH, Windham MD, Britton DM, Robinson SJ. 1985. Triploidy and its evolutionary significance in *Cystopteris protrusa. Canadian Journal of Botany* **63:** 1855–1863.

Haufler CH, Moran RC, Windham MD. 1993. *Cystopteris*. Flora of North America, **Vol. 2.** New York: Oxford University Press, 263–270.

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A. 2005. Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* **25:** 1965–1978.

Hovenkamp P. 1990. The significance of rhizome morphology in the systematics of the Polypodiaceous ferns (*sensu stricto*). *American Fern Journal* **80:** 33–43.

Hülber K, Sonnleitner M, Suda J, et al. 2015. Ecological differentiation, lack of hybrids involving diploids, and asymmetric gene flow between polyploids in narrow contact zones of *Senecio carniolicus* (syn. *Jacobaea carniolica*, Asteraceae). *Ecology and Evolution* **5:** 1224–1234.

Husband BC. 2000. Constraints on polyploid evolution: a test of the minority cytotype exclusion principle. *Proceedings of the Royal Society B: Biological Sciences* **267:** 217–223.

Husband BC, Ozimec B, Martin SL, Pollock L. 2008. Mating consequences of polyploid evolution in flowering plants: current trends and insights from synthetic polyploids. *International Journal of Plant Sciences* **169:** 195–206.

Kawakami SM, Kawakami S, Kato J, Kondo K, Smirnov SV, Damdinsuren O. 2010. Cytological study of a fern *Cystopteris fragilis* in Mongolian Altai. *Chromosome Botany* **5:** 1–3.

Kolář F, Čertner M, Suda J, Schönswetter P, Husband BC. 2017. Mixed-ploidy species: progress and opportunities in polyploid research. *Trends in Plant Science* **22:** 1041–1055.

Kreiner JM, Kron P, Husband BC. 2017. Evolutionary dynamics of unreduced gametes. *Trends in Genetics* **33:** 583–593.

Landis JB, Soltis DE, Li Z, Marx HE, Barker MS, Tank DC, Soltis PS. 2018. Impact of whole-genome duplication events on diversification rates in angiosperms. *American Journal of Botany* **105:** 348–363.

Laport RG, Minckley RL, Ramsey J. 2016. Ecological distributions, phenological isolation, and genetic structure in sympatric and parapatric populations of the *Larrea tridentata* polyploid complex. *American Journal of Botany* **103:** 1358–1374.

Leitch IJ, Bennett MD. 2004. Genome downsizing in polyploid plants. *Biological Journal of the Linnean Society* **82:** 651–663.

Levin DA. 1975. Minority cytotype exclusion in local plant populations. *Taxon* **24:** 35–43.

Levin DA. 2002. *The role of chromosomal change in plant evolution*. Oxford: Oxford University Press.

Liu HM, Russell SR, Vogel J, Schneider H. 2018. Inferring the potential of plastid DNA-based identification of derived ferns: a case study on the *Asplenium trichomanes* aggregate in Europe. *Plant Systematics and Evolution* **304:** 1009–1022.

Manton I. 1950. *Problems of cytology and evolution in the Pteridophyta*. Cambridge: Cambridge University Press.

Manzaneda AJ, Rey PJ, Bastida JM, Weiss-Lehman C, Raskin E, Mitchell-Olds T. 2012. Environmental aridity is associated with cytotype segregation and polyploidy occurrence in *Brachypodium distachyon* (Poaceae). *New Phytologist* **193:** 797–805.

McAllister C, Blaine R, Kron P, *et al.* 2015. Environmental correlates of cytotype distribution in *Andropogon gerardii* (Poaceae). *American Journal of Botany* **102:** 92–102.

Moran RC. 1982. The *Asplenium trichomanes* complex in the United States and adjacent Canada. *American Fern Journal* **72:** 5–11.

Nakato N, Kato M. 2005. Cytogeography of the *Adiantum pedatum* complex (Pteridaceae, Subfamily Adiantoideae). *Acta Phytotaxonomica et Geobotanica* **56:** 85–96.

Parks JC, Dyer AF, Lindsay S. 2000. Allozyme, spore and frond variation in some Scottish populations of the ferns *Cystopteris dickieana* and *Cystopteris fragilis*. *Edinburgh Journal of Botany* **57:** 83–105.

Petit C, Lesbros P, Ge X, Thompson JD. 1997. Variation in flowering phenology and selfing rate across a contact zone between diploid and tetraploid *Arrhenatherum elatius* (Poaceae). *Heredity* **79:** 31–40.

Petit C, Bretagnolle F, Felber F. 1999. Evolutionary consequences of diploid–polyploid hybrid zones in wild species. *Trends in Ecology & Evolution* **14:** 306–311.

PPG I. **2016.** A community-derived classification for extant lycopods and ferns. *Journal of Systematics and Evolution* **54:** 563–603.

R Core Team. **2016.** *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Ramsey J. 2007. Unreduced gametes and neopolyploids in natural populations of *Achillea borealis* (Asteraceae). *Heredity* **98:** 143–150.

Ramsey J, Schemske DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics* **29:** 467–501.

Rothfels CJE. 2012. *Phylogenetics of Cystopteridaceae: reticulation and divergence in a cosmopolitan fern family*. PhD Thesis, Duke University, Durham.

Rothfels CJ, Windham MD, Pryer KM. 2013. A plastid phylogeny of the cosmopolitan fern family Cystopteridaceae (Polypodiopsida). *Systematic Botany* **38:** 295–306.

Rothfels CJ, Johnson AK, Windham MD, Pryer KM. 2014. Low-copy nuclear data confirm rampant allopolyploidy in the Cystopteridaceae (Polypodiales). *Taxon* **63:** 1026–1036.

Rothfels CJ, Pryer KM, Li F-W. 2017. Next-generation polyploid phylogenetics: rapid resolution of hybrid polyploid complexes using PacBio single-molecule sequencing. *New Phytologist* **213:** 413–429.

Schneider H, Liu HM, Chang YF, *et al.* 2017. Neo- and paleopolyploidy contribute to the species diversity of *Asplenium*–the most species-rich genus of ferns. *Journal of Systematics and Evolution* **55:** 353–364.

Sessa EB, Testo WL, Watkins JE. 2016. On the widespread capacity for, and functional significance of, extreme inbreeding in ferns. *New Phytologist* **211:** 1108–1119.

Shinohara W, Ushio Y, Seo A, *et al.* 2010. Evidence for hybrid origin and segmental allopolyploidy in eutetraploid and aneutetraploid *Lepisorus thunbergianus* (Polypodiaceae). *Systematic Botany* **35:** 20–29.

Soltis DE, Soltis PS, Tate JA. 2003. Advances in the study of polyploidy since plant speciation: research review. *New Phytologist* **161:** 173–191.

Soltis DE, Visger CJ, Marchant DB, Soltis PS. 2016. Polyploidy: pitfalls and paths to a paradigm. *American Journal of Botany* **103:** 1223–1235.

Tayalé A, Parisod C. 2013. Natural pathways to polyploidy in plants and consequences for genome reorganization. *Cytogenetic and Genome Resources* **140:** 79–96.

ter Braak C, Šmilauer P. 2012. *Canoco reference manual and user's guide: software for ordination, version 5.0*. Ithaca: Microcomputer Power.

Trávníček P, Kubátová B, Čurn V, *et al.* 2011. Remarkable coexistence of multiple cytotypes of the *Gymnadenia conopsea* aggregate (the fragrant orchid): evidence from flow cytometry. *Annals of Botany* **107:** 77–87.

Trewick SA, Morgan-Richards M, Russell SJ, *et al.* 2002. Polyploidy, phylogeography and Pleistocene refugia of the rockfern *Asplenium ceterach*: evidence from chloroplast DNA. *Molecular Ecology* **11:** 2003–2012.

Vida G. 1974. Genome analysis of the European *Cystopteris fragilis* complex: 1. Tetraploid taxa. *Acta Botanica Academiae Scientiarum Hungaricae* **20:** 181–192.

Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH. 2009. The frequency of polyploid speciation in vascular plants. *Proceedings of the National Academy of Sciences USA* **106:** 13875–13879.

Yamauchi A, Hosokawa A, Nagata H, Shimoda M. 2004. Triploid bridge and role of parthenogenesis in the evolution of autopolyploidy. *The American Naturalist* **164:** 101–112.

Paper 13

Ekrt L., Košnar J., Rothfels C., Hanušová K., Hornych O. & Urfus T. (2022). Cytogenetic, geographical, spore type and plastid haplotype data reveal cryptic patterns of species diversity in the cosmopolitan *Cystopteris fragilis* complex (Polypodiopsida: Cystopteridaceae). – Botanical Journal of Linnean Society 199: 728–739.

Cytogenetic, geographical, spore type and plastid haplotype data reveal cryptic patterns of species diversity in the cosmopolitan *Cystopteris fragilis* complex (Polypodiopsida: Cystopteridaceae)

LIBOR EKRT[1,*,][•], JIŘÍ KOŠNAR[1], CARL J. ROTHFELS[2], KRISTÝNA HANUŠOVÁ[3], ONDŘEJ HORNYCH[1] and TOMÁŠ URFUS[3]

[1]*Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 1760, CZ-370 05 České Budějovice, Czech Republic*
[2]*University Herbarium and Department of Integrative Biology, University of California, Berkeley, CA 94720, USA*
[3]*Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 00 Praha, Czech Republic*

The delimitation of lineages in the *Cystopteris fragilis* complex is complicated by the presence of multiple cytotypes and a lack of defining morphological characters. One character, the production of rugose instead of regular spiny spores, is sometimes associated with a potential Scottish endemic, *C. dickieana*; however, whether this character is associated with a distinct lineage is uncertain. To better understand the diversity in the *C. fragilis* complex, we selected 87 *C. fragilis* samples of known ploidy (4*x*, 5*x*, 6*x*) for sequencing of two plastid loci and we assessed their spore types. These samples represent the variability found in Northern Hemisphere populations, including the type locality of *C. dickieana* in Scotland. Our analyses revealed two haplotype lineages, which we label the *hemifragilis* and *reevesiana* clades, based on their potential relationship to the two presumed diploid parents of *C. fragilis*. Hexaploids and tetraploids were both polyphyletic. Rugose spores were rarer overall (26% of samples), but five times more prevalent in the *hemifragilis* clade. Although proper delimitation and understanding of *C. fragilis* remains a challenge, this study further describes great genotypic and cytotypic complexity present in this complex. Furthermore, rugose-spored plants are widely distributed and should not be associated with a single name.

ADDITIONAL KEYWORDS: chloroplast DNA – cryptic species – cytotypes – ferns – hybridization – polyploidy – pteridophytes – spores.

INTRODUCTION

The tools of molecular phylogenetics have fundamentally rewritten our understanding of the broad relationships among fern lineages (PPG I, 2016) and proved invaluable in elucidating complex patterns of fine-scale evolution, such as with species complexes (Testo *et al.*, 2019; Chen, Hyvönen & Schneider, 2020; Kao *et al.*, 2020; Kuo *et al.*, 2020). However, one particularly notable species complex remains, the cosmopolitan *Cystopteris fragilis* (L.) Bernh group.

In the *C. fragilis* complex, many species have a history of recognition, including diploids [e.g. *C. protrusa* (Weath.) Blasdell, *C. reevesiana* Lellinger] and polyploids [e.g. *C. alpina* Desv., *C. diaphana* (Bory) Blasdell, *C. douglasii* Hook., *C. tasmanica* Hook., *C. tenuis* Desv.; Moran, 1983; Haufler & Windham, 1991; Paler & Barrington, 1995; Rothfels *et al.*, 2014). Additionally, several polyploid taxa [*C. laurentiana* (Weath.) Blasdell, *C. tennesseensis* Shaver and *C. utahensis* Windham & Haufler] arose from the hybridization of *C. bulbifera* (L.) Bernh. with members of the *C. fragilix* complex (Haufler, Moran & Windham, 1993; Rothfels, Pryer & Li, 2017). Individuals not falling into one of these more-or-less recognizable taxa

*Corresponding author. E-mail: libor.ekrt@gmail.com

are typically lumped into an amorphous *C. fragilis*. Therefore, *C. fragilis* represents a loose group of as-yet unresolved taxa/lineages rather than a cohesive species, and much of our understanding of *C. fragilis* is still limited. Further complications arise from the fact that recent molecular studies have shed light on the internal relationships in *Cystopteris* and revealed substantial complexity in *C. fragilis* (Rothfels, Windham & Pryer, 2013; Rothfels *et al*., 2014, 2017). Plastid markers have demonstrated the presence of two main clades (Rothfels *et al*., 2013), apparently corresponding to the diploid *C. reevesiana* and an undiscovered or extinct taxon named '*C. hemifragilis*' (Haufler, 1985), respectively; these two diploids have been previously hypothesized to be the parental lineages of some *C. fragilis*, based on isozyme data (Haufler, 1985; Haufler & Windham, 1991). The presence of two haplotype lineages in *C. fragilis* is seen as an indication of reciprocal crossing between at least the two diploids giving rise to one or probably more tetraploid *C. fragilis* lineages. These tetraploids were described as functionally diploidized, indicating an ancient origin (Haufler & Windham, 1991; Parks, Dyer & Lindsay, 2000). The analysis of nuclear DNA showed an even greater degree of complexity: the six *C. fragilis* accessions sampled by Rothfels *et al.* (2014), for example, represented five unique genomic combinations. However, the above-mentioned

molecular analyses were not designed to fully address the complexity in the *C. fragilis* complex, and a study with a greater representation of samples worldwide is needed.

Recent research has also revealed substantial cytotype diversity in *C. fragilis*. Tetraploids, pentaploids, hexaploids and even an octoploid were found, although tetraploids and hexaploids dominated (Hanušová *et al*., 2019). The existence of hexaploids has been known for some time (Manton, 1950) and may further complicate the phylogenetic structure of *C. fragilis*. Studies of chromosome pairing in meiosis indicate an allopolyploid origin (Vida, 1974; Vida & Mohay, 1980; Kawakami *et al*., 2010), possibly involving the diploid *C. protrusa* (Vida, 1974). A broader sampling of tetraploid and hexaploid *C. fragilis* is necessary to assess the origin of the hexaploids and to infer their relationships with the rest of the species complex.

As the *C. fragilis* complex exhibits complicated patterns of morphological variation, the potential value of a seemingly simple feature, perispore ornamentation, has been the object of considerable attention. The perispore of most *Cystopteris* spp. protrudes into characteristic spines (Blasdell, 1963). However, *C. dickieana* R.Sim (Fig. 1E), a member of the *C. fragilis* complex described from Scotland (Sim, 1848), has, in addition to an unusual leaf morphology, a rugose instead of an echinate perispore. Rugose-spored
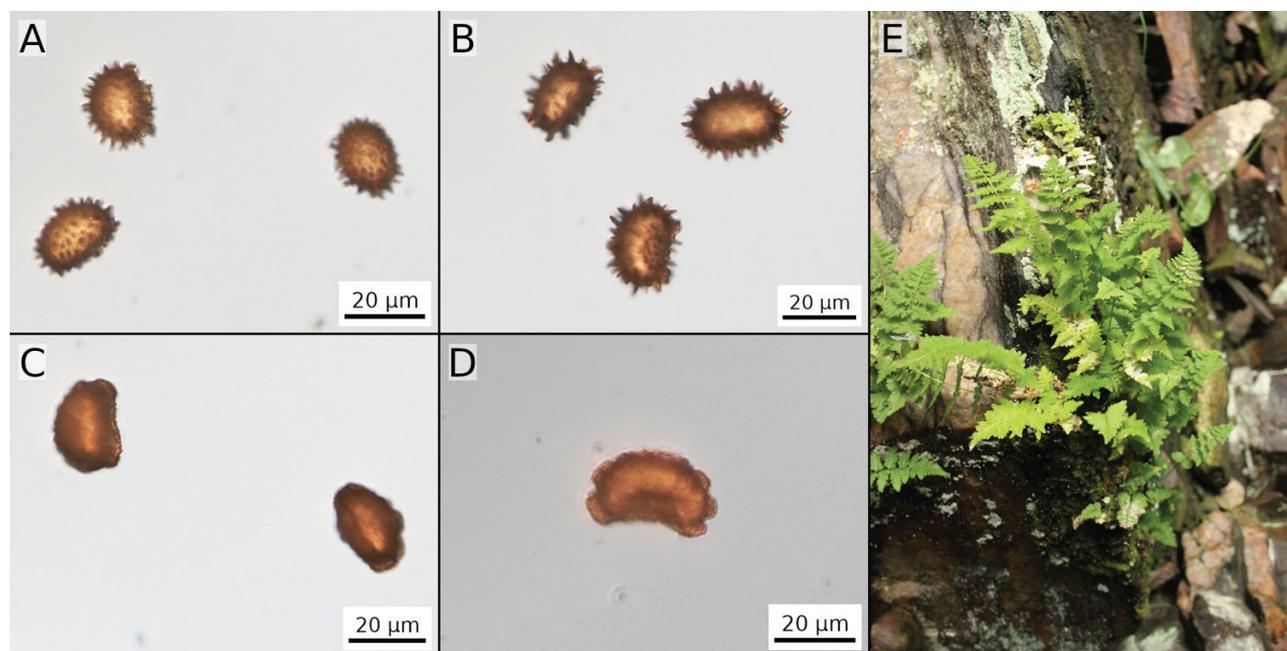
Figure 1. Spore micrographs and a living sporophyte of *Cystopteris fragilis*, all tetraploid. A, spiny spores (haplotype H1, plant ID 3); B, spiny spores (haplotype R1, plant ID 55); C, rugose spores (haplotype H2, plant ID 85); D, rugose spores (haplotype R1, plant ID 7); E, living plant of haplotype H2 from the type locality of *C. dickieana* from Cove Bay Cave, Aberdeen (Scotland, UK).

plants were subsequently found outside Scotland and across the world (e.g. by Hagenah, 1961; Breckle, 1987; Hörandl, 1989; Haufler & Windham, 1991; Berg, 1992; Ołlgaard & Tind, 1993), prompting further debate about the taxonomic recognition of *C. dickieana*, and its circumscription. Based on several lines of evidence (e.g. lack of corroborating features), the validity of *C. dickieana* as a species has been frequently questioned (Blasdell, 1963; Haufler & Windham, 1991; Haufler *et al*., 1993; Parks *et al*., 2000), and rugose-spored plants will be treated as belonging to *C. fragilis* in this study. Nevertheless, several authors still treat *C. dickieana* as a species separate from *C. fragilis* (Wang, 1983; Breckle, 1987; Jermy, 1993; Frey *et al*., 2006; Wang *et al*., 2013). Some evidence that the Scottish plants are unique holds up today. Parks *et al*. (2000) recognized a distinct pattern of isozyme variation compared to other rugose-spored plants. The existence of sterile hybrids between spiny- and rugose-spored plants (including ones from the type locality) also supports the notion that *C. dickieana* may be a separate species (Manton & Reichstein, 1965; Vida, 1974; Prada & Salvo, 1985). Although a recent molecular analysis included rugose-spored plants, deeming them no different from other *C. fragilis* samples (Rothfels *et al*., 2013), plants from the type locality were last studied based on isozyme analysis (Parks *et al*., 2000). A more contemporary analytical method used on specimens from the type locality may provide new insights into this as-yet unresolved potential species. Furthermore, it is possible that rugose spores could serve as a defining characteristic for other lineages in *C. fragilis*. Such a possibility is tantalizing as spore type could be an easily observable and determinable characteristic in an otherwise largely cryptic complex.

To address the lack of knowledge about the enigmatic *C. fragilis* group, we attempt to answer the following questions. (1) What is the phylogenetic pattern among the members of the *C. fragilis* complex worldwide, based on phylogenetic analyses of plastid DNA? (2) What is the relationship of hexaploid *C. fragilis* to tetraploid *C. fragilis* and other related taxa? (3) Are rugose spores a reliable characteristic defining any group, e.g. *C. dickieana*?

MATERIAL AND METHODS

PLANTS AND SPORES USED FOR THE STUDY

We chose a representative subgroup of 96 *Cystopteris* samples, each of known ploidy, from the study of Hanušová *et al*. (2019). We selected 87 plants of *C. fragilis* from 44 populations covering a broad geographical scale (Supporting Information, Table S1; Fig. 1). Our sampling covered Europe (55 plants, 28

localities), the northern Arctic regions Svalbard and Greenland (four plants, three localities), the central part of North America (17 plants, six localities), and western and central Asia (11 plants, seven localities). Our selected samples cover the variation in ploidy as analysed by Hanušová *et al*. (2019): 50 (57.5%) were tetraploid, five (5.8%) were pentaploid and 32 (36.7%) were hexaploid. Additionally, *C. alpina* (seven plants, four localities) and *C. diaphana* (two plants, two localities) were analysed for comparative purposes. The analysed *C. alpina* samples were tetraploid (one plant), pentaploid (one plant), hexaploid (three plants) and octoploid (two plants); *C. diaphana* was hexaploid. Herbarium vouchers for the plants under study are deposited in CBFS.

Spore type was examined under a microscope (Olympus CX31) by observing the perispore in specimens with ripe and developed spores (86 of 96 specimens). Spores were determined as spiny (perispore with numerous spines all around the spore, Fig. 1A, B), rugose (without spines and with an irregularly smoothly undulated perispore, Fig. 1C, D) or aborted (perispore shrivelled and spore malformed). Ten specimens were collected outside of their phenological optimum, resulting in an absence of spores needed for assessment. Thus, their spore type was labelled as unresolved.

MOLECULAR PROTOCOLS

Total genomic DNA of all our samples was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Two plastid loci described by Rothfels *et al*. (2013) were selected for the analysis: *matK* region and *trnG-R* intergenic spacer, respectively. PCRs were performed according to the protocol of Rothfels *et al*. (2013), except that Plain PP Master Mix (Top-Bio, Prague, Czech Republic) was used for amplification. Successful amplifications were sent for Sanger sequencing (GATC Biotech, Constance, Germany). The amplification primers were used as sequencing primers.

MOLECULAR DATA ANALYSIS

Sequences were edited using BioEdit v.7.0.9.0 (Hall, 1999) and aligned manually following Rothfels *et al*. (2013). The raw alignments of the two plastid loci were trimmed according to the shortest sequence in the dataset and concatenated into the final matrix. Accessions were assigned to haplotypes using FaBox 1.41 (Villesen, 2007; available online at http://users-birc.au.dk/biopv/php/fabox/).

Phylogenetic relationships were assessed using Bayesian inference as implemented in MrBayes v.3.1.2. (Huelsenbeck, 2001). To incorporate our data into a

broader phylogenetic context, we included sequences of other *Cystopteris* and related taxa obtained by Rothfels *et al*. (2013). For taxa that formed well-supported monophyletic clades in that study, one representative accession was selected per clade. Ambiguously aligned positions in *trnG-R* were excluded. To enhance the phylogenetic resolution, aligned sequences of the *rbcL* plastid region were added to the matrix for the accessions provided by Rothfels *et al*. (2013). Gaps were coded as missing data. The best-fit model of sequence evolution was selected using the Akaike information criterion calculated in jModelTest v.0.1.1 (Posada, 2008). The HKY model (Hasegawa, Kishino & Yano, 1985) with a discrete gamma distribution and proportion of invariable sites was selected for *matK*, and the general time-reversible model (Rodríguez *et al*., 1990) with a discrete gamma distribution was selected for *rbcL* and *trnG-R*. Two runs, with 910 000 generations, starting with a random tree and employing four simultaneous chains each (one hot, three cold) were executed. The temperature of a hot chain was set to 0.01, and every 500th tree was saved. The analysis was considered complete when the average standard deviation of split frequencies dropped below 0.01. The first 455 trees (25%) were discarded as the burn-in phase, and the remaining 1365 trees were used to construct a majority-rule consensus tree. Based on previous a phylogenetic study (Rothfels *et al*., 2013), *Gymnocarpium robertianum* (Hoffm.) Newman was used as the outgroup. The aligned sequences used for Bayesian inference had a length of 3639 bp, of which 541 characters were variable and 244 potentially parsimony-informative.

TCS v.1.18 (Clement, Posada & Crandall, 2000) was used to produce a parsimony network of *matK–trnG-R* haplotypes with a 95% confidence limit. The analysis involved a representative selection of the *C. fragilis* complex *sensu* Rothfels *et al*. (2013), excluding *C. protrusa*, as this taxon forms a well-supported clade, sister to the rest of the complex. The alignment showed no ambiguously aligned positions. Gaps were treated as missing data, but potentially informative indels were scored (present/absent) using the simple coding method (Simmons & Ochoterena, 2000) implemented in SeqState v.1.4 (Müller, 2005) and the data were added to the matrix. The aligned sequences used for haplotype network analysis had a length of 2240 bp, of which 51 characters were variable and 48 potentially parsimony-informative.

RESULTS

MOLECULAR ANALYSES

In the plastid DNA analyses, all taxa of the *C. fragilis* complex formed a well-supported [posterior probability (PP = 1.00)] clade (Fig. 2), with *C. protrusa* sister to

the remainder of the complex. Apart from *C. alpina*, all the taxa of the complex with more than one accession were non-monophyletic. Beside the *C. protrusa* clade, the *C. fragilis* complex included two distinct and well-supported clades, with accessions of *C. fragilis* being more-or-less equally distributed among them. The first major clade, hereafter called the '*reevesiana* clade' (PP = 0.99), included all three accessions of *C. reevesiana*, a single *C. laurentiana* accession, one of the two *C. tenuis* accessions, and the clade of *C. diaphana* with nested accessions of *C. membranifolia* Mickel and *C. millefolia* Mickel & Tejero. These results indicate that the last two taxa could be merged into *C. diaphana*, but our limited sampling does not allow for a definite conclusion. The second major clade, hereafter called the '*hemifragilis* clade' (PP = 0.99), included one accession of *C. tenuis*, *C. sandwicensis* Brack. and *C. douglasii*. This clade also includes plants from the type locality of *C. dickieana*, which have a haplotype unique to Scotland in our sampling. The remaining accessions (*C. alpina*, *C. tasmanica* and the H6 haplotype of *C. fragilis* – rugose-spored types from the Himalayas) were poorly resolved among the two main clades.

The haplotype network for *C. fragilis* includes three dominant haplotypes. Haplotype H1 (*hemifragilis* clade) was found worldwide, from North America to Asia. Haplotypes R1 and R6 (*reevesiana* clade) were found only in Europe and Asia. In the entire *reevesiana* clade, no overlap in haplotypes was found between North America and Eurasia (Fig. 3A). In Europe, representatives of the *hemifragilis* clade were found in Scotland, the north (northern Norway and Svalbard) and in montane regions of the south (Macedonia, Spain and Turkey).

The tetraploid, pentaploid and hexaploid accessions did not form monophyletic groups; they were each distributed in both major clades of the *C. fragilis* complex. Of the 16 haplotypes found in *C. fragilis* accessions (82 plants) of known ploidy, six haplotypes were shared by both tetraploids and hexaploids, indicating at least six independent polyploidization events leading from tetraploids to hexaploids. Six haplotypes were found exclusively in tetraploids, and four exclusively in hexaploids. However, three of the four unique hexaploid haplotypes differed by a single point mutation from the closest haplotype found in tetraploids (Fig. 4), generally suggesting a close relationship between tetraploid and hexaploid haplotypes. The haplotypic diversity in populations with multiple samples indicates a local differentiation between tetraploids and hexaploids: one of five (20%) populations with just tetraploids contained multiple haplotypes, whereas in eight of 18 (44%) mixed-ploidy populations the hexaploids had a haplotype
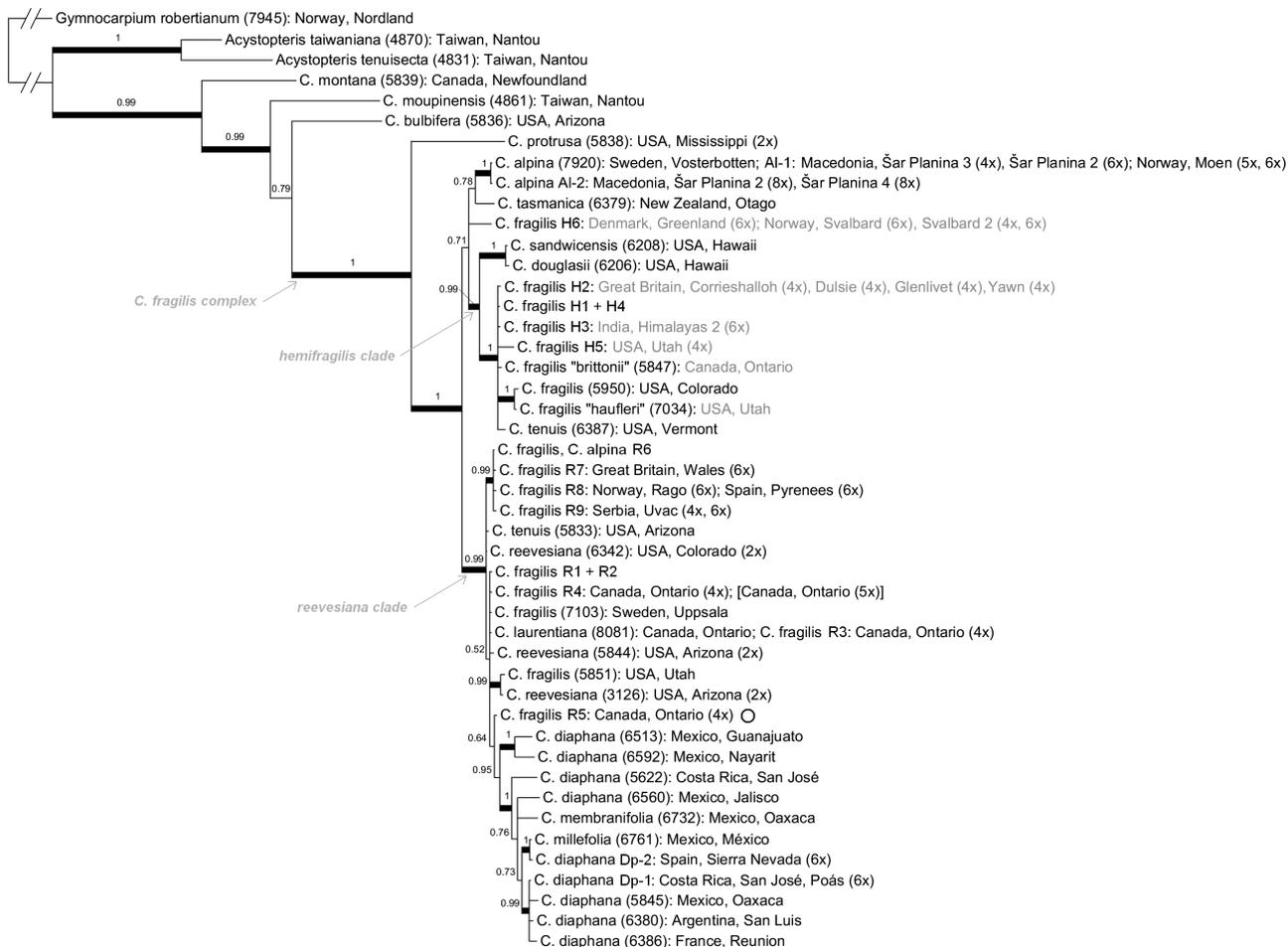
Figure 2. Phylogenetic tree of the *Cystopteris fragilis* complex and related taxa based on plastid DNA data. The tree was constructed using Bayesian inference and was rooted with *Gymnocarpium robertianum*. Numbers on branches indicate posterior probabilities. Bold lines indicate branches with posterior probabilities > 0.95. Accessions with rugose spores corresponding to *C. dickieana* are indicated by grey colour. Accessions with aborted spores are given in square brackets. Two pairs of distinct haplotypes (H1 and H4, R1 and R5, respectively) were collapsed due to exclusion of variable sites which occurred in ambiguously aligned positions in *trnG-R*. For detailed voucher information, see Supporting Information, Table S1. Lineage H1 included the following samples: Canada, British Columbia 2 (4*x*); Macedonia, Šar Planina (4*x*, 6*x*); Norway, Birtavarre (4*x*), Rago (4*x*); Spain, La Rioja (4*x*); Turkey, Kackar (4*x*); USA, Utah (4*x*, 6*x*), Washington (4*x*, 6*x*); Canada, Ontario (6*x*), Ontario 2 (6*x*); Norway, Birtavarre (4*x*); Spain, Sierra Nevada (4*x*); USA, Utah (6*x*); [Canada, Ontario (4*x*, 5*x*); France, Pyrenees (4*x*); Norway, Birtavarre (4*x*); Spain, La Rioja (4*x*)]; H4: Canada, British Columbia (4*x*). Lineage R1 included the following samples: Armenia (4*x*, 6*x*); Czech Republic, Brtnice (4*x*), Krkonoše (4*x*); France, Annot (4*x*), Pyrenees 2 (4*x*); Germany, Neudahn (4*x*); Poland, Karkonosze (4*x*); Serbia, Kladovo (4*x*); Spain, Pyrenees (4*x*), Sierra Nevada (4*x*); Armenia, Garni (4*x*); Kyrgyzstan, Kyzart (6*x*); Turkey, Kackar (4*x*); [Armenia, Garni (6*x*); France, Pyrenees (6*x*); Italy, Lillaz (4*x*, 5*x*, 6*x*)]; R2: Czech Republic, Krkonoše (6*x*); India, Himalayas (4*x*, 6*x*); [Czech Republic, Oheb (5*x*); Norway, Rondane (4*x*)]. Lineage R6 included the following samples: France, Annot (6*x*), Pyrenees 2 (6*x*); Georgia, Vardzia (6*x*); UK, Malham (4*x*, 6*x*), Smoo Cave (6*x*); Romania, Ungarn Mare (6*x*); Serbia, Tara (6*x*); [Czech Republic, Oheb (5*x*)]; C. alpina R6: Macedonia, Šar Planina 4 (6*x*).

different from any of the tetraploids present. The diversity found in pentaploids (five haplotypes) showed no unique haplotypes and overlapped with variation found in tetraploids and hexaploids. The limited sampling for *C. alpina* (six plants) revealed two closely related haplotypes differing by a single substitution (Al-1 in tetraploids and hexaploids, and Al-2 in octoploids, respectively). Furthermore, one hexaploid individual also possessed the haplotype of the *reevesiana* clade (R6), probably resulting from crossing with *C. fragilis*. This result confirms the correct identification of *C. alpina* by Hanušová *et al.* (2019), which revealed new cytotypes in this species (4*x*, 5*x*, 6*x* and 8*x*). Due to the haplotype and cytotype
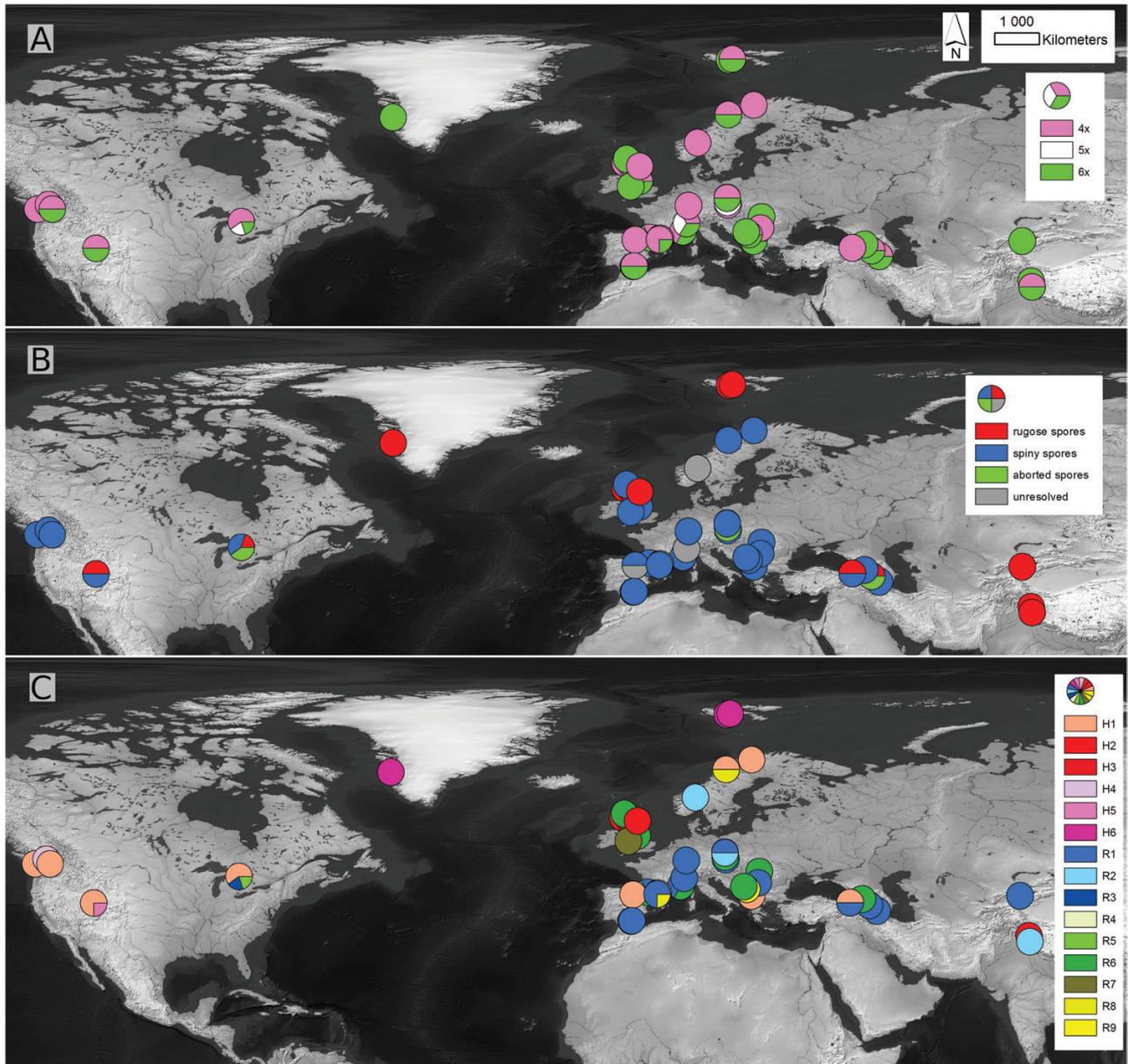
Figure 3. Spatial distribution of (A) cytotypes, (B) spore types and (C) haplotypes (based on the *matK* region and the *trnG-R* intergenic spacer of plastid DNA) in the 87 samples of *Cystopteris fragilis* used in this study. The labels H# and R# refer assignment to the *hemifragilis* and *reevesiana* clades, respectively.

diversity, the situation in *C. alpina* is probably complicated and deserving of further study.

SPORE TYPES

Of the examined plants, approximately half (54%) had spiny spores, 26% had rugose spores and 8% had aborted spores (12% of plants did not have mature spores). Haplotypes of abortive plants were always a subset of non-abortive plants in their population, except for population 33, in which only abortive

plants were sampled. In this case, both haplotypes were present in tetraploids and hexaploids elsewhere. Abortive samples were tetraploid (two samples), pentaploid (four samples) and hexaploid (one sample).

Neither the rugose-spored nor spiny-spored plants were monophyletic ([Fig. 2](#)). In the *hemifragilis* clade, 18 of 30 (60%) of the plants with mature non-abortive spores produced rugose spores, compared to five of 40 (12.5%) in the *reevesiana* clade. Moreover, *c.* 64% of rugose-spored samples shared haplotypes with spiny-spored plants. Three of five haplotypes that were well resolved in the

hemifragilis clade were found exclusively in plants with rugose spores. In the four central Asian samples, only rugose spores were observed; elsewhere, spiny spores dominated (Fig. 3B). In Europe, rugose spores were found only in samples from Scotland (including the *C. dickieana* type locality) and Svalbard. In the broader *C. fragilis* complex, the examined *C. alpina* and *C. diaphana* produced only spiny spores, except for one aborted *C. alpina* sample. No individuals with aborted spores had unique haplotypes.

Seven populations included three or more samples, all with variation in haplotype or spore type or both. Three of these populations included abortive plants. In population 1, all samples were tetraploid and possessed the H1 haplotype, but spiny, rugose and aborted spores were found. In the other two cases, abortive plants were matched with either spiny-spored (population 13, 5*x* aborted) or rugose-spored (population 28, 4*x* and 5*x* aborted) plants of the population, even though both developed spore types were present in each one.

DISCUSSION

RELATIONSHIPS WITHIN *CYSTOPTERIS FRAGILIS*

Our analysis of 87 *C. fragilis* samples, spanning the majority of Northern Hemisphere collections, revealed several patterns. Sister to the rest of the *C. fragilis* complex is *C. protrusa*, as observed by Rothfels *et al.* (2013, 2014, 2017). Also matching published results (Rothfels *et al.*, 2013, 2014), core *C. fragilis* is split into two main clades. First, the *reevesiana* clade contains most of the sampled *C. fragilis* and several other taxa, notably *C. diaphana* and *C. reevesiana*. The last is presumed to be one of the diploid progenitors of *C. fragilis* (Haufler & Windham, 1991). In contrast to North America, members of this clade seem to be predominantly found in Europe and Asia. In this clade, there was no overlap between North America and Eurasia in our study (Fig. 2). However, additional sampling is needed to further clarify the situation; only four haplotypes of this clade were from North America. Second, the *hemifragilis* clade is named after a presumed extinct or undiscovered diploid taxon, '*C. hemifragilis*', first suggested by Haufler (1985). This clade dominates in North America but is also found throughout Europe, including its most frequent haplotype. A representative of this clade was also found in Taiwan (Rothfels *et al.*, 2014), but was not included in our analyses due to a lack of data for one of the loci.

These results may provide insight into the origin of and relationships in the *C. fragilis* group. The observed dichotomous haplotypic pattern is thought to reflect the bidirectional origin of *C. fragilis*. The members of this species are presumed to be allopolyploids, as an apogamously formed diploid *C. fragilis* was sterile, unless having undergone somatic polyploidization

(Vida, 1974). The putative parents are related to *C. reevesiana* and '*C. hemifragilis*' (Haufler & Windham, 1991). Both parents have served as plastid DNA donors (maternal parents), leading to the observed pattern of plastid dichotomy. Nevertheless, this hypothesis may not fully explain the complexity of this group.

As one possible interpretation of our data, we propose an expansion of the above-mentioned hypothesis. First, there are at least two distinct (non-interfertile) species groups in *C. fragilis*, differing in their plastid origin. Second, both species groups share the genome of '*C. hemifragilis*', one having it as the maternal parent and vice versa. Third, the second genomic complement of the two species groups are either two lineages of *C. reevesiana* or *C. reevesiana* and an unnamed closely related taxon. This hypothesis is based on the presence of hybrids between haplotype groups and the relationship of various members of the *C. fragilis* complex in our and other phylogenetic analyses.

Hybridization between the two possible species groups of *C. fragilis* has been observed by several authors (Manton & Reichstein, 1965; Vida, 1974; Prada & Salvo, 1985), usually as a cross between spiny- and rugose-spored plants. Specifically, Vida (1974) crossed a rugose-spored individual from the type Scottish locality of *C. dickieana* with a spiny-spored *C. fragilis* from Poland, both tetraploid. Based on our haplotype analysis, we are confident that the Scottish plant possessed the H2 haplotype from the *hemifragilis* group. The Polish plant probably contained one of the common haplotypes from the *reevesiana* clade, due to its geographical location. The resultant cross was abortive, which has long been considered a sign of hybridization in ferns (Wagner & Chen, 1965; Reichstein, 1982; Hornych & Ekrt, 2017). In summary, non-interfertile lineages exist in *C. fragilis*, and they may possibly be indicated by plastid haplotype.

The hybrid raised during the experiments of Vida (1974) formed 54 bivalents and 60 univalents in meiosis ($2n = 4x = 168$ in total), indicating that the two parental tetraploids share a diploid progenitor in common. However, 12 extra bivalents were formed, so the two other parents are probably closely related (Vida, 1974). Molecular analyses of biparental markers are generally consistent with these results, although they were based on only a small number of samples (Rothfels *et al.*, 2014, 2017). In these studies, nearly all European and North American samples of *C. fragilis* derived their genomic composition from three clades, labelled B, E and F in Rothfels *et al.* (2014) and *C. fragilis*1, *C. fragilis*2 and *C. fragilis*3 in Rothfels *et al.* (2017). The 'F'/'*C. fragilis*3' nuclear lineage was found in all samples regardless of their plastid haplotype lineage (*hemifragilis* or *reevesiana*) and is possibly the shared genome type leading to the many bivalents of Vida's hybrid.

The shared genome of the two species groups is probably that of the yet undiscovered or extinct '*C. hemifragilis*'. This enigmatic taxon (possibly composed of multiple species) is not only considered to be the parent of *C. fragilis*, but also that of the allotetraploid *C. tenuis* (Haufler, 1985; Haufler *et al.*, 1985; Haufler & Windham, 1991; Rothfels *et al.*, 2014, 2017). One parental lineage of the *C. tenuis* analysed by Rothfels *et al.* (2014, 2017) was related to *C. protrusa*, and the other was a member of the 'F'/'*C. fragilis*3' clade. As mentioned above, the F/*C. fragilis*3 clade is the one shared by individuals of both haplotype groups. Furthermore, both genomes of the Icelandic sample in Rothfels *et al.* (2014) were found in the F/*C. fragilis*3 clade, and the plastid belonged to the *hemifragilis* lineage, indicating a connection between the F/*C. fragilis*3 clade and '*C. hemifragilis*'.

As discussed above, the second genomic complement of *C. fragilis* includes at least two related lineages. If the '*C. hemifragilis*' genome is indeed shared by both haplotype clades, *C. reevesiana* may be one of the other species involved in *C. fragilis*, as its plastid has been found in *reevesiana* clade members. The other taxon involved may be an extinct or undiscovered diploid species closely related to *C. reevesiana*. A hint may be found in the internal structure of the *reevesiana* clade. Two main haplotype subgroups, differing by approximately three substitutions, were observed (R1 and R6, Fig. 4), possibly representing the two *C. reevesiana* lineages. In summary, the two different *C. fragilis* species groups share one ancestor taxon, '*C. hemifragilis*', and one of the other ancestors is *C. reevesiana* or another closely related diploid.

Although the presented 'two-species-group hypothesis' explains our results, the story of *C. fragilis* worldwide is probably more complicated, as indicated by other genome types found by Rothfels *et al.* (2014, 2017). For example, the existence of some autopolyploid *C. fragilis* individuals could also explain the chromosome behaviour of the hybrid raised by Vida (1974) and would affect our interpretation. Additionally, some lineages in the *C. fragilis* complex may have developed reproductive isolation regardless of their plastid haplotype, as is probably the case for *C. diaphana*. A more thorough sampling, especially in Asia, would greatly improve our understanding of the complex. Our hypothesis should also be further tested by more widely encompassing analyses of nuclear DNA.

Hexaploid *Cystopteris fragilis*

The presence of hexaploids further complicates the patterns of reticulation in the *C. fragilis* complex. Two important observations may be inferred from the haplotypes of hexaploids. First, the haplotype of

hexaploids frequently differed from the haplotype of tetraploids with which they co-occurred (i.e. in the same population). Second, the overall diversity of haplotypes in hexaploids is mostly a subset of the diversity of the tetraploids. All three unique hexaploid haplotypes differed by a single substitution from major haplotypes found in both tetraploids and hexaploids. Thus, hexaploids originated from within the overall variation found in tetraploids but may subsequently have dispersed between populations. Observing chromosome behaviour during meiosis, Vida & Mohay (1980; samples from central and eastern Europe) and Kawakami *et al.* (2010; samples from Mongolia) led to the conclusion that hexaploid *C. fragilis* is probably of allopolyploid origin. *Cystopteris protrusa* was suggested as a possible diploid parent (Vida, 1974; Vida & Mohay, 1980), but that species is restricted to eastern North America, and no hexaploid samples possessed the plastid genome of *C. protrusa* in our study. Such an observation does not preclude the possibility that some hexaploid populations formed from a cross between diploid *C. protrusa* and a tetraploid member of the *C. fragilis* complex. The tetraploid being the predominant maternal parent (plastid donor) in triploid hybrids (which may turn into hexaploids) was previously observed in ferns (Xiang *et al.*, 2000; Testo, Watkins & Barrington, 2015; but see Hornych *et al.*, 2019 for opposite results). Therefore, *C. protrusa* may be the paternal parent of hexaploid *C. fragilis*. Considering our two-species-group concept explained above, hexaploids may also have arisen through hybridization between tetraploid *C. fragilis* of one haplotype lineage and a parental diploid of the other haplotype lineage. Congruent with our results, the genetic variation of hexaploids would be more or less included in that of tetraploids. A comprehensive survey of ploidy in *C. fragilis* did not find triploids (Hanušová *et al.*, 2019), indicating that the hexaploids would have emerged a long time ago or their triploid progenitors would be incredibly rare. Alternatively, the formation of hexaploids may involve the union of an unreduced and a reduced spore from one or more tetraploid *C. fragilis* lineages. Under this scenario, the haplotype diversity of the hexaploids would also fall within the diversity of tetraploids. Possibly, no single scenario may fully explain the origin of hexaploid *C. fragilis* due to its polyphyletic nature. We have found evidence for at least six polyploidization events that gave rise to hexaploids. Similar results have been found in other fern complexes in active evolution, with taxa originating from different genetic lineages through multiple hybridization events in different geographical areas showing no difference in frond morphology (Ranker, Floyd & Trapp, 1994; Hunt *et al.*, 2011; Chao *et al.*, 2012; Beck *et al.*, 2012; Williams, Farrar & Henson, 2016).
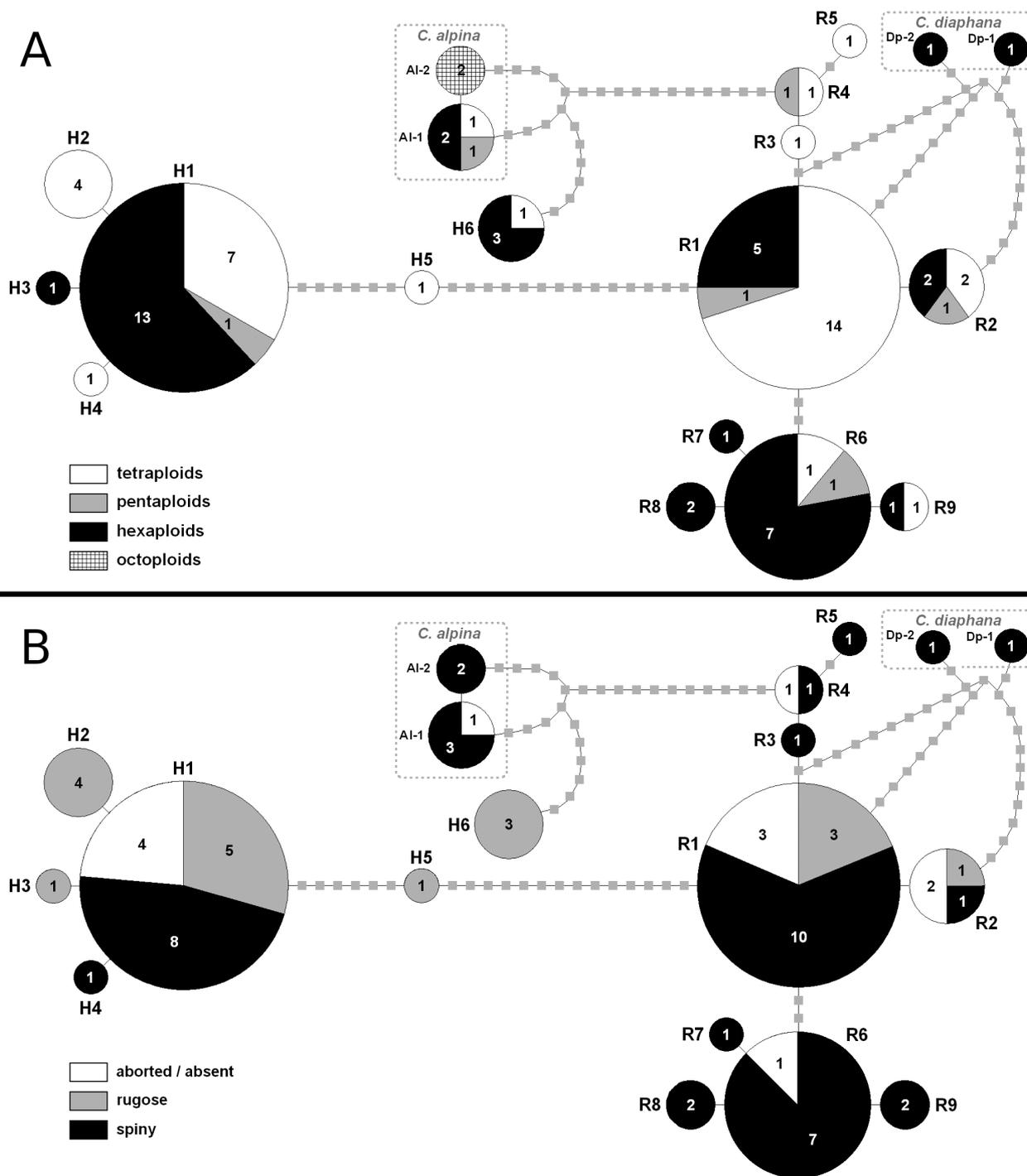
Figure 4. TCS haplotype network of plastid DNA haplotypes involving accessions of *C. fragilis* and related taxa with (A) known ploidy, (B) spore types. Lines represent parsimonious connections between haplotypes with probability > 95%; each grey square on a line represents one mutation step. Numbers within haplotype symbols represent the number of accessions of given ploidy/spore type.

Spore characteristics in light of haplotypic variation

Our study confirms the presence of rugose-spored plants across the Northern Hemisphere, although spiny-spore populations dominated (92%) in the thorough sampling of Hanušová *et al*. (2019; see Supporting Information, Table S1). Rugose-spored samples were polyphyletic and found in both haplotype lineages/species groups. Nevertheless, the frequency of rugose-spored plants was about five times higher among our sampled *hemifragilis* clade accessions than in the *reevesiana* clade, although uneven sampling (especially in Scotland) may have skewed the results. Therefore, the presence of rugose spores may not be used to accurately distinguish the *hemifragilis* and *reevesiana* clades but seems to be more prevalent in the former.

In contrast, a clearer pattern emerged among plants with aborted spores. Abortive plants always shared a haplotype with at least one non-aborted individual from the same population, if present (for one population, all sampled individuals had aborted spores), suggesting that they are hybrids formed *in situ*. These results confirm the generally assumed sterility of fern hybrids severely limiting or preventing dispersal and subsequent growth (Sigel, 2016; Hornych & Ekrt, 2017; Shepherd *et al*., 2019).

Status of *Cystopteris dickieana*

Over the years, much has been written about *C. dickieana*, seemingly without satisfactory conclusion (reviewed by Dyer, Parks & Lindsay, 2000). Several authors still consider it a separate species (Wang, 1983; Breckle, 1987; Jermy, 1993; Frey *et al*., 2006; Wang *et al*., 2013), but others see it as a variant of *C. fragilis* at best (Blasdell, 1963; Haufler & Windham, 1991; Haufler *et al*., 1993; Parks *et al*., 2000). The most discussed defining character of the presumed *C. dickieana* is the presence of rugose spores. Our sampling has corroborated earlier studies that demonstrate that rugose-spored plants also occur outside Scotland, as mentioned above. Thus, spore morphology may not serve as the defining characteristic, not even for one of the haplotypic lineages/species groups. However, all Scottish plants of the *hemifragilis* clade, including that of the *C. dickieana* type locality near Aberdeen, share a single and unique haplotype (H2), differing by a single mutation from the most common *hemifragilis* haplotype. Similarly, Parks *et al*. (2000) found a unique isozyme pattern in the *C. dickieana* type population and nearby. Our study further elucidates the taxonomic and evolutionary complexity in the *C. fragilis* complex and demonstrates that *C. dickieana*, as typically used (for all rugose-spored plants), is not a monophyletic unit. However, further research may yet show that the Scottish plants belong to an unrecognized tetraploid lineage, in which case the name *C. dickieana* would apply.

ACKNOWLEDGEMENTS

REFERENCES

Beck JB, **Allison JR**, **Pryer KM**, **Windham MD. 2012.** Identifying multiple origins of polyploid taxa: a multilocus study of the hybrid cloak fern (*Astrolepis integerrima*; Pteridaceae). *American Journal of Botany* **99:** 1857–1865.

Berg RY. 1992. Sporevariasjon i skandinaviske populasjoner av *Cystopteris fragilis*. (Spore variation in Scandinavian populations of *Cystopteris fragilis*.). *Blyttia* **50:** 143–157.

Blasdell RF. 1963. A monographic study of the genus *Cystopteris*. *Memoirs of the Torrey Botanical Club* **21:** 1–102.

Breckle SW. 1987. Distribution and ecology of *Cystopteris* (Athyriaceae) species within the *Flora Iranica* region. *Plant Systematics and Evolution* **155:** 59–65.

Chao YS, **Dong SY**, **Chiang YC**, **Liu HY**, **Chiou WL. 2012.** Extreme multiple reticulate origins of the *Pteris cadieri* complex (Pteridaceae). *International Journal of Molecular Sciences* **13:** 4523–4544.

Chen CC, **Hyvönen J**, **Schneider H. 2020.** Exploring phylogeny of the microsoroid ferns (Polypodiaceae) based on six plastid DNA markers. *Molecular Phylogenetics and Evolution* **143:** 106665.

Clement M, **Posada D**, **Crandall KA. 2000.** TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9:** 1657–1659.

Dyer AF, **Parks JC**, **Lindsay S. 2000.** Historical review of the uncertain taxonomic status of *Cystopteris dickieana* R. Sim (Dickie's bladder fern). *Edinburgh Journal of Botany* **57:** 71–81.

Frey W, **Frahm JP**, **Fischer E**, **Lobin W. 2006.** *The liverworts, mosses and ferns of Europe* (English edition revised and edited by T. L. Blockeel). Colchester: Harley Books.

Hagenah DJ. 1961. Spore studies in the genus *Cystopteris*. I. The distribution of *Cystopteris* with non-spiny spores in North America. *Rhodora* **63:** 181–193.

Hall T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucleic Acids Symposium Series* **41:** 95–98.

Hanušová K, **Čertner M**, **Urfus T**, **Koutecký P**, **Košnar J**, **Rothfels CJ**, **Jarolímová V**, **Ptáček J**, **Ekrt L. 2019.** Widespread co-occurrence of multiple ploidy levels in fragile

ferns (*Cystopteris fragilis* complex; Cystopteridaceae) probably stems from similar ecology of cytotypes, their efficient dispersal and inter-ploidy hybridization. *Annals of Botany* **123:** 845–855.

Hasegawa M, **Kishino H**, **Yano T. 1985.** Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22:** 160–174.

Haufler CH. 1985. Pteridophyte evolutionary biology: the electrophoretic approach. *Proceedings of the Royal Society of Edinburgh. Section B. Biological Sciences* **86:** 315–323.

Haufler CH, **Windham MD**, **Britton DM**, **Robinson SJ. 1985.** Triploidy and its evolutionary significance in *Cystopteris protrusa*. *Canadian Journal of Botany* **63:** 1855–1863.

Haufler CH, **Moran RC**, **Windham MD. 1993.** *Cystopteris*. In: Haufler CH, Moran RC, Windham MD, eds. *Flora of North America, Vol. 2*. New York: Oxford University Press, 263–270.

Haufler CH, **Windham MD. 1991.** New species of North American *Cystopteris* and *Polypodium*, with comments on their reticulate relationships. *American Fern Journal* **81:** 7–23.

Hörandl E. 1989. *Cystopteris dickieana* R. Sim (Pteridophyta)—neu für Österreich und andere Gebiete. *Verhandlungen der Zoologisch-Botanischen Gesellschaft in Wien* **126:** 179–183.

Hornych O, **Ekrt L**, **Riedel F**, **Koutecký P**, **Košnar J. 2019.** Asymmetric hybridization in Central European populations of the *Dryopteris carthusiana* group. *American Journal of Botany* **106:** 1477–1486.

Hornych O, **Ekrt L. 2017.** Spore abortion index (SAI) as a promising tool of evaluation of spore fitness in ferns: an insight into sexual and apomictic species. *Plant Systematics and Evolution* **303:** 497–507.

Huelsenbeck JP. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* **294:** 2310–2314.

Hunt HV, **Ansell SW**, **Russell SJ**, **Schneider H**, **Vogel JC. 2011.** Dynamics of polyploid formation and establishment in the allotetraploid rock fern *Asplenium majoricum*. *Annals of Botany* **108:** 143–157.

Jermy AC. 1993. *Cystopteris* Bernh. In: Tutin TG, Burges NA, Chater AO, Edmondson JR, Heywood VH, Moore DM, Valentine DH, Walters SM, Webb DA, eds. *Flora Europaea, Vol.1, 2nd edn*. Cambridge: Cambridge University Press, 24–25.

Kao T, **Rothfels CJ**, **Melgoza-Castillo A**, **Pryer KM**, **Windham MD. 2020.** Infraspecific diversification of the star cloak fern (*Notholaena standleyi*) in the deserts of the United States and Mexico. *American Journal of Botany* **107:** 658–675.

Kawakami SM, **Kawakami S**, **Kato J**, **Kondo K**, **Smirnov SV**, **Damdinsuren O. 2010.** Cytological study of a fern *Cystopteris fragilis* in Mongolian Altai. *Chromosome Botany* **5:** 1–3.

Kuo L, **Chang Y**, **Huang Y**, **Testo W**, **Ebihara A**, **Rouhan G**, **Quintanilla LG**, **Watkins JE**, **Huang Y**, **Li F. 2020.** A global phylogeny of *Stegnogramma* ferns (Thelypteridaceae): generic and sectional revision, historical biogeography and evolution of leaf architecture. *Cladistics* **36:** 164–183.

Manton I. 1950. *Problems of cytology and evolution in the Pteridophyta*. Cambridge: Cambridge University Press.

Manton I, **Reichstein T. 1965.** Die Chromosomenzahlen von *Cystopteris sudetica* A. Br. et Milde von Berchtesgaden (Bayern) sowie von *Cystopteris dickieana* Sim. *s. l.* vom Foostock (Kanton Glarus, Schweiz). *Bauhinia* **2:** 307–312.

Moran RC. 1983. *Cystopteris tenuis* (Michx.) Desv.: a poorly understood species. *Castanea* **48:** 218–223.

Müller K. 2005. SeqState: primer design and sequence statistics for phylogenetic DNA datasets. *Applied Bioinformatics* **4:** 65–69.

Øllgaard B, **Tind K. 1993.** *Scandinavian ferns: a natural history of the ferns, clubmosses, quillworts, and horsetails of Denmark, Norway, and Sweden*. Copenhagen: Rhodos.

Paler MH, **Barrington DS. 1995.** The hybrid *Cystopteris fragilis × C. tenuis* (Dryopteridaceae) and the relationship between its tetraploid progenitors. *Systematic Botany* **20:** 528–545.

Parks JC, **Dyer AF**, **Lindsay S. 2000.** Allozyme, spore and frond variation in some Scottish populations of the ferns *Cystopteris dickieana* and *Cystopteris fragilis*. *Edinburgh Journal of Botany* **57:** 83–105.

Posada D. 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25:** 1253–1256.

PPG I. **2016.** A community-derived classification for extant lycophytes and ferns: PPG I. *Journal of Systematics and Evolution* **54:** 563–603.

Prada C, **Salvo ÁE. 1985.** Notulae taxonomicae, chorologicae, nomenclaturales, bibliographicae aut philologicae in opus *Flora Iberica* intendentes. Notas sobre *Cystopteris* Bernh. *Anales del Jardín Botánico de Madrid* **1985:** 466.

Ranker TA, **Floyd SK**, **Trapp PG. 1994.** Multiple colonizations of *Asplenium adiantum-nigrum* onto the Hawaiian archipelago. *Evolution* **48:** 1364–1370.

Reichstein T. 1982. Hybrids in European Aspleniaceae (Pteridophyta). *Botanica Helvetica* **91:** 89–139.

Rodríguez F, **Oliver JL**, **Marín A**, **Medina JR. 1990.** The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology* **142:** 485–501.

Rothfels CJ, **Johnson AK**, **Windham MD**, **Pryer KM. 2014.** Low-copy nuclear data confirm rampant allopolyploidy in the Cystopteridaceae (Polypodiales). *Taxon* **63:** 1026–1036.

Rothfels CJ, **Pryer KM**, **Li FW. 2017.** Next-generation polyploid phylogenetics: rapid resolution of hybrid polyploid complexes using PacBio single-molecule sequencing. *New Phytologist* **213:** 413–429.

Rothfels CJ, **Windham MD**, **Pryer KM. 2013.** A plastid phylogeny of the cosmopolitan fern family Cystopteridaceae (Polypodiopsida). *Systematic Botany* **38:** 295–306.

Shepherd LD, **Brownsey PJ**, **Stowe C**, **Newell C**, **Perrie LR. 2019.** Genetic and morphological identification of a recurrent *Dicksonia* tree fern hybrid in New Zealand. *PLoS One* **14:** e0216903.

Sigel EM. 2016. Genetic and genomic aspects of hybridization in ferns: genomic aspects of hybrid ferns. *Journal of Systematics and Evolution* **54:** 638–655.

Sim R. 1848. *Cystopteris dickieana*. *The Gardeners & Farmers* **2:** 308.

Simmons MP, **Ochoterena H. 2000.** Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* **49:** 369–381.

Testo WL, **Field AR**, **Sessa EB**, **Sundue M. 2019.** Phylogenetic and morphological analyses support the resurrection of *Dendroconche* and the recognition of two new genera in Polypodiaceae subfamily Microsoroideae. *Systematic Botany* **44:** 737–752.

Testo WL, **Watkins JE**, **Barrington DS. 2015.** Dynamics of asymmetrical hybridization in North American wood ferns: reconciling patterns of inheritance with gametophyte reproductive biology. *New Phytologist* **206:** 785–795.

Vida G. 1974. Genome analysis of the European *Cystopteris fragilis* complex: 1. Tetraploid taxa. *Zeitschrift für Pilzkunde* **20:** 181–192.

Vida G, **Mohay J. 1980.** Cytophotometric DNA studies in polyploid series of the fern genus *Cystopteris* Bernh. *Acta Botanica Academiae Scientiarum Hungaricae* **26:** 455–461.

Villesen P. 2007. FaBox: an online toolbox for fasta sequences. *Molecular Ecology Notes* **7:** 965–968.

Wagner WH, **Chen KL. 1965.** Abortion of spores and sporangia as a tool in the detection of *Dryopteris* hybrids. *American Fern Journal* **55:** 9–29.

Wang ZR. 1983. *Cystopteris dickieana* Sim in China. *Acta Phytotaxonomica Sinica* **21:** 102–104.

Wang ZR, **Haufler CH**, **Pryer KM**, **Kato M. 2013.** *Cystopteris*. In: Wu ZY, Raven PH, Hong DY, eds. *Flora of China, Vol. 2-3*. Beijing: Science Press; St. Louis: Missouri Botanical Garden Press, 261–266.

Williams EW, **Farrar DR**, **Henson D. 2016.** Cryptic speciation in allotetraploids: lessons from the *Botrychium matricariifolium* complex. *American Journal of Botany* **103:** 740–753.

Xiang L, **Werth CR**, **Emery SN**, **McCauley DE. 2000.** Population-specific gender-biased hybridization between *Dryopteris intermedia* and *D. carthusiana*: evidence from chloroplast DNA. *American Journal of Botany* **87:** 1175–1180.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Samples of *Cystopteris* used in this study with their haplotypic, cytotypic and spore type presented. Links to vouchers may be found in Hanušová *et al*. (2019) (doi: 10.1093/aob/mcy219).

Appendix 2
Professional Curriculum vitae

Professional Curriculum vitae

Personal data

RNDr. Libor Ekrt, Ph.D.

Date and place of birth: 3. 4. 1978, Náchod, Czech Republic
Address: nám. Bratří Čapků 264, Telč, 58856
E-mail: libor.ekrt@gmail.com, ekrt@prf.jcu.cz
Phone (university): +420 387 772 303
www profile: https://botanika.prf.jcu.cz/systematics/en/libor-ekrt-2/
Research Gate: https://www.researchgate.net/profile/Libor-Ekrt

Education and Employment

Course of education and acquisition of scientific degrees:

1997–2000 bachelor study; Faculty of Biological Sciences, University of South Bohemia in České Budějovice, BSc. thesis: Komplex *Dryopteris carthusiana* agg. na Šumavě a Předšumaví (supervisor: Ing. Milan Štech Ph.D., defended 29.6. 2000)

2000–2003 master study; Faculty of Biological Sciences, University of South Bohemia in České Budějovice, MSc. Thesis: Revize polyploidního komplexu *Asplenium trichomanes* agg. v ČR (supervisor: Ing. Milan Štech, Ph.D., defended 3.6. 2003)

2003–2009 PhD study; Faculty of Sciences, University of South Bohemia in České Budějovice, PhD thesis: Diversity, variability and distribution of polyploid groups of ferns in Central Europe (supervisor: Ing. Milan Štech Ph.D., defended 11.12. 2009)

Employment

2003–2004   Správa CHKO Broumovsko, botanist, Police nad Metují
2005–2009   Šumava National Park, botanist, Vimperk
2009–now   Faculty of Sciences, University of South Bohemia in ČB, assistant professor

Pedagogical activity

Teaching at the Faculty of Sciences, University of South Bohemia in České Budějovice

KBO/132 Botanika vyšších rostlin M (lecture 3 hours/week). – LS 2011, LS 2012, LS 2013, LS 2014, LS 2015, LS 2016, LS 2017, LS 2018, LS 2019, LS 2020, LS 2021, LS 2022, LS 2023

KBO/296 Biologie kapraďorostů (lecture 2 hours/week + practices 2 hours/week). – ZS 2010, ZS 2014, ZS 2016, ZS 2019, ZS 2021

KBO/430 Letní kurz vegetačního mapování (practices, 2 hours/week). – LS 2011, LS 2013, LS 2015, LS 2017, LS 2021

KBO/122 Management a monitoring v ochraně přírody (practices, 2 hours/week). – LS 2014, LS 2018, LS 2022

KBO/138 Botanika vyšších rostlin V-I (practices, 2 hours/week). – ZS 2009, ZS 2010, ZS 2011, ZS 2012, ZS 2013, ZS 2014, ZS 2015, ZS 2017, ZS 2018, ZS 2019, ZS 2020, ZS 2021, ZS 2022

KBO/139 Botanika vyšších rostlin V-II (practices, 2 hours/week). – LS 2010, LS 2011, LS 2012, LS 2013, LS 2014, LS 2015, LS 2016, LS 2018, LS 2019, LS 2020, LS 2021, LS 2022

KBO/191 Mezioborová exkurze k ekologii biomu (practices, 2 hours/week). – LS 2010, LS 2011, LS 2012, LS 2014, LS 2017, LS 2019

KBO/140 Seminář systematické botaniky 1 (practices together with 2 others, 2 hours/week). – ZS 2017, ZS 2018, ZS 2019, ZS 2020, ZS 2021, ZS 2022

KBO/141 Seminář systematické botaniky 2 (practices together with 2 others, 2 hours/week). – LS 2017, LS 2018, LS 2019, LS 2020, LS 2021, LS 2022, LS 2023

KBO/308 Květena a vegetace střední Evropy (2 lectures, 3 hours/week). – ZS 2016, ZS 2018, ZS 2020, ZS 2022

KBE/354 Biotopy ČR (1 lecture, 2 hours/week). – LS 2013, LS 2015, LS 2019

Supervising of graduates and students of undergraduate and doctoral studies

Bachelor thesis

J. Hurtová – Populačně-ekologická studie invazního druhu *Lupinus* polyphyllus v oblasti Šumavy, defended 2007

B. Jonášová – Faktory ovlivňující fertilitu hasivky orličí (*Pteridium aquilinum*), defended 2015

A. Férová – Hodnocení výtrusů u kříženců sexuálních a apomiktických druhů rodu *Dryopteris*, defended 2018

J. Krejčí – Cytotypová variabilita *Huperzia selago* ve střední Evropě, defended 2018

O. Šimeček – Stabilita velikosti genomu vysušených tkání u vybraných rodů kapraďorostů, ongoing.

Master thesis

O. Hornych – Analysis of spore abortion in ferns, defended 2016

J. Podroužek – Četnost výskytu fertilních a sterilních populací hasivky orličí (*Pteridium aquilinum*) s porovnáním jednotlivých cytotypů, defended 2017

J. Krejčí – Rozšíření a diverzita *Huperzia selago* agg. V Evropě, defended 2020

A. Férová – Reprodukční mechanismy u apomiktických a sexuálních druhů kapradin, defended 2021

L. Šternerová – Diverzifikace a polyploidní speciace plavuní (*Lycopodium*) v Evropě, ongoing.

PhD thesis

O. Hornych – Reproduction and hybridization in ferns, defended 2021

K. Vejvodová – Microspeciation processes in spore-bearing vascular plants, ongoing.

Scientific research activities

Scientific publications

Papers in international journals with impact factor

Ekrt L., Košnar J., Rothfels C., Hanušová K., Hornych O. & Urfus T. (2022): Cytogenetic, geographical, spore type and plastid haplotype data reveal cryptic patterns of species diversity in the cosmopolitan *Cystopteris fragilis* complex (Polypodiopsida: Cystopteridaceae). – Botanical Journal of Linnean Society 199: 728–739.

Hornych O., Férová A., Hori K., Košnar J. & **Ekrt L.** (2022): Apomictic fern fathers: An experimental approach to the reproductive characteristics of sexual, apomict and hybrid fern gametophytes. – American Journal of Botany 109(4):628–644.

Hornych O., Černochová L., Lisner A., **Ekrt L.** (2022): An experimental assessment of competitive interactions between sexual and apomictic fern gametophytes using Easy Leaf Area. – Applications in Plant Sciences 10: e11466.

Hornych O., Černochová L., Košnar J. & **Ekrt L.** (2022): Biotic interactions between the gametophytes of wall rue (*Asplenium ruta-muraria*) and other fern species. – International Journal of Plant Sciences 183(1): 10–17.

Langhansová L., Pumprová K., Haisel D., **Ekrt L.**, Pavicic A., Zajíčková M., Vaněk T. & Dvořáková M. (2021): European ferns as rich sources of antioxidants in the human diet. – Food Chemistry 356: 129637.

Dvořáková M., Pumprová K., Antonínová Ž., Rezek J., Haisel D., **Ekrt L.**, Vaněk T., Langhansová L. (2021): Nutritional and antioxidant potential of fiddleheads from European ferns. – Foods 10: 460.

Ekrt L., Podroužek J., Hornych O., Košnar J. & Koutecký P. (2021): Cytotypes of bracken (*Pteridium aquilinum*) in Europe: widespread diploids and scattered triploids of likely multiple origin. – Flora 274: 151725.

Hornych O., Testo W., Sessa E., Watkins J., Campany C., Pittermann J. & **Ekrt L.** (2021): Insights into the evolutionary history and widespread occurrence of antheridiogen systems in ferns. – New Phytologist 229: 607–619.

Kaplan Z., Danihelka J., **Ekrt L.**, Štech M., Řepka R., Chrtek J., Grulich V., Rotreklová O., Dřevojan P., Šumberová K. & Wild J. (2020): Distributions of vascular plants in the Czech Republic. Part 9. – Preslia 92: 255–340.

Hornych O., **Ekrt L.**, Riedel F., Koutecký P. & Košnar J. (2019): Asymmetric hybridization in the Central European populations of the *Dryopteris carthusiana* group. – American Journal of Botany 106(11): 1477–1486.

Liu H-M., **Ekrt L.**, Koutecký P., Pellicer J., Hidalgo O., Marquardt J., Pustahija F., Ebihara A., Siljak-Yakovlev S., Gibby M., Leitch I. & Schneider H. (2019): Polyploidy does not control all: lineage-specific average chromosome length constraints genome size evolution in ferns. – Journal of Systematics and Evolution 57: 418–430.

Kaplan Z., Danihelka J., Chrtek J. Jr., Zázvorka J., Koutecký P., **Ekrt L.**, Řepka R., Štěpánková J., Jelínek B., Grulich V., Prančl J. & Wild J. (2019): Distributions of vascular plants in the Czech Republic. Part 8. – Preslia 91: 257–368.

Hanušová K., Čertner M., Urfus T., Koutecký P., Košnar J., Rothfels C. J, Jarolímová V., Ptáček J., **Ekrt L.** (2019): Widespread co-occurrence of multiple ploidy levels in fragile ferns (*Cystopteris fragilis* complex; Cystopteridaceae) likely stems from similar ecology of cytotypes, their efficient dispersal and inter-ploidy hybridization. – Annals of Botany 123: 845–855.

Wild J., Kaplan Z., Danihelka J., Petřík P., Chytrý M., Novotný P., Rohn M., Šulc V., Brůna J., Chobot K., **Ekrt L.**, Holubová D., Knollová I., Kocián P., Štech M., Štěpánek J. & Zouhar V. (2019): Plant distribution data for the Czech Republic integrated in the Pladias database. – Preslia 91: 1–24.

Kaplan Z., Danihelka J., Chrtek J., Prančl J., Ducháček M., **Ekrt L.**, Kirschner J., Brabec J., Zázvorka J., Trávníček B., Dřevojan P., Šumberová K., Kocián P., Wild J. & Petřík P. (2018): Distributions of vascular plants in the Czech Republic. Part 7. – Preslia 90: 425–531.

Langhanzová L., Pumprová K., Haisel D., **Ekrt L.**, Vaněk T. (2018): Antioxidant capacity of selected European fern species. – In Vitro Cellular & Developmental Biology-Plants 54: S29 (Meeting abstract).

Kaplan Z., Koutecký P., Danihelka J., Šumberová K., Ducháček M., Štěpánková J., **Ekrt L.**, Grulich V., Řepka R., Kubát K., Mráz P., Wild J. & Brůna J. (2018): Distributions of vascular plants in the Czech Republic. Part 6. – Preslia 90: 235–346.

Kaplan Z., Danihelka J., Šumberová K., Chrtek J., Rotreklová O., **Ekrt L.**, Štěpánková J., Taraška V., Trávníček B., Prančl J., Ducháček M., Hroneš M., Kobrlová L., Horák D. & Wild J. (2017): Distributions of vascular plants in the Czech Republic. Part 5. – Preslia 89: 333–439.

Kaplan Z., Danihelka, J., Koutecký P., Šumberová K., **Ekrt, L.**, Grulich V., Řepka R., Hroudová Z., Štěpánková J., Dvořák V., Dančák M., Dřevojan P. & Wild J. (2017): Distributions of vascular plants in the Czech Republic. Part 4. – Preslia 89: 115–201.

Hornych O. & **Ekrt L.** (2017): Spore abortion index (SAI) as a perspective tool of evaluation of spore fitness in ferns: An insight into sexual and apomictic species. – Plant Systematics and Evolution 303(4): 497–507.

Kaplan Z., Danihelka J., Lepší M., Lepší P., **Ekrt L.**, Chrtek J. Jr., Kocián J., Prančl J., Kobrlová L., Hroneš M. & Šulc V. (2016): Distributions of vascular plants in the Czech Republic. Part 3. – Preslia 88: 459–544.

Kaplan Z., Danihelka J., Štěpánková J., **Ekrt L.**, Chrtek J. Jr., Zázvorka J., Grulich V., Řepka R., Prančl J., Ducháček M., Kúr P., Šumberová K. & Brůna J. (2016): Distributions of vascular plants in the Czech Republic. Part 2. – Preslia 88: 229–322.

Ekrt L. & Koutecký P. (2016): Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the Dryopteris affinis agg. (Dryopteridaceae). – Annals of Botany 117: 97–106.

Hanušová K., **Ekrt L.**, Vít P., Kolář F., Urfus T. (2014): Continuous morphological variation correlated with genome size indicates frequent introgressive hybridization among *Diphasiastrum* species (Lycopodiaceae) in Central Europe. PLOS ONE 9(6): e99552.

Ekrt L. & Hrivnák R. (2010): *Asplenium platyneuron*, a new pteridophyte for Europe. – Preslia 82: 357–364.

Ekrt L., Holubová R., Trávníček P. & Suda J. (2010): Species boundaries and frequency of hybridization in the *Dryopteris carthusiana* (Dryopteridaceae) complex: A taxonomic puzzle resolved using genome size data. – American Journal of Botany 97: 1208–1219.

Ekrt L., Trávníček P., Jarolímová V., Vít P. & Urfus T. (2009): Genome size and morphology of the *Dryopteris affinis* group in Central Europe. – Preslia 81: 261–280.

Ekrt L. & Štech M. (2008): A morphometric study and revision of the *Asplenium trichomanes* group in the Czech Republic. – Preslia 80(3): 325–347.

Ekrt L., Lepší M., Boublík K. & Lepší P. (2007): *Dryopteris remota* rediscovered for the flora of the Czech Republic. – Preslia 79: 69–82.

Other scientific papers in journals without impact factor

Ekrt L. (2016): Znovuobjevená kapradina bodlinkatá (*Polystichum setiferum*) pro květenu České republiky. – Zpr. Čes. Bot. Společ. 51: 211–220.

Ekrt L. (2015): Revize populace podmrvky hadcové (*Notholaena marantae*) na Mohelenské hadcové stepi: rozšíření, početnost, stanovištní preference. – Acta Rerum Naturalium 18: 1–8.

Ekrtová E., **Ekrt L.** & Roleček J. (2014): Květena a vegetace Přírodní památky Dubová stráň u Dačic. – Acta Rerum Naturalium 17: 47–64.

Ekrt L., Linhartová R. & Štech M. (2013): Rozlišování a revize rozšíření kapradě podobné (*Dryopteris expansa*) a jejího křížence D. ×ambroseae v České republice. – Zpr. Čes. Bot. Společ. 48: 263–291.

Ekrt L. & Ekrtová E. (2013): Floristický a vegetační průzkum unikátní šumavské lokality Zhůří-střelnice u Nové Hůrky. – Silva Gabreta 19(1): 25–50.

Ekrt L., Štech M., Lepší M. & Boublík K. (2010): Rozšíření a taxonomická problematika skupiny *Dryopteris affinis* v České republice. – Zpr. Čes. Bot. Společ. 45: 25–52.

Ekrt L., Ekrtová E. & Čech L. (2010): Botanický průzkum lokality Malý Pařezitý v Jihlavských vrších. – Acta Rer. Natur. 8: 17–30.

Ekrt L. & Půbal D. (2009): Novinky v květeně cévnatých rostlin české Šumavy a přiléhajícího Předšumaví. II. – Silva Gabreta 15(3): 173–196.

Vydrová A., Grulich V, **Ekrt L.** & Ekrtová E. (2009): Řídká blana u Zahájí na Českobudějovicku – významná lokalita vodní a mokřadní flóry a vegetace. – Muzeum a současnost, Roztoky, ser. natur., 24: 27–54.

Ekrt L. & Půbal D. (2008): Novinky v květeně cévnatých rostlin české Šumavy a přiléhajícího Předšumaví. I. – Silva Gabreta 14(1): 19–38.

Ekrt L. (2008): Rozšíření a problematika taxonů skupiny *Asplenium trichomanes* v České republice. – Zpr. Čes. Bot. Společ. 43(1): 17–65.

Ekrtová E. & **Ekrt L.** (2008): Floristický a vegetační průzkum horní části údolí potoka Strouha u Telče. – Acta Rer. Natur. 4: 17–30.

Ekrt L. & Ekrtová E. (2008): Květena a vegetace Přírodní památky Toužínské stráně u Dačic. – Acta Rer. Natur. 5: 207–228.

Ekrtová E., **Ekrt L.**, Košnar J., Zapomělová E. & Čejková A. (2008): Míčovka kulkonosná (*Pilularia globulifera*) znovu objevena v České republice. – Zpr. Čes. Bot. Společ. 43(2): 193–208.

Ekrt L. (2008): Revize rozšíření sleziníku střídavolistého (*Asplenium ×alternifolium*) v České republice. – Zpr. Čes. Bot. Společ. 43(2): 231–250.

Hofhanzlová E. & **Ekrt L.** (2007): Floristický a vegetační inventarizační průzkum Národní přírodní rezervace Velký Špičák. – Acta Rer. Natur. 3: 11–20.

Strnad L. & **Ekrt L.** (2007): Nález nové lokality kyvoru lékařského (*Asplenium ceterach*; *Aspleniaceae*, *Pteridophyta*) a přehled jeho rozšíření na území České republiky. – Zpr. Čes. Bot. Společ. 42(2): 221–229.

Hofhanzlová E. & **Ekrt L.** (2006): Floristický a vegetační inventarizační průzkum Národní přírodní rezervace Zhejral. – Acta Rer. Natur. 2: 19–37.

Hofhanzlová E., **Ekrt L.** & Štechová T. (2005): Floristický a vegetační průzkum rašeliniště Na Klátově. – Acta Rer. Natur. 1: 45–52.

Chán V., Růžička V., Lepší P., Boublík K., Doležal P., **Ekrt L.**, Hofhanzlová E., Lepší M., Lippl L., Štech M., Švarc J. & Žíla V. (2005): Floristický materiál ke květeně Dačicka. – Acta Rer. Natur. 1: 17–44.

Ekrt L. (2004): Významný nález *Asplenium adiantum-nigrum* v Českém ráji. – Zpr. Čes. Bot. Společ. 39 (1): 37−40.

Stolinová Š. & **Ekrt L.** (2001): Výskyt kapradě rezavé v Pekelském údolí. – Panorama 9: 99–100.

Contributions to books

Čech L., **Ekrt L.**, Ekrtová E., Juřička J. & Jelínková J. (2021): Červená kniha květeny Vysočiny. – Pobočka České společnosti ornitologické na Vysočině, Jihlava, 537p., ISBN 978-80-88242-05-5

Kubát K. & **Ekrt L**. (2019): Kapradiny – Polypodiophyta, pp. 107–125. – Kaplan Z., Danihelka J., Chrtek J. jun., Kirschner J., Kubát K., Štech M. & Štěpánek J. (eds) (2019) Klíč ke květeně České republiky [Key to the flora of the Czech Republic]. Ed. 2. – Academia, Praha.

Boublík K., Brabec J., Bureš P., Danihelka J., Douda J., Ducháček M., **Ekrt L**., Ekrtová E., Grulich V., Hans V., Havlíček P., Hesoun P., Hroudová Z., Chán V., Chrtek J., Janáková J., Kaplan Z., Kirschnerová L., Kolář F., Košnar J., Koutecký P., Kúr P., Lepší M., Lippl L., Lučanová M., Paulič R., Pavlíčko A., Průša D., Průšová M., Půbal D., Rektoris L., Řepka R., Soukup M., Štech M., Štěpánková J., Šumberová K., Trávníček B., Vaněček

Z., Vydrová A. & Žíla V. (2013): Červená kniha květeny jižní části Čech. – Jihočeské muzeum v Českých Budějovicích. 503 s., ISBN 978-80-87311-35-6.

Hroudová Z., **Ekrt L.,** Ekrtová E. & Šumberová K. (2011): *Scirpetum radicantis* Nowiński 1930. – In: Chytrý M. [ed.], Vegetace České republiky – 3. Vodní a mokřadní vegetace, Academia, Praha, pp. 462–465.

Ekrt L. (2011): Flóra a vegetace. – In: Dimter T. & Lisák P. [eds], Adršpašsko, Juko, Náchod, 391p. ISBN:978-80-86213-45-3.

Ekrt L. & Mráz P. (2007): Revision of caryological data to the genera *Asplenium* L., pp. 116 – In: Marhold K., Mártonfi P, Mereďa jun. & Mráz P. [eds], Chromosome number survey of the ferns and flowering plants of Slovakia, Veda, Bratislava.

Horváthová V., **Ekrt L.** & Skolek M. (2007): Bezlesí Národního parku Šumava – ochrana bezlesí a jeho management. – Správa NP a CHKO Šumava, Vimperk, 52 p., ISBN 978-80-239-9566-4.

Citation to own papers

Total number of citations (WOS) without autocitations: **349**
H-index (WOS): **13**

Projects solved in the last 5 years

2022 Plant diversification facilitated by climate change: evolution of ancient lycopods connected with arctic, post glaciated and boreal regions, INTERACT 2022/716, remote access, principal investigator, total 15,000 CZK.

2019–2021 War of dominance between sexuals and apomicts: the mechanisms and consequences of the conflict between the two types of reproduction in wood ferns, Czech Grant Foundation (GAČR) no. 19-17379S, principal investigator 3 331,000 CZK.