University of South Bohemia
in České Budějovice
Faculty of Fisheries
and Protection
of Waters

Taxonomic revision of homocytous cyanobacteria

Otakar Strunecký

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Appendixes:

1. Bohunická, M., Mareš, J., Hrouzek, P., Urajová, P., Lukeš, M., Šmarda, J. et al. (2015) A combined morphological, ultrastructural, molecular, and biochemical study of the peculiar family Gomontiellaceae (Oscillatoriales) reveals a new cylindrospermopsin-producing clade of cyanobacteria. J. Phycol. 51:1040-1054. doi:10.1111/jpy.12354

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Introduction

The taxonomic system of cyanobacteria has been developing for more than a century. Although it classifies prokaryotic organisms, it has remained closely attached to the Darwinian concept of a tree of life for the whole period. Individual species were assigned to the genera, grouped into families and orders according to the most recognizable morphological features such as cell shape and size, mucilage or sheath structure; trichome width, tapering, branching type, presence of akinetes, and heterocytes in cyanobacteria forming filaments, or colony architecture and structure in species forming complex thalli.

Geitler (1925) divided cyanobacteria into seven orders: Chroococcales, Entophysalidales, Pleurocapsales, Dermocarpales, Siphononematales, Nostocales, and Stigonematales, synthesizing species and genera context created by preceding authors such as Gomont (1892), Borzì (1914), or Schaffner (1922). For the next 80 years, the original Geitler's division runs like a red thread through the following taxonomic changes, with repeated merging, splitting, and moving of individual orders, families, genera, and species into, in principle, very similar categories (Elenkin 1938, Geitler 1942, Desikachary 1959, Starmach 1966, e.g. Bourrelly 1970). Today, these classification systems are viewed as constrained by the assumption of a fundamental distinction between unicellular and filamentous (trichal) cyanobacterial forms

Fundamental taxonomic changes, driven by the availability of new genetic data, were introduced in Bergey's Manual of Systematic Bacteriology. This reclassification divided cyanobacteria into five provisional subsections instead of orders, with the expectation of formal revisions as more data becomes available. These were: I (= Chroococcales), II (= Pleurocapsales), III (= Oscillatoriales), IV (= Nostocales), and V (= Stigonematales) (Castenholz 2001). This proposal was later transformed to classical taxonomic categories to comply with the International Code of Nomenclature for algae, fungi, and plants (ICN) with several updates based on the 16S rRNA gene phylogeny considering the thylakoids arrangement (Hoffmann et al. 2005). In particular, the order Gloeobacterales Cavalier-Smith was proposed to harbor primitive thylakoid-less cyanobacteria. Synechococcales Hoffmann, Komárek et Kaštovský and Pseudanabaenales Hoffmann, Komárek et Kaštovský contained basal groups of coccoid and filamentous cyanobacteria with parietal thylakoids, and the definition of Nostocales was expanded to include the true-branching Stigonematales to retain monophyly (Hoffmann *et al.* 2005a) of heterocystous types. The last comprehensive review of the cyanobacterial system by Komárek et al. (2014) introduced a cyanobacterial phylogenomic tree for taxonomic purposes. The authors have proposed a couple of entirely new orders to contain cyanobacterial lineages apparently distant from the existing ones (Spirulinales, Chroococcidiopsidales) but did not revise the obviously polyphyletic groups such as Synechococcales and Osciallatoriales, leaving this to follow-up studies. Since then, efforts to initiate this difficult task have been made and resulted in the creation of two new orders, Cyanomargaritales Moreira and Thermostichales Komárek & Strunecký (Moreira *et al.* 2017, Komárek *et al.* 2020). However, the taxonomical system of cyanobacteria has recently undergone restructuring, employing a multigene approach as its foundation. The following text outlines the process of proposing an up-to-date taxonomic system for cyanobacteria, including the key steps that have led to this proposal.

Chapter I: challenges in current cyanobacterial taxonomy

Morphological variations observed under different natural and laboratory conditions, as documented by researchers like Šabacká (2001), and Raabová *et al.* (2019), have posed significant challenges in microscopic evaluation and species assignment for cyanobacteria. To discern differences between genera, meticulous examination of numerous strains within specific lineages is essential. Nevertheless, species recognition remains difficult for certain cyanobacterial species, even with a polyphasic approach.

Unicellular cyanobacteria
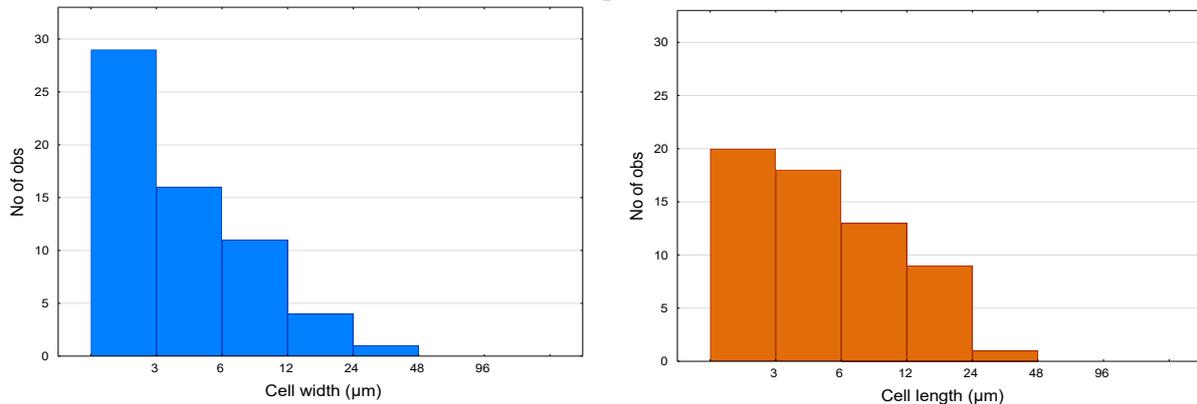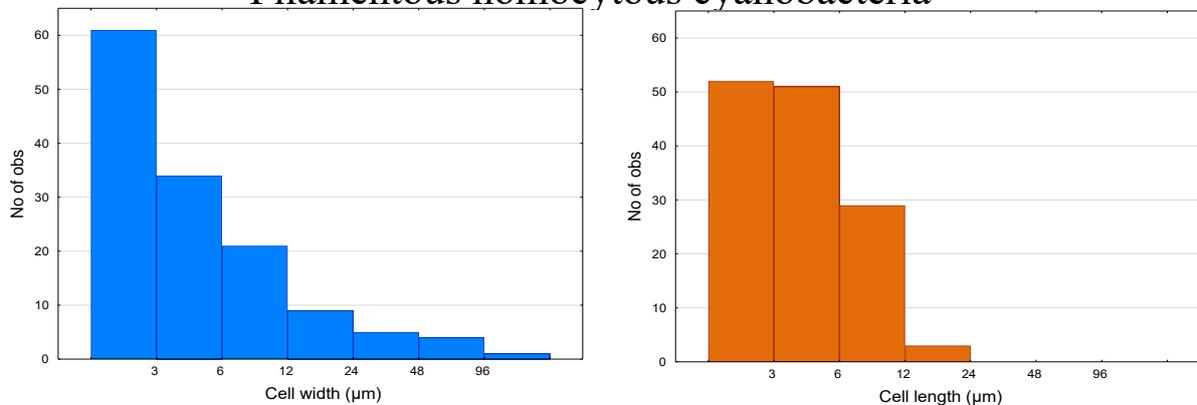

Filamentous homocytous cyanobacteria

Figure 1 Histogram of the cell sizes of cyanobacteria. One observation represents the dimensions given for one described type species as in Strunecký et al. (2023)

The relatively limited morphological diversity, making the morphological determination extremely challenging, is common in a vast number of cyanobacterial species, which can be attributed to their evolutionary history and adaptation to nutrient-deprived environments. Majority of cyanobacterial species retain a small size (Fig. 1), which offers a favorable surface area-to-volume ratio (Strunecký *et al.* 2023). This unique characteristic enables efficient nutrient absorption and waste removal per unit of cell mass, enhancing their survival in challenging conditions. Smaller cyanobacteria's rapid growth and reproduction confer a competitive advantage in fluctuating environments and facilitate efficient dispersal through liquids and surfaces. Despite these advantages, cyanobacteria cannot reach the diminutive sizes of many other bacterial species due to the spatial requirements of their photosynthetic apparatus. The thylakoid membrane pair, essential for establishing the electron gradient in photosynthesis, typically has a thickness ranging from 100 to 500 nm (Liberton *et al.* 2013). This requirement, combined with the presence of phycobilisomes, imposes a lower size limit on cyanobacteria, preventing them from achieving the small scale seen in some other bacteria. With three or more thylakoid layers and a nucleoplasm with a diameter of around 500 nm, typical lower size limit for cyanobacteria, their cell size generally ranges from approximately 0.6 mm to 2 mm in diameter. This lower size limit is also influenced by the presence or absence of complex phycobilisomes with phycoerythrin, enabling smaller cell sizes in types limited to phycocyanins (Fig. 2). Furthermore, environmental constraints, particularly in planktic species, tend to favor smaller sizes due to their lower sedimentation rates. These factors collectively restrict the sizes and shapes of the majority of planktic cyanobacterial species to either having gas vesicles or simple spherical or slightly elongated bacilli-like

shapes. This morphology is especially common in species equipped with pili, further emphasizing the constraints imposed by their environment and ecological niche.

In the late 1990s, the advent of a reliable genetic marker, the 16S rRNA gene, greatly facilitated the recognition and identification of consistent traits for morphological determination of cyanobacterial species. By the 2010s, the use of the 16S rRNA gene in cyanobacterial taxonomy had become well-established, although the number of isolated and sequenced strains remained relatively limited. Assigning specific cyanobacterial strains to the type species of traditional genera based on genetic traits emerged as a promising approach to advance cyanobacterial taxonomy (Komárek 2016).

While the 16S rRNA gene has proven to be a valuable tool in resolving taxonomic issues, it is not without limitations. Its use must be approached cautiously, particularly regarding the potential for strain contamination by ubiquitous or cryptic cyanobacteria. Particularly in the studies on ecological diversity, the overreliance on the 16S rRNA gene as the sole trait in environmental studies may lead to an underestimation of species diversity. This issue was explicitly addressed by Strunecký et al. (2019) in their study of a Rupite hot spring, where they found that species composition might be influenced by: A) The efficiency of DNA isolation, especially in species that produce a high amount of extracellular polymeric substance and B) by the the selectivity of different primer pairs (even those considered as universal primers) in Next Generation Sequencing (NGS) technologies. Additionally, optical microscopy analyses tend to underestimate species with low frequencies.
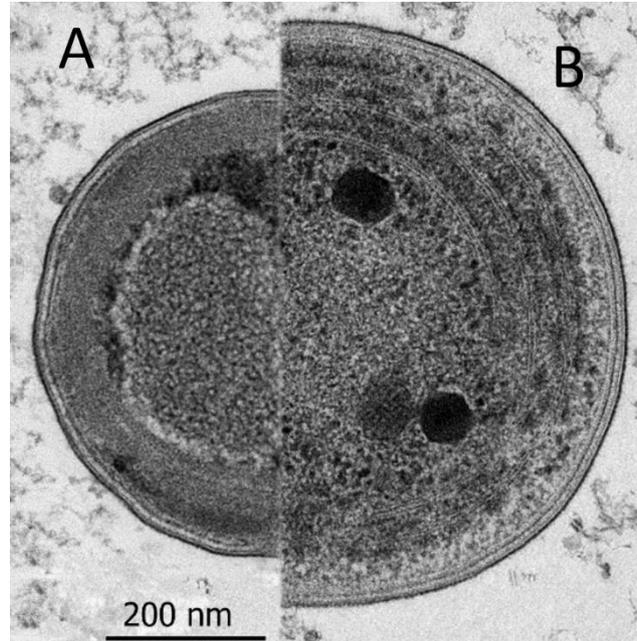
Figure 2 Prochlorococcus marinus (A) and Synechococcus marinus (B). The ultrastructure of both species is similar, with a characteristic centroplasma and radial thylakoids showing extreme cell size minimalization.

Despite the challenges mentioned earlier, the rapid expansion of the 16S rRNA gene sequence database and the widespread adoption of the polyphasic approach workflow (e. g. Johansen & Casamatta 2005, Osorio-Santos *et al.* 2014) have fueled the creation of numerous new genera. Between 2014 and 2021 alone, approximately 80 new genera emerged. Similarly, this progress has led to the establishment of new families, including Oculatellaceae and Trichocoleusaceae (Mai *et al.* 2018a), Laspinemaceae (Zimba et al. 2020), Geminocystaceae (Tuji et al. 2021).

The assessment of phylogenetic and taxonomic relationships among these numerous, often highly divergent new entities is an urgent task to prevent increasing taxonomic confusion. However, it's worth noting that the classification of the majority of taxa established in the 20th century still relies on hierarchical relationships inherited from classical morphological taxonomy. As previously mentioned, Synechococcales have been identified as polyphyletic in various orders, consisting of small coccal or thin filamentous cyanobacteria. This phenomenon occurs primarily but not exclusively at the bottom part the cyanobacterial phylogeny (Mareš 2018, Komárek et al. 2020). Oscillatoriales encompassed most filamentous genera previously not classified under Synechococcales. However, recent research has unveiled their notable phylogenetic divergence (Mareš *et al.* 2019, Galica *et al.* 2021). While Nostocales constituted a cohesive group, their internal relationships remained obscured due to morphological convergence and limited diversity in the 16S rRNA loci. This was evident from the presence of numerous semi-cryptic *Nostoc*-like taxa (Cai *et al.* 2019, Soares *et al.* 2020).

Remarkably, the arrangement of thylakoids, previously regarded as an essential trait in modern cyanobacterial taxonomies (Castenholz *et al.* 2001b, Hoffmann *et al.* 2005b, Komárek *et al.* 2014), has recently been discovered to provide only limited taxonomic information (Mareš et al. 2019). The

absence of thylakoids in Gloeobacterales and the special form of parallel thylakoid arrangement in Geminocystaceae seem to represent the only true phylogenetically informative traits. Various parietal and fascicular architectures are dispersed throughout the cyanobacterial tree of life and exhibit considerable homoplasy. In this respect, the thylakoid patterns resemble other primitive or convergent morphological traits such as rod-shaped unicellular morphology, multicellularity, filament branching, production of the mucilaginous sheath, gas vacuoles, etc., which recurrently appear in cyanobacterial evolution (Schirrmeister *et al.* 2013, Dvorak *et al.* 2014).

The high number of whole-genome sequences in clades with picocyanobacteria led to the proposal of another type of artificial so-called ecogenomic classification (Walter *et al.* 2017, Salazar *et al.* 2020, Tschoeke *et al.* 2020). Creating tens of new cyanobacterial taxa based merely on multilocus phylogenies with no connection to validly described species effectively disconnects the traditional and genome-based taxonomies, which we consider highly unfavorable.

These seemingly contradicting approaches must be thus combined to achieve a reliable cyanobacterial taxonomy linked to the literature record throughout the last 130 years. Similarly, the official starting point for classifying cyanobacteria by the International Code of Nomenclature for algae, fungi, and plants has to be still accepted to avoid misconceptions among professionals in the field. While the higher-level taxonomy of prokaryotes tends to be, to some extent, artificial and arbitrary, the consensus on species and generic names ordered into families and orders is fundamental to enable specialists to study and compare cyanobacterial traits ranging from the function of a single protein found in evolutionary close cyanobacteria to identification in drinking water management. Moreover, hierarchical taxonomy is required by all current metagenomics workflows (Parks *et al.* 2020).

Chapter II: Taxonomy and ecology of simple filamentous homocytous cyanobacteria

The conspicuously thick filaments observed in *Oscillatoria* and similar genera, such as *Lyngbya*, have historically represented a prominent focal point within the realm of morphological criteria. This characteristic likely found expression in the initial conceptualization of the Oscillatoriales order, which encompassed filamentous cyanobacteria lacking heterocytes and forming well-defined trichomes (Schaffner 1922), especially considering the earlier microscopes with less than perfect resolution of the finest details of the cell connections in filaments. Interestingly, according to the original description by Schaffner (1922): "free filaments commonly massed into flat layers, sometimes several filaments enclosed in one common sheath," the Oscillatoriales were likely primarily founded on a widespread species renowned for its tendency to aggregate multiple filaments within a single sheath exhibiting morphological features that mostly approximated *Oscillatoria* sensu stricto, recently known as *Microcoleus* (Strunecký et al. 2013). An additional significant challenge arose with *Oscillatoria*'s role as the cornerstone type species within the Oscillatoriales order. Isolating, cultivating, and maintaining strains of *Oscillatoria* in laboratory cultures has earned a reputation for being exceptionally problematic. Nonetheless, a breakthrough emerged in 2018 when Mühlsteinová *et al.* (2018) overcame these challenges and conclusively placed the genetic traits of *Oscillatoria* sensu stricto within a distinct clade.

The newly acquired knowledge was possible to match with previous data based on morphological diversity of natural material. Preceding comparative analyses of both the formerly described genera and microscopic specimens during the 20th century (Boone *et al.* 2001, Komárek & Anagnostidis 2005) indicated striking diversity of morphological characteristics spanning various genera, families and orders within the formerly conceived Oscillatoriales and suggested their potential polyphyly, even when solely considering their morphological traits (Komárek & Anagnostidis 2005). According to Komárek and Anagnostidis (2005) particularly enigmatic genera with intriguingly high morphological variability included *Phormidium* Kützing ex Gomont (Strunecký *et al.* 2011), *Microcoleus* Gomont (Strunecký et al. 2013), and *Geitlerinema* Gomont (Chatchawan *et al.* 2012, Strunecký *et al.* 2014, Strunecký *et al.* 2017). Other filamentous taxa such as Leptolyngbyaceae Komárek et al. as an example (Raabová et al. 2019) with very thin trichomes were supposed to have a puzzled polyphyletic phylogeny possibly containing a number of cryptic genera (Mai *et al.* 2018b).

One of the biggest problems of the current taxonomy remains the vague determination of numerous type species stretching like a red thread from the late 19th century set as an official starting

point for naming filamentous cyanobacteria by the International Code of Nomenclature for algae, fungi, and plants (Strunecký et al. 2023). A good example of still unresolved type species of a historically prominent genus can be found in *Phormidium*. Gomont (1892) involved the description of 29 species of *Phormidium*. Following taxonomic studies, including Geitler's comprehensive monograph in 1932 and Drouet's exhaustive list in 1968, significantly augmented the record of the officially recognized *Phormidium* species to 85 and, astonishingly, to more than 260, respectively (Geitler 1932, Drouet 1968). In 2005, Komárek and Anagnostidis conducted a significant taxonomic refinement by validly describing a total of 163 species. Furthermore, they reclassified certain species with trichomes tapering toward their ends or possessing apical cells with calyptra from the *Oscillatoria* and *Lyngbya* genera into the *Phormidium* genus. This set of species was presumed to be present in various biotopes globally, including marine environments. However, comparing them with the type species of the genus *Phormidium* is crucial for correct taxonomic evaluation, as outlined by Geitler (1932). His description of *Phormidium* type species, *P. lucidum*, followed the original descriptions by Kützing (1843) and Gomont (1892), which were based on Agardh's (1827) "*Oscillatoria lucida*." Interestingly, the specimen of *O. lucida* was originally collected from aerophytic mats found on the walls of a thermal spring in Carlsbad (Karlovy Vary, Czech Republic) on locality, which was later destroyed by city development (pers. com. J. Kaštovský). Further resampling to resolve the specific strain on type locality thus became impossible. However, the thermal biotope is considered as a critical feature of *Ph. lucidum* by Komárek and Anagnostidis (2005).

Nonetheless, the thermal biotopes in central Europe have undergone a substantial transformation, with many evolving into spa resorts or experiencing extensive channelization of thermal springs. These changes have not only altered the nature of these biotopes but have also hindered the development of the original cyanobacterial communities. In light of these challenges, a comprehensive survey was conducted across more than 90 thermal biotope sites in Czech Republic, Germany, Iceland, Slovakia, Hungary, and Bulgaria (Strunecký, unpublished). Optical microscopy was employed in a meticulous examination with the specific objective of identifying the *P. lucidum* morphotype. Regrettably, despite these concerted efforts, no specimens exhibiting the morphology characteristic of *P. lucidum* (Fig. 1) were discovered within the examined thermal sites. Given the stringent ecological and morphological criteria required for the precise identification of *P. lucidum*, it appears increasingly improbable that this species will be successfully characterized using conventional molecular biology tools. Up to now, the closest appearance to *P. lucidum* documented Heidari *et al.* (2018) in the Iranian hot springs (Fig. 1) was classified as *Laspinema* Heidari et Hauer.

Figure 3 P. lucidum Gomont (iconotype) with width 5-7 um (A). The short celled filaments are rare in thin Oscillatoriales. The short celled Laspinema thermale is 3- 5 um wide (B).

Also, it is important to acknowledge the striking extent of morphological variability observed in species that thrive in high-temperature environments. This variability can be so pronounced that it may even encompass the transformation of filamentous cyanobacteria with disc-shaped cells into unicellular forms with a coccal shape (Strunecký *et al.* 2019). Correspondingly, heterocytes are not formed at temperatures higher than 50 degrees making the hot spring cyanobacterial species determination challenging.

Nevertheless, extensive studies of hot springs have provided valuable insights into the presence of specific thermophilic cyanobacteria (Strunecký et al. 2019), even though the confirmation of *P. lucidum* remains elusive. Investigations conducted in high-temperature biotopes shed light on the existence of unique cyanobacterial species in hot water environments, namely identifying worldwide a limited number of genera such as *Thermoleptolyngbya* Sciuto et Moro, *Desertifilum* Dadheech et al., *Thermosynechococcus* Katoh et al., *Thermostichus* (Copeland) Komárek et Strunecký, *Geitlerinema/Klisinema* Heidari & Hauer, repeatedly identified worldwide in biotopes above 50 °C (McGregor & Rasmussen 2008, Heidari et al. 2018, Cheng *et al.* 2020).

Contrasting aspects of cyanobacterial occurrence might be found in cold areas. In these extreme conditions, on the other side of the environmental spectrum, cyanobacteria are superbly adapted to
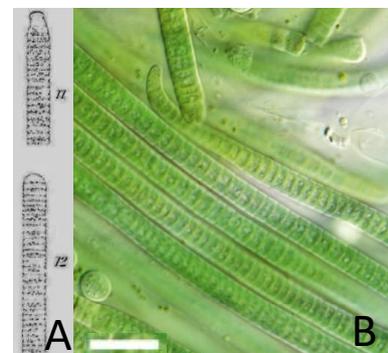
freezing and desiccation while restoring an active metabolism minutes after rehydration (Rajeev *et al.* 2013). Cyanobacteria belong to the main primary producers in the polar regions and play an essential role in carbon and nitrogen economy in polar soils, supplying the ecosystem with 2/3 of the total nitrogen input (Stewart *et al.* 2011). Non-photosynthetic bacteria living in polar areas do not merely endure such extremely inhospitable conditions, but they are highly adapted to these environments. Decades of polar research based mainly on morphological considerations showed that polar cyanobacteria could not be considered psychrophilic, having a growth optimum from ~15 to 30 °C (Tang & Vincent 1999). Later, Kleinteich *et al.* (2012) showed that polar cyanobacterial mats cultivated from 4 to 23°C formed different communities according to cultivation temperature, particularly Leptolyngbyaceae and *Nostoc* Vaucher ex Bornet et Flahault strains growing only in the coldest treatments.

Moreover, numerous Leptolyngbyaceae species were found only in polar regions (Raabová et al. 2019, Strunecký *et al.* 2020), suggesting psychrophyly in these species. However, further research is essential to substantiate the earlier ecological findings and gain a deeper understanding of the unique metabolic traits that underlie the successful growth of individual cyanobacterial species within these extreme ecological niches. Additionally, the exploration of how distinct populations and strains have evolved to thrive in specific natural conditions holds immense potential. Such investigations not only enhance our comprehension of the unique genetic and metabolic attributes within these bacteria but also open up promising opportunities for their potential utilization in various biotechnological and industrial applications.

During these specific ecological studies (Strunecký *et al.* 2010, Strunecký *et al.* 2012, Mareš et al. 2019, Raabová et al. 2019), hundreds of collected individual strains backed the comparative material for taxonomic generalization (combined with purchased strains) in non-heterocystous cyanobacteria (Fig. 4). From these studies a state of art understanding of several traditional taxa emerged using the polyphasic approach. This methodology established a foundation for further categorization of these organisms, with 16S rRNA gene serving as a pivotal anchor for subsequent studies. Additionally, novel simple filamentous non-heterocystous species and genera have surfaced through phylogenetic analysis mainly amidst of cyanobacterial tree of life (Fig. 3), to a certain extent ecologically and morphologically distinct.

Fascinatingly, as depicted in the cyanobacterial tree of life (Fig. 3), the Prochlorothrichales Strunecký et Mareš represent a first order of filamentous cyanobacteria characterized by possessing a contiguous peptidoglycan cell wall structure connecting adjacent cells. In stark contrast, the Nodosilineales Strunecký et Mareš, situated within the same cluster of orders (Fig. 4), display a distinct feature, with genera exhibiting dual axes of division, resulting in the formation of convoluted and uneven filaments encapsulated within a common sheath retaining an observable outer membrane between adjacent cells. While the ultrastructural portrayal using transmission electron microscopy is currently unavailable for *Gibliniella* Strunecký et al. (Nodosilineales), it is reasonable to infer that it shares analogous characteristics with its counterparts. Except intensive motility *Gibliniella* is notable among the slenderest documented filamentous cyanobacteria, boasting a cellular width measuring 0.6–1 μm and a cellular length spanning from 1.4 to 4.3 μm (Strunecký et al. 2020).

The Leptolyngbyales Strunecký et Mareš constitutes the subsequent order within the cyanobacterial phylogenetic framework (Fig. 4). Analogous to the Prochlorothrichales, this order also exhibits an interconnected peptidoglycan cell wall architecture between adjacent cells. Despite the occasional uneven cell division exceptionally found in species such as *Phormidesmis arctica* Raabová et al. or *P. communis* Raabová et al., the Leptolyngbyales are primarily characterized by a distinctive trait setting it apart from the Nodosilineaceae: their cells are typically shorter than they are wide.

Ubiquitous and ecologically very successful *Leptolyngbya* Anagnostidis et Komárek, boasting an expansive repository of documented cultures numbering several hundred, in addition to an even greater abundance of 16S rRNA gene sequences, showcases notable heterogeneity within the 16S rRNA marker gene. An unpublished analysis reveals the presence of over 50 clades within this taxonomic group, distinguished by variations of more than 5% in their 16S rRNA gene sequences. Although the majority of genera remain cryptic, concerted efforts have been directed towards ameliorating this complexity. An endeavor was undertaken to condense nearly one-third of the known sequences into the genus *Phormidesmis* Turicchia et al., and also to one morphologically very special species of traditional of genus *Pseudophormidium* (Forti) Anagnostidis et Komárek (Raabová et al. 2019). In response to the
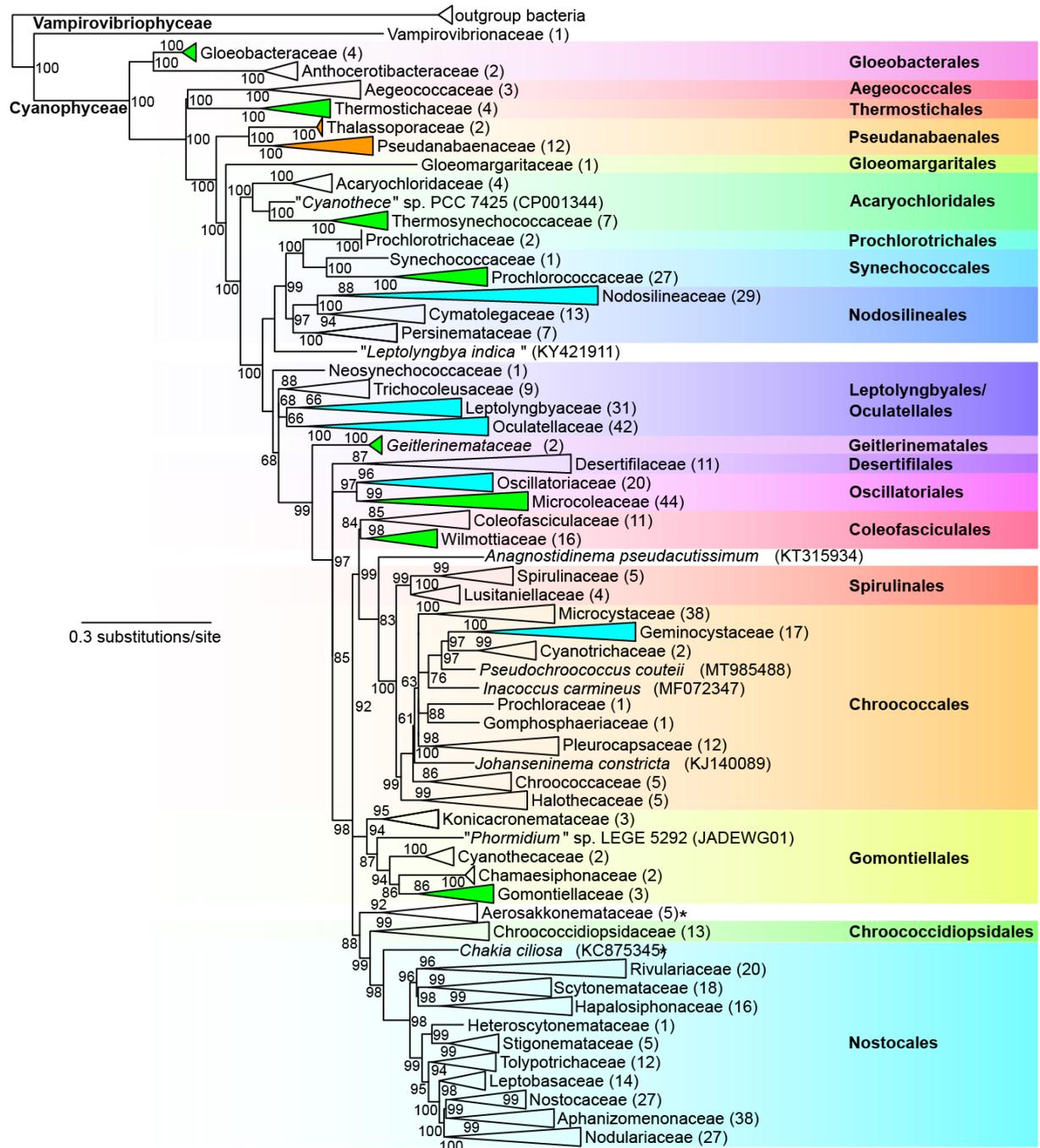

Figure 4 16S rRNA gene phylogenetic tree of cyanobacterial families and orders. The maximum likelihood tree search was constrained by the topology of a phylogenomic tree inferred from a subset of taxa for which whole genome sequences were available. Bootstrap values >50 are given at the nodes. Colored branches indicate taxa described in this MS, green indicates significant taxonomic revision of a taxon, blue contains newly described genera, and orange current proposals

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recommendations of reviewers, a solitary additional genus, *Leptodesmis* Raabová et al., was subsequently established. However,r researchers continue the description of cultivated strains creating cryptic taxa to *Leptolyngbya* (e.g. Tang *et al.* 2022), and the creation of several dozens of cryptic genera can be expected in the future.

The Oculatellales Strunecký et Mareš stands as the immediate sister order to the Leptolyngbyales. Despite its relatively recent description by Zammit *et al.* (2012), genus *Oculatella*, characterized by the distinctive presence of an "oculus" or bacterial eye within its terminal cell driving its phototactic motility, has catalyzed the generation of 16 novel genera. Remarkably, within this taxonomic context, a closely related polar genus, *Shackletoniella* Strunecký, Raabová et Bernardová, and a thermophilic hot-spring genus, *Thermoleptolyngbya* Sciuto et Moro, lack the characteristic eyespot. Nonetheless, they exhibit a noteworthy light-responsive gliding motility, as evidenced in the works of Strunecký et al. (2019, 2020). The morphological diversity in Oculatellales is remarkably extensive. Exploring this intriguing group further, through comprehensive investigations spanning genomics, metabolism, and morphology, undoubtedly holds the promise of unveiling a wealth of novel insights into cyanobacterial processes.

Following taxon on the cyanobacterial tree of live (Fig. 4) comprises the specific Geitlerinematales Strunecký et Mareš with a single known genus. It was taxonomically divided from other similar thin motile filamentous cyanobacteria by Strunecký et al. (2017). Thin filamentous cyanobacterium with cells around 2.0 um wide and 3.5–8.0 μm long similarly as the other studied cyanobacteria was described in 19th century *as Oscillatoria splendida* Greville ex Gomont and later transferred to *Geitlerinema splendidum* (Anagnostidis 1989). The main diacritical marker is the typical highly flexible terminal cell up to 15 μm long (Fig. 5) associated with clear gliding motility, which combined with cyanophycin granules close to external cell walls visible by optical microscopy as light bodies makes distinct diacritic markers.

Oscillatoriales Schaffner were currently narrowed to three families (Oscillatoriaceae (Gray) Kirchner, Microcoleaceae Strunecky, Johansen et Komárek, and Aerosakkonemataceae Strunecký et Mareš) that shifted the taxonomic placement of the majority of filamentous cyanobacteria (Strunecký et al. 2023). Such a shift in the concept of Oscillatoriales, which had previously been impeded by the absence of a universally accepted reference strain representing typical *Oscillatoria*, has been made possible by the recent identification of an epitype that closely aligns with the original description of the taxon (Mühlsteinová et al. 2018).

Following previous studies based on molecular analyses already indicating the polyphyly of *Phormidium* (Taton *et al.* 2006, Palinska & Marquardt 2008), further work was focused on the genetic diversity of *Phormidium* according to Komárek & Anagnostidis (2005). The *Microcoleus* (formerly including species such as *P. autumnale* Gomont transferred to *Microcoleus*) study included ninety-two strains and witnessed their high morphological and phylogenetic diversity (Fig. 5), however, it also found common generic features (Strunecký et al. 2013). The most important are: trichome width 4–10 um, cells isodiametric, occasionally as short as 1/3 as long as wide, often with characteristic cyanophycin bodies located close to cell walls between cells. *Microcoleus* have a characteristic raft structure of thylakoids, which can be also seen by optical microscopy as green field areas within the cells. Multiple trichomes in a common sheath can be found present facultatively in many but not all species. It is ubiquitous out of saltwater areas.
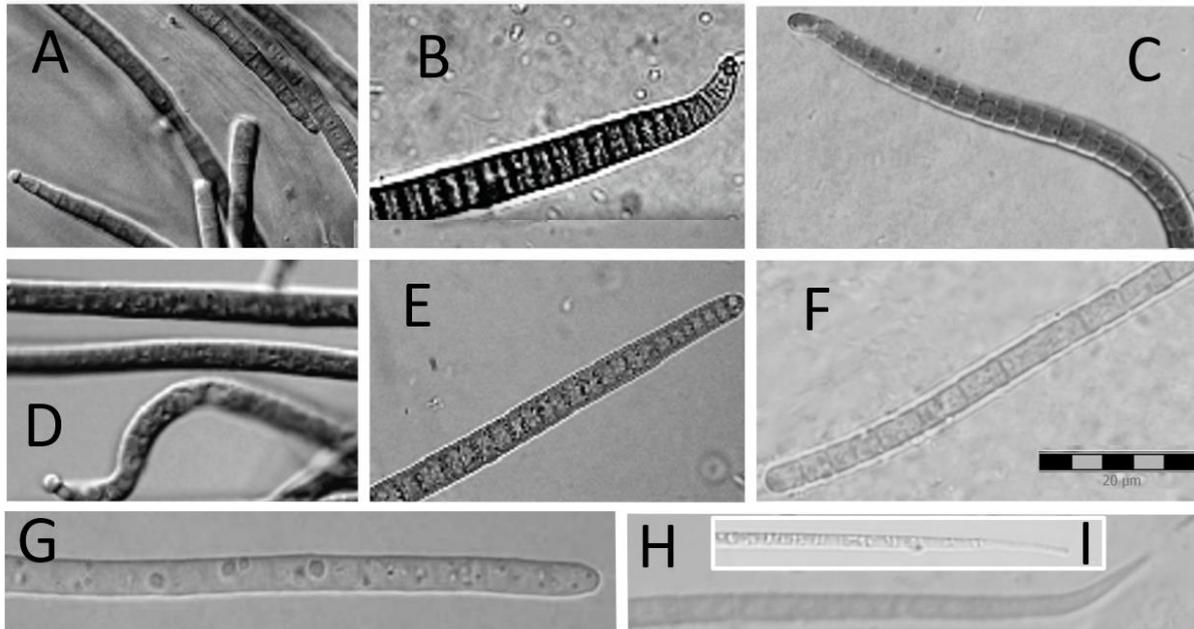
Figure 5 Revised filamentous species: Microcoleus vaginatus (A) , M. autumnalis (B), Kamptonema formosum (C), M. fonticulus (D), M. attenuatus (E), Wilmottia murrayi (F), Anagnostidinema formosum (G), Oxynema thaianum (H), Geitlerinema splendidum (I)

Another surprising addition to the Oscillatoriales was the description of thin cyanobacterial genus *Oxynema* (Gomont) Chatchawan et al. from a saline environment (Chatchawan et al. 2012) (Fig. 5). Cells are 7.5-9 mm wide, 2.2-3 mm long with characteristic long and triangular apical cell. A similar morphotype was observed by author in Israel salterns near Eilat gliding on the top of salt saturated water.

Phylogenetically distinct Wilmottiaceae Strunecký et Mareš, belonging in newly formed Coleofasciculales Strunecký et Mareš (Figs. 4, 5) contains two other described genera, *Wilmottia* Strunecký et al. and *Anagnostidinema* Strunecký et al. (Fig. 5) which were followed by four other genera described by various authors (e.g. Heidari et al. 2018). These genera have common features as they consist of solitary, soft, flexuous trichomes with rounded apices are enclosed in thin hyaline sheaths, trichomes are composed of more or less isodiametric cells with widths and lengths between 3.0–10.0 μm. Generally freshwater cyanobacteria, probably of worldwide occurrence, because their identification by sequencing rises from more and more localities around the world.

The above-mentioned genera exhibit significant variability and impressive ecological adaptability to specific environmental conditions, a characteristic particularly evident in Oscillatoriales. For instance, a noteworthy illustration of this adaptability can be observed in the lacustrine species *Oscillatoria kawamurae* Negoro, where the cyanobacterial population can reach a thickness exceeding 100 μm, as documented by Thu *et al.* (2020). In stark contrast, the highly motile saline cyanobacterium *Oxynema thaianum* Chatchawan et al. is approximately ten times thinner while still adhering to the conventional parameters defining Oscillatoriales, characterized by very short cells. Another compelling example can be found within the recently classified soil genus *Argonema* Skoupý & Dvořák, belonging to the Oscillatoriales Skoupý *et al.* (2022). *Argonema* exhibits a cryptic nature compared to various *Microcoleus autumnale* soil strains, emphasizing the intricate interplay between cyanobacterial morphology and environmental factors.

These instances highlight the notion that the morphological attributes of cyanobacteria are subject to constraints imposed by their surrounding environmental conditions, resulting in adjustments to their physical characteristics. For instance, in the case of filamentous cyanobacteria inhabiting soil ecosystems, a filament diameter of approximately 10 μm, as seen in soil boring *Microcoleus* and *Argonema*, may probably confer distinct advantages in specific soil structure. Conversely, cyanobacteria with narrower filaments, such as *Wilmottia*, *Coleofascicullus*, Siegesmund, Johansen et Friedl (5 μm

wide) (Siegesmund *et al.* 2008), or even thinner phylogenetically distant *Leptolyngbya*, *Trichotorquatus* Pietrasiak et Johansen (Pietrasiak *et al.* 2021), and *Oculatella* (below 2.5 µm), might exhibit preferences for different soil textures. It is essential to underscore that further investigation into both the different and shared physiological and metabolic properties among the studied genera, including horizontal gene transfer, is necessary to elucidate the specific conditions favoring different taxa.

Simultaneously, these findings highlight the limitations of the current taxonomy paradigm, which predominantly relies on the vertical inheritance of a limited set of genes primarily associated with protein translation and transcription. This suggests the need for a more comprehensive and dynamic approach to taxonomy that considers the ecological adaptations and morphological diversity exhibited by cyanobacteria in response to their varying environmental niches.

Chapter II: Ecology and taxonomy of coccal/unicellular cyanobacteria

The relatively limited morphological diversity of vast number of unicellular cyanobacterial species (Figs. 1, 6) can be almost certainly attributed to their evolutionary history in natural nutrient-deprived environments. Cyanobacteria maintain a small size, which provides them with a favorable surface area-to-volume ratio. This typical bacterial feature allows for enhanced nutrient absorption and waste removal per unit of cell mass, facilitating their survival in challenging conditions. The rapid growth and reproduction of smaller cyanobacteria offer a competitive advantage in fluctuating environments and enable efficient dispersal through liquids and surfaces. As discussed previously minimal diameter of 0.6 mm is evolutionarily connected with photosynthesis in cells without complex phycobilisomes to 2 mm diameter in cells with phycoerythrin containing phycobilisomes (Fig. 2). The other important feature linked to diameter are the environmental constraints and particularly planktic species tend to be smaller due to their lower sedimentation rate. These parameters limit the sizes and shapes of most planktic cyanobacterial species to a simple spherical or slightly prolonged bacilli-like shape that is particularly adopted by species with pili.

Variability is an inherent aspect of nature, and it is important to understand that the exact timing of subsequent cellular processes cannot always be consistently replicated. The processes involved in cell division are highly complex, involving an intricate machinery of proteins responsible for cell and chromosome division. These proteins need to be translated from specific genes and meticulously regulated. Currently, our knowledge about the comprehensive network that influences these processes remains relatively limited. The whole continuum of forms ranges from strictly spherical shape (which is relatively rare) to relatively long rods of varying lengths. Moreover, morphology transitions from slightly oval to elongated forms change under specific environmental conditions or within the context of the culture cycle, causing these predominantly unicellular organisms to form short chains of divided cells. Finding the diacritical markers in such a fuzzy spectrum of shapes that ranges from the relatively uncommon strictly spherical shape to the appearance of short rods in varying lengths is described further. Consideration must be therefore given to the fact that the morphology varies, and the goal is to distinguish between natural variations and traits that remain relatively consistent. However, the utilization of genetic characteristics has facilitated this process, enabling the discrimination of many of these uncomplicated unicellular cyanobacteria to valid taxonomic groups.

Basal taxonomical groups

The genus *Gloeobacter* Rippka, Waterbury et Cohen-Bazire, is considered the most primitive among living cyanobacteria with a unique ancestral cell organization. Notably, it lacks inner photosynthetic membranes (thylakoids) and exhibits an unusual configuration of the photosynthetic apparatus. Historically, this species has been assumed to possess a limited ecological range, attributable to its rudimentary photosynthetic machinery. In contrast to prior assumptions, the research conducted by Mareš *et al.* (2013) has illuminated a new perspective, revealing that *Gloeobacter* is far more prevalent than previously believed, gaining recognition as a ubiquitous cyanobacterium inhabiting rocky environments.

This finding also offered a potential explanation for the origin of cyanobacteria, viable for the cyanobacterial phylum as a whole. It suggested that alkaline rock-associated biofilms, the primary habitat for *Gloeobacter*, may have served as the ancestral environment for these microorganisms. However, recently, a new morphologically matching family Anthocerotibacteraceae (Fig. 6) proposed by Strunecký and Mareš in 2023 after the description of several newly identified strains, including tropical epiphyte *Anthocerotibacter panamensis* Rahmatpour et al. and Candidatus *Aurora vandensis* Grettenberger et al. from an Antarctic lake. These phylogenetically distinct cyanobacteria, which also lack thylakoids, cover divergent environments, testifying to the evolutionary divergence of Gloeobacterales. Anthocerotibacteraceae, therefore show that the claim of the origin of cyanobacteria in a particular environment cannot be verified. However, it can be assumed that even phylogenetically more original organisms basal to current Gloeobacterales will be found due to the rapid development in the identification and documentation of environmentally diverse cyanobacteria.

One of the fundamental challenges in cyanobacterial taxonomy has been understanding the variability followed by the classification of small planktic unicellular cyanobacteria with generally spherical or slightly oval appearances, usually having very similar pale green color and lack of diacritical traits. Assessment of natural material is even more challenging due to natural diversity. Contemporary amplicon studies have revealed the existence of a substantial number (>10) of closely related subpopulations within lentic aquatic ecosystems exhibiting 16S rRNA nucleotide differences of approximately 1-2% (unpublished data). This diversity in genetic makeup likely contributes to morphological diversity, as it is logical to infer that each of these subpopulations have evolved to exploit a particular combination in the physical and chemical conditions of their respective habitats. The formidable challenge of morphological classification for these types eventually led to the majority of these forms being categorized under the *Synechococcus* Nägeli and Synechocystis Sauvageau according to the cell division type. *Synechococcus* had elongated oval or rod-shaped cells dividing in one crosswise, perpendicular plane, whereas *Synechocystis* Sauvageau was defined by more or less spherical cells dividing in two perpendicular planes.

Synechococcus was initially conceptualized by Nägeli as a rod-like cyanobacterium originating from benthic freshwater environments. However, it was later discovered that this genus has a broader ecological range and throughout the 20th century, members of the *Synechococcus* genus were identified in nearly every imaginable habitat hosting cyanobacteria, including vast marine provinces and hot springs with many additional species described in various monographs (e. g. Starmach 1966). Although the polyphyly of *Synechococcus* was presumed for more than 20 years according to 16S rRNA gene phylogenies (e.g. Dvořák *et al.* 2014), the consortium of strains from distant phylogenetic groups and environments was gathered and the taxonomic revision was published relatively recently by Komárek et al. (2020). Following a comprehensive polyphyletic analysis, a proposal was made to designate a neotype for *Synechococcus elongatus* in accordance with the recommendation put forth by Rippka and Cohen-Bazire as outlined in Bergey's Manual (Herdman *et al.* 2001). The reference strain *Synechococcus elongatus* PCC 6301 from
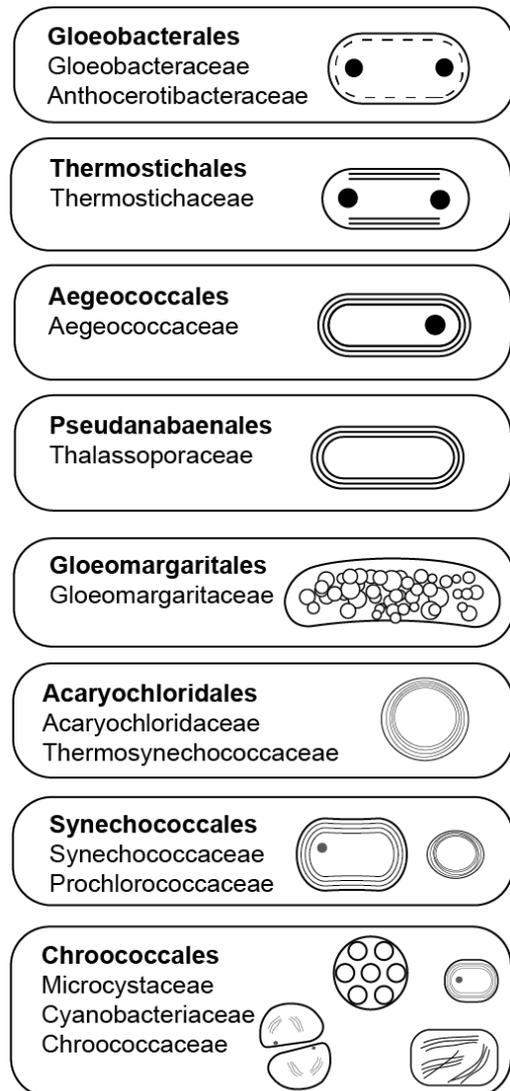

Figure 6 Graphic presentation of prevailing morphotypes in non-filamentous cyanobacteria.

the freshwater environment was selected to establish the anchor point for phylogenetic studies of genuine *Synechococcus*.

Accurate and consistent identification, along with stable naming of individuals in communities and stable naming of individual groups is vital for the mere comparability of research results. This objective provided by the taxonomic revision of Komárek et al. (2020) enabled further taxonomic revisions and the creation of an order of Aegeococcales Strunecký and Mareš as the successive phylogenetic lineages following Gloeobacterales on cyanobacterial tree of life; based on most recent description of *Aegeococcus* Konstantinou and Gkelis from the sublittoral zone of Mediterranean Sea (Konstantinou *et al.* 2021) accompanied by "Synechococcus" sp. PCC 7336 isolated 50 years ago by J. Waterbury.

Interestingly, the phylogenetic lineage following Aegeococcales was, according to the available data 16S rRNA gene in GenBank, found exclusively in hot springs in Europe, Asia, and North America. The Thermostichales Komárek and Strunecký belong to the true thermophiles thriving in temperatures from (35) 40 to 65 (70) °C (Strunecký et al. 2019, Strunecký et al. 2023).

Figure 7 Phylogenetic tree of cyanobacteria inferred by maximum likelihood method in RAxML. Pseudanabaenales are expanded.

Unicellular Pseudanabaenales

Multilocus phylogenetic analysis has identified the subsequent group of cyanobacteria as Pseudanabaenales Anagnostidis & Komárek traditionally conceived as filamentous (Komárek & Anagnostidis 2005). Even though a high number of species from this group form short filaments of up to 10 cells (Castenholz *et al.* 2001a), they are recognized as the earliest group of filamentous cyanobacteria in the tree of life (Strunecký et al. 2023). Moreover, current meticulous, even yet unpublished study documented that this crucial milestone in cyanobacterial evolution requires comprehensive investigation, and it is a subject of intensive research. The two major groups identified in Pseudanabaenales differentiated into two major clusters, the Pseudanabaenaceae Anagnostidis & Komárek, and Thalassoporaceae Strunecký & Mareš. The filamentous Thalassoporaceae morphologically cryptic to Pseudanabaenaceae were described only from benthic marine environments. However, there is a gap in the description of both marine and freshwater unicellular Pseudanabaenales whose occurrence in phylogenies was already reported (Konstantinou et al. 2021), previously considered to belong to *Synechococcus* (Korelusová *et al.* 2009). Among the unresolved strains isolated

Figure 8 Optical microscopy of Pseudanbaenococcus habilitatis SAG 2387 under 1000x magnification.

quite long ago are the *Synechococcus* sp. PCC 7502 and *Synechococcus* sp. SAG 2387 actually falls into Thalassoporaceae (Fig. 7). These strains belong to a distinct clade (Fig. 7), and their 16S rRNA gene
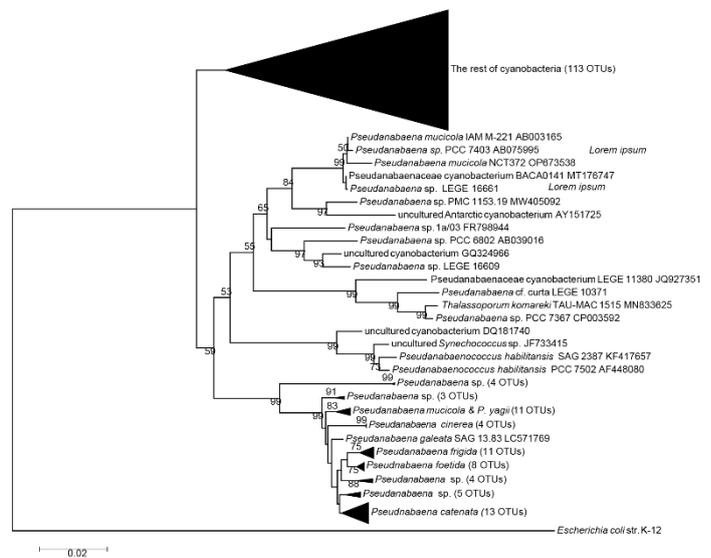
shows 9% difference to adjacent clades (Tab. 1), supporting their generic separation. Therefore, a new taxon *Pseudanabaenococcus* can be proposed with a type species *Pseudanabaenococcus habilitatis*.

Pseudanabaenococcus gen. nov.

Description: Phylogenetically separated clade within Thalassoporaceae. Freshwater or epiphytic. Cells solitary or in agglomerations formed by cylindrical cells with rounded or filleted ends, longer than wide, 1.5 to 3 μm wide, up to 7 μm in length, strictly cylindrical or slightly arcuated, with visible sheaths. Reproduction by binary fission, perpendicular to the long axis of the cell in subsequent generations, cells are more there 3 times longer than wide before division, not forming chains of divided cells. Thylakoids parietal, with seven concentric layers and clearly delimited centroplasma.

Habitat: Biofilm in highland or mountain streams and lakes, most likely also epilithic on smothered rocks. Sequences belonging to this clade were identified in European mountain ranges and the Antarctica, suggesting probable worldwide occurrence.

Type species: *Pseudanabaenococcus habilitatis* sp. nov.

Etymology: *Pseudanabaenococcus habilitatis*, a coccal cyanobacterium from Pseudanabaenales described in a habilitation thesis.

Description: Unicellular photosynthetic prokaryotes with cells 2.0 mm wide (in range from 1.5 to 2.7 mm) and 4.5 μm long (in the range from 2.9 to 6.6 μm) (Fig. 8). Cells solitary, or in irregular agglomerations, blue–green or green with rounded ends, longer than wide, straight, strictly cylindrical, or slightly arcuated, with visible sheaths. Reproduction by binary fission, perpendicular to the long axis of the cell in subsequent generations, symmetrical or slightly asymmetrical, daughter cells separating soon after division. Gas vesicles have not been observed. Thylakoids parietal, with about seven concentric layers and clearly delimited centroplasma.

Holotype here designated: CBFS A–023–X, dried material of the strain *Pseudanabaenococcus habilitatis* SAG 23.87, deposited in the herbarium collection of the University of South Bohemia.

Reference strain: *Pseudanabaenococcus habilitatis* SAG 2387. Other prominent strain belonging to this species is "*Synechococcus* sp". PCC 7502 isolated by R. Rippka from sphagnum bog, near Kastanienbaum, Vierwald stattersee, Switzerland, 1972.

Type locality: Harz Mountains, Westerhoefer Creek, Germany

Narrowed Synechococcales

The research on so-called picocyanobacteria, i.e. in the size scale of picoplankton (< 2 μm), is still attracting considerable attention due to their high ecological significance. They have been increasingly observed in diverse planktic environments, and their substantial contribution to primary production has been highlighted over the past few decades. These most prominent picocyanobacteria belong to Prochlorococcaceae Komárek et Strunecký with the metabolically and genetically highly diverse marine *Prochlorococcus* Chisholm et al., *Parasynechococcus* Coutinho et al. and freshwater *Cyanobium* Rippka et Cohen–Bazire (Strunecký *et al.* 2021). Komárek et al. (2020) also validated the correct reclassification of the marine "*Synechococcus marinus*" to *Parasynechococcus.* Although these marine picocyanobacteria are phylogenetically adjacent to freshwater *Synechococcus elongatus,* marine *Prochlorococcus* and *Parasynechococcus* have quite different ecology and genetic inventory, making them superbly accommodated to oligotrophic oceanic provinces (Strunecký et al. 2021), and proving them essential players in the planetary metabolism. Marine picocyanobacteria *Prochlorococcus* and *Parasynechococcus* are responsible for at least one-quarter of the global primary production (Garcia-Pichel *et al.* 2003, Whitton 2012). These organisms are believed to be crucial in the food web of many ecosystems, serving as a primary producer of organic matter and as a food source for higher trophic levels. Their small size and rapid growth rates allow them to thrive in a wide range of aquatic habitats, making them an important contributor to the diversity and resilience of these environments.

Table 1 Similarity of 16S rRNA gene of P. habilitatis with other strains within Pseudanabaenales and close phylogenetic clades. A part of 16S rRNA gene with a length of 996 bp was used for comparison.

	strain:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*Pseudosynechococcus habilitatis* SAG 2387		90.6	90.5	99.2	92.8	91.8	90.5	89.1	90.7	90.1	90.1	90.7	90	87.7	86.2	87.2
2	*Pseudanabaena_catenata* SAG 254.80			98.4	90.9	91.9	91.7	98.9	89.3	99	99	99	98	89.2	88.2	88.8	89.3
3	*Pseudanabaena galeata* SAG 13.83				90.8	91.8	91.5	99	89.1	98.7	98.5	98.5	98.7	89.2	88.1	88	88.8
4	*Pseudosynechococcus habilitatis* PCC 7502					92.9	91.7	90.8	89.2	91	90.4	90.4	91	89.9	87.9	86.7	87.2
5	*Pseudanabaena* sp. PCC 7403						94.8	91.8	88.4	92	92	92	91.8	91.6	87.8	88.3	89.2
6	*Pseudanabaena* sp. PCC 6802							91.3	87.9	91.3	91.8	91.8	91	92	86.8	88	89
7	*Pseudanabaena minima* GSEPSE2005C								89.1	98.6	98.4	98.4	98.6	88.7	88.1	87.8	88.4
8	*Limnothrix redekei* 165a									89.5	89.5	89.5	89.3	87.7	89	86.6	87.4
9	*Limnothrix* sp. Sai002										98.9	98.9	98.5	88.9	88.2	88.4	89
10	*Pseudanabaena* sp. PCC 6903												98.3	89.2	88	89.1	89.5
11	*Pseudanabaena* sp. PCC 7402												98.3	89.2	88	89.1	89.5
12	*Pseudanabaena* sp. CCALA 873													88.9	88.4	88.2	88.4
13	*Pseudanabaena* sp. PCC 7367														88.1	88.8	88.8
14	*Leptolyngbya boryana* UTEX B488															86.9	86.9
15	*Thermostichus* sp. JA-2-3B'a																90.4
16	*Gloeobacter violaceus* PCC 9601																

Due to their importance in oceanic and freshwater ecosystems, more than 2300 papers have been published up to day to understand their biological properties and ecological functions. Hence, the taxonomic validity of "*Synechococcus marinus*" and particularly the morphological generic classification to *Synechococcus* was problematic due to reliance on formerly acquired morphological names. High variety of marine isolates and strains that were unambiguously phylogenetically distinct from freshwater *Synechococcus* sensu stricto throughout the cyanobacterial tree of life were used as key models for physiological and biochemical studies comparing their physiological and genomic properties. Unfortunately, their earlier classification attempts were unsuccessful due to not implementing all formal disclosures. Therefore, in 2020 the type species of *Synechococcus*, *S. elongatus* Nägeli was defined according to neotype material that morphologically, cytologically, and ecologically matched Nägeli's freshwater epipelic type (Komárek et al. 2020). The revised genus *Synechococcus* formed a distinct clade and provides the exact phylogenetic position distinguished from the other coccal morphologically similar *Synechococcus*–like cyanobacteria. (Fig. 4).

Picocyanobacteria in Chroococcales

In the research conducted by Komárek et al. (2020), another *Synechococcus*-like species was also detailed, clustering in the order Chroococcales, far away from other picocyanobacteria (Strunecký et al. 2023). This species was initially isolated from hot springs in Rupite, as reported by Strunecký et al. (2019), and was designated as *Picosynechococcus fontinalis* Komárek, Johansen, and Strunecký. The freshwater genus *Picosynechococcus* includes exceptionally small planktic piliated picocyanobacteria, measuring approximately 1.2–3.0 × 0.8–2.0 μm. These minute organisms may have often been overlooked in previous floristic and ecological studies due to their diminutive size and morphological resemblance to other picocyanobacteria, such as *Cyanobium*. While *Picosynechococcus fontinalis* is known to thrive within a temperature range of 20 to 40 °C, there are indications that the extensive variability in morphotypes within this genus exists across a wide array of environments. These environments encompass planktonic, periphytic, and even soil ecosystems. It is expectable that ongoing and future research efforts may uncover a rich diversity of *Picosynechococcus* morphotypes in various habitats, thereby expanding our understanding of their ecological significance.

Conclusions and further perspectives

- Our research, as documented in the publications listed, has made substantial contributions to elucidating numerous taxa through effectively consolidating five traditionally recognized species. This conservative approach, backed by modern genetic methods, offered a solution to address the exceptional variability observed in cyanobacterial species since the advent of optical microscopy.

- Contrary to previous studies, based on multigene and 16S rRNA gene phylogenies, we were able to deduce that thylakoid arrangements exhibit notable instability and are not prone to

convergence, rendering them unsuitable as taxonomic markers for distinguishing cyanobacterial orders, families, and, in some cases, even genera. Regrettably, we concluded that this situation mirrors challenges encountered with other phenotypic traits, such as multicellularity, true branching, and heteropolarity. Our findings provided support for the concept of recurrent convergent evolution of specific cyanobacterial phenotypes throughout their exceptionally extended evolutionary timeline.

- Our research was pivotal in reevaluating the taxonomic system based on the latest genomic data. By utilizing multigene phylogenies, we introduced a contemporary taxonomic framework, which resulted in the establishment of ten new orders and fifteen families. Utilizing the monophyly criterion to all taxonomic ranks above the genus level in our analysis, we have finally been able to suggest the much-anticipated dismantling and division of polyphyletic traditional orders. This was especially significant for the paraphyletic foundational branches of "Synechococcales sensu lato." Although the proposed changes to the taxonomic framework may appear extensive, it's crucial to highlight that the resulting system maintains strong ties to previous taxonomies. Within this context, our current study was crafted to stand as a robust milestone in the journey toward establishing a comprehensive genomic polyphasic taxonomy of cyanobacteria, rather than representing a fundamental shift in the taxonomic paradigm. However, there is still great unresolved diversity in cyanobacteria, either in the described genera and family level as well as cryptic species will probably rise. Similarly, a number of new orders will probably be recognized based both on newly obtained cultures as well as on metagenomic studies. From this point of view, we will continue to contribute to the knowledge of cyanobacterial diversity and try to combine the traditional morphological approach with newly gathered information.

Nonetheless, significant diversity within cyanobacteria remains unresolved, both at the level of described genera and families, as well the probability of discovering new species, genera and families is high. Additionally, it is foreseeable that new orders will be delineated, leveraging insights gained from both recently isolated cultures and metagenomic explorations. Guided by this perspective, our ongoing endeavors will persist in advancing our comprehension of cyanobacterial diversity, with the goal of harmonizing the traditional morphological approach with the expanding repository of newly acquired data. It is also imperative to address the emerging challenge posed by cryptic species.

Another significant challenge in the realm of cyanobacterial phylogeny is the identification of gene sets responsible for specific traits, particularly those linked to morphology, such as formation of filaments, specific cell width and length or other basal features as adaptation of photosynthesis to specific environmental conditions. It remains a pivotal inquiry to ascertain whether these traits are governed by a limited set of gene combinations and sequences or if nature tends to favor a reverse trajectory characterized by gene convergence or horizontal gene transfer. These inquiries have been recently addressed through extensive collaboration with colleagues from Czech scientific institutions and abroad. These efforts are expected to generate a deeper understanding of the mechanisms that have ensured the persistence of a crucial environmental component that has thrived on Earth for billions of years, the cyanobacteria.

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