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Contribution to the taxonomic revision of the order Oscillatoriales

Ph.D. Thesis

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Annotation

With the increased use of molecular data, especially the 16S rRNA gene and the following 16S-23S ITS region, many new species and genera have been described in recent years. At the same time, numerous traditional taxa have been recognized as polyphyletic and subsequently subjected to the revisionary process leading to their smaller size and narrower definition. However, there are many species and genera for which no molecular data are available as they were described long before the invent of the sequencing techniques. Thus, it is difficult to allocate their position in the modern phylogenies and move forward with their revisions. This thesis focuses on several simple filamentous cyanobacteria currently classified in the order Oscillatoriales and on the detail study of their type species. The connection of traditional descriptions with corresponding organism results in possibility to obtain reliable molecular data and study the taxa's relationship with other cyanobacteria. Thus, the reference phylogenetic points were established in this thesis for the traditional genera *Arthrospira* and *Oscillatoria*, and subsequently two new genera *Limnospira* and *Tenebriella* were introduced together with their reference strains. Clarification of the phylogenetic position of the type species of *Oscillatoria*, *O. princeps*, means first step towards resolving the polyphyletic nature of the whole order Oscillatoriales and its taxonomic revision.

Declaration

I hereby declare that I am the author of this dissertation and that I have used only those sources and literature detailed in the list of references.

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List of papers and author's contributions

The thesis is based on the following papers (listed chronologically):

1. Mühlsteinová R., Hauer T., De Ley P. & Pietrasiaak N. (2018): Seeking the true *Oscillatoria*: a quest for a reliable phylogenetic and taxonomic reference point. *Preslia* 90: 151–169.
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Radka Hauerová (RH, née Mühlsteinová) was responsible for strains isolation and cultivation, morphological documentation, molecular methods, and preparation of the manuscript draft including the figures and tables. RH is the first and corresponding author of this paper and in total contributed 70 % of the work.

2. Nowicka-Krawczyk P., Mühlsteinová R. & Hauer T. (2019): Detailed characterization of the *Arthrospira* type species separating commercially grown taxa into the new genus *Limnospira* (Cyanobacteria). *Scientific Reports* 9: 694.
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RH contributed to morphological analyses, molecular methods, as well as manuscript writing, and prepared the figures and plates for the manuscript; the overall contribution was 33 %.

3. Hauerová, R., Hauer T., Kaštovský J., Komárek J., Lepšová-Skácelová O. & Mareš J. (manuscript): *Tenebriella* gen. nov. – the dark twin of *Oscillatoria*.

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Consent of the co-authors

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As a senior author I agree that Radka Hauerová contributed to this manuscript in the above-mentioned extent.

Tomáš Hauer, the senior author:

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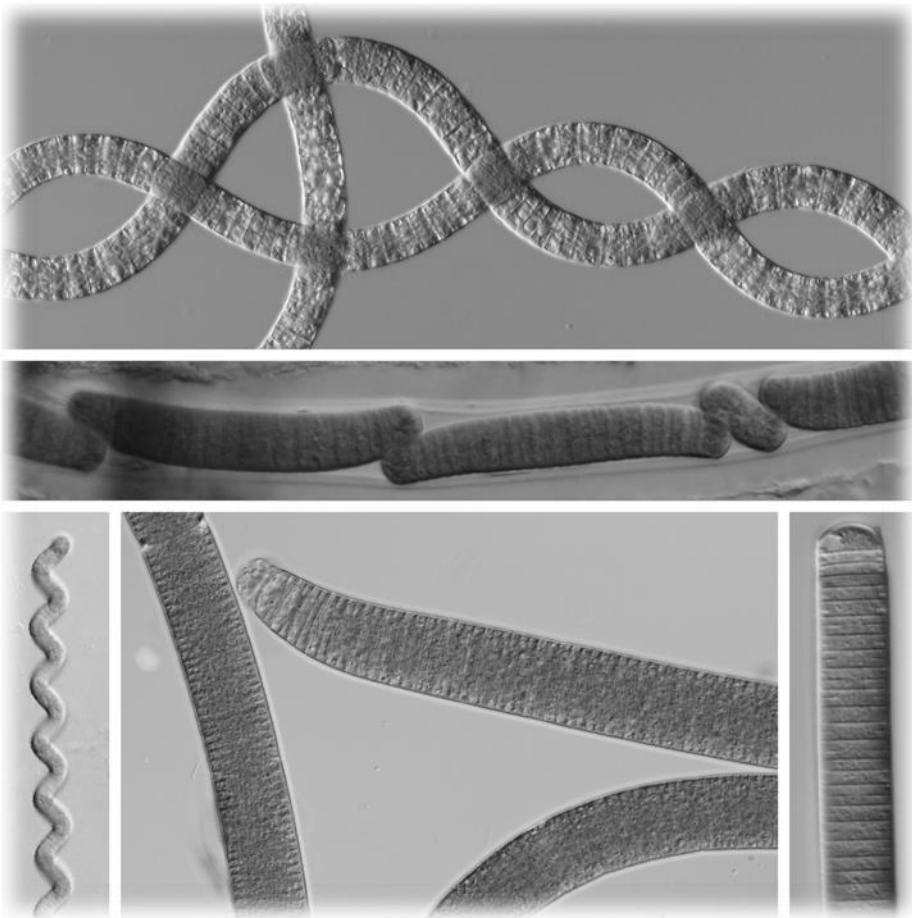
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Chapter 1

Introduction



1. Introduction

1.1 *Cherchez la...cyanobactérie*

Cyanobacteria are photosynthetic prokaryotic organisms that have been around for about 3,500 million years (Schopf and Packer, 1987). Due to their ability to release oxygen through photosynthesis, the life as we know it today could develop. Not only they produced enough oxygen to change the atmosphere composition in a process called the Great Oxidation Event (Whitton, 2012), but through endosymbiosis they enabled the development of eukaryotic photosynthetic organisms such as algae and plants (Keeling, 2013; Ponce-Toledo et al., 2017).

When compared to other bacterial groups, cyanobacteria possess conspicuously variable morphology ranging from tiny naked rods or spheres to multiseriate trichomes covered in thick and colorful sheaths. Their size can reach up to several hundreds of micrometers and they can be visible by naked eye. Besides the ability to create multicellular filaments, some cyanobacteria form also specialized cells devoted to nitrogen fixation – heterocytes (Komárek, 2013; Komárek and Anagnostidis, 1998, 2005). Schirrmeister et al. (2013) suggested that the development of multicellularity in cyanobacteria was an important step on a way to the Great Oxidation Event by allowing cyanobacteria to inhabit wider range of habitats, to diversify, and subsequently to accelerate the amount of released oxygen.

Also today the wide range of cyanobacterial adaptations including the fixation of nitrogen, formation of colonies, production of sheaths, and synthesis of specific compounds and pigments, allow these organisms to inhabit many different environments in various parts of the world (Whitton, 2012). In many of those habitats, cyanobacteria are not only merely present, but play crucial ecological roles. The marine picocyanobacteria such as members of the genera *Synechococcus* and *Prochlorococcus* are among the most abundant organisms on Earth contributing crucially to the primary production in the oligotrophic parts of the oceans (Biller et al., 2015; Flombaum et al., 2013). On various substrates cyanobacteria are among the first pioneering organisms and rare primary producers that are able to survive and participate in nutrient accumulation, and thus potentially allow further colonization. This is the case for example in desert soils (Flechten et al., 2008; Schwabe, 1960; Řeháková et al., 2007), on rock walls and caves (Hauer et al., 2015), as well as in polar regions and high

mountains (Čapková et al., 2016; Kaštovská et al., 2005). And of course, cyanobacteria are crucial part of freshwater phytoplankton of ponds and lakes, where they usually attract the most attention among public by their ability to create extensive water blooms generally accompanied by the presence of toxins (Briland et al., 2020; Kust et al., 2020; Plaas and Paerl, 2021). To simply sum up the worldwide distribution of cyanobacteria in only a few words, one can use part of Nick Cave's lyrics: *"From the deepest ocean to the highest peak..."*.

1.2 *Cyanobacterial nomenclature*

Traditionally, due to their ability to photosynthesize, cyanobacteria were classified together with algae under the botanical approach, often named as blue-green algae. More than 40 years ago Stanier et al. (1978) suggested classification of cyanobacteria among other bacteria based on the prokaryotic nature of their cells. This resulted in their partial separation from the botanical system to newly established bacteriological one and thus, currently, there are two approaches to the classification of cyanobacteria:

The bacteriological approach follows the International Code of Nomenclature of Prokaryotes (Parker et al., 2019), where new taxa need to be described in the International Journal of Systematic and Evolutionary Microbiology (formerly International Journal of Systematic Bacteriology or International Bulletin of Bacteriological Nomenclature and Taxonomy) and with the living culture of the given organism representing the nomenclatural type for newly described species. According to the Prokaryotic Code, the scientists describing new taxa need to avoid names used under either the International Code of Zoological Nomenclature or the International Code of Nomenclature for Algae, Fungi, and Plants (Oren, 2011; Parker et al., 2019). This all makes describing new cyanobacterial species under these rules quite complicated, and thus, most of the phycologists dealing with the nomenclature and systematics of cyanobacteria are working under the botanical approach.

The botanical approach follows the rules summed up by the International Code of Nomenclature for Algae, Fungi, and Plants (Turland et al., 2018). In contrast with the Prokaryotic Code, no particular journal is designated for publishing new taxa, and the nomenclatural type must be represented by an organism in a metabolically inactive state (often herbarium specimen); to use living culture as a type is not possible. By being independent of zoological and prokaryotic nomenclature, the Botanical Code accepts names of taxa described

under these codes (Turland et al., 2018). From historical reasons the majority of cyanobacterial taxa is described under the Botanical Code and mostly they continue to be described under this set of rules still today. However, several exceptions exist, such as genera *Planktothricoides* (Suda et al., 2002) or *Halospirulina* (Nübel et al., 2000) for example that were described under the Prokaryotic Code. Nevertheless, within this work the rules of the Botanical Code were followed.

1.3 *Cyanobacterial species differentiation and definition*

Regardless of the different systems and set of rules used for the nomenclature of cyanobacteria, species serves as the basic category in a taxonomic system. For a long time, the morphological characteristics were the only ones used for taxa species. However, with the development of electron microscopy, use of molecular methods, as well as with the bacteriological approach to the cultured cyanobacteria and use of axenic strains, much new data became available during the second half of the 20th century and keeps accumulating today. Thus, many taxonomists gradually adopted the polyphasic approach that has been regarded as critical today for characterization and delimitation of species. This approach considers all the data available including molecular, morphological, ecological, and any other data at hand, and have played an important role in formation of the modern approach to cyanobacterial classification (Anagnostidis and Komárek, 1985; Komárek, 2003; Komárek, 2005; Komárek et al., 2014).

The debate on the species differentiation has been often connected with the debate on the species definition, i.e. the species concept (Castenholz et al., 1992; Dvořák et al., 2015; Johansen and Casamatta, 2005). Johansen and Casamatta (2005) overviewed various species concepts and discussed their applicability to cyanobacteria. The authors advocated the use of Monophyletic Species Concept sensu Mishler and Theriot (2000), where species are defined as “the smallest monophyletic groups worthy of taxonomic recognition”. This concept encourages the use of phylogenetic analyses based on the molecular data, but also emphasizes the need of polyphasic approach (Johansen and Casamatta, 2005). According to Dvořák et al. (2015) most of the cyanobacterial species descriptions under the botanical approach in recent years have been based on this species concept. The concept of monophyletic species characterized with the use of polyphasic approach is also followed in this work.

1.4 *Cyanobacterial taxonomy – brief historical overview*

It is probably impossible to date exactly the beginning of the cyanobacterial research, however it is well documented already at the beginning of the 19th century (e.g. Vaucher, 1803). As many other groups of organisms, cyanobacteria were originally characterized mainly based on their morphological traits such as cell and trichome shape, size, and color, sheath properties, movement, branching, etc. Toward the end of the 19th century important phycological works organizing the then available knowledge to more complex units were published and their importance increased as they later became considered nomenclature starting points for certain groups of cyanobacteria. For simple filamentous cyanobacteria it is the work of Gomont (1892), whereas for the heterocytous taxa there are the works of Bornet & Flahault (1886a; 1886b, 1887, 1888). For the non-filamentous cyanobacteria Carl Linné's *Species Plantarum* (Linné, 1753) serves as the starting point, even though these cyanobacteria are not even listed there (Anagnostidis and Komárek, 1985).

During the 20th century the efforts to organize the system of cyanobacteria further continued and very different approaches to the cyanobacterial taxonomy were taken. Probably the most striking difference can be demonstrated by the approach of F. Drouet on one hand, who attributed most of the observed morphological variability purely to the influence of the environment and proposed over simplified system of cyanobacteria with highly reduced number of genera – for example in *Oscillatoriaceae* only 6 genera were left (Drouet, 1968). On the other hand, K. Anagnostidis and J. Komárek advocated “small” (and subsequently numerous) genera representing monophyletic units (Anagnostidis and Komárek, 1985). One of the most important works of the 20th century was performed by L. Geitler, who organized all the then known cyanobacteria to extensive determination key (Geitler, 1932) that was later widely used in various parts of the world.

Since the 20th century cyanobacterial taxonomy has been affected by the development and gradually more common application of new methods such as the electron microscopy allowing observation of cell ultrastructure including the thylakoid pattern, and the molecular methods – particularly the sequence of the 16S rRNA gene (Giovannoni et al., 1988; Nübel et al., 1997; Ris and Singh, 1961; Yarza et al., 2014). As the methods continue to improve, more data have been available, and thus, most of the traditional traits have been constantly re-evaluated and the taxonomic system of cyanobacteria has been continuously

revised. The latest most complex evaluation of the taxonomic system of cyanobacteria was published by Komárek et al. (2014) and Mareš (2018). With the ongoing updates of the system of cyanobacteria, it is not always easy to quickly navigate through all the changes. The databases that are trying to keep up with the current state of knowledge such as Algaebase (Guiry and Guiry, 2021) or CyanoDB (Hauer and Komárek, 2021) play crucial role in summarizing and mediating the newest taxonomic changes to the interested scientists.

1.5 *Cyanobacterial taxonomy – the current state*

Despite the complications brought by the ongoing revisions, the correct taxonomy is important not only for cataloging the diversity of organisms, but also for understanding their ecological requirements, biochemical properties, changes over time and geographical ranges, as well as for bringing the common language to researchers. Even though the last point is currently often not true for the taxa described before the invention of molecular techniques, it is true for the newly described species, and it is the goal towards which the revisions aim to.

During the recent years, the world of cyanobacterial taxonomy has been changed significantly by easier accessibility of molecular data, especially the sequence of the 16S rRNA gene. This gene has been used for more than 30 years to reconstruct cyanobacterial phylogenies and over that time enormous amount of its sequences have been collected in the public databases (Giovannoni et al., 1988; Nübel et al., 1997; Yarza et al., 2014). For the cultured and over all well studied organisms, the sequence adds another piece to the puzzle and provides information about the organism's phylogeny (e.g. Caires et al., 2018a; Mühlsteinová et al., 2014; Osorio-Santos et al., 2014). However, nowadays, the development of high-throughput sequencing and its affordability has allowed obtaining large amounts of sequences directly from the environmental samples and for these organisms the sequence together with ecology is often the only information available (e.g. Pushkareva et al., 2015; Vogt et al., 2019). This makes the credible identification of sequences recovered in metagenomic studies possible only if there already exists reference sequence of recently described or revised taxon. Unfortunately, the amount of available correctly named reference sequences is much smaller than the amount of newly gained environmental sequences (Dvořák et al., 2018).

From the 301 genera listed in Komárek et al. (2014) for only 118 was a 16S rRNA gene sequence of the type species available and for only 62 of them

the sequence originated from the type material (Komárek et al., 2014). The category of taxa for which the 16S sequence of type species is missing includes both little known cyanobacteria described long time ago and rarely seen afterwards such as *Chamaesiphonopsis*, as well as rather well-known genera including for example *Calothrix*. The unrevised taxa for which molecular data are not available thus represent one of the biggest challenges of the current taxonomy (De Clerck et al., 2013). Without connecting the existing cyanobacterial names to their reference sequences, the discovery of the cyanobacterial diversity is slowed down by the repeated necessity to decide, whether the studied organism represents taxon yet undescribed, or taxon already described, but not sequenced. Due to the application of botanical approach to cyanobacterial taxonomy, for many taxa dry type specimens are available in herbaria. In the best-case scenario, the type of all unrevised species would be simply sequenced, and the names would thus be connected directly to their reference phylogenetic points. And indeed, several studies have successfully used the herbaria specimens to obtain the DNA sequence (either of the 16S rRNA gene or the whole metagenome) that then helped to clarify the taxonomic situation of the studied organisms (Dvořák et al., 2020; Mareš et al., 2013; Palinska et al., 2006). Nevertheless, not for all species awaiting revisions the herbarium specimens are available or in a good enough condition to allow the DNA analyses. Unfortunately, in many cases the only clues connecting the currently studied cyanobacteria with the names originating more than a hundred years ago are represented by their description and a drawing. Thus, detail comparison of morphological, geographical, and ecological fit of the available data is then necessary to decide whether it is possible to assign newly gained DNA sequence to an existing name. This is time and energy consuming, however, connecting the old names with current data is crucial for revisions of numerous traditional taxa and moving the cyanobacterial taxonomy forward.

Until recently, cyanobacterial cultures were necessary to obtain sufficient amount of biomass for the DNA extraction and subsequent amplification of the 16S rRNA gene. Regarding the limited cultivability of prokaryotes in general, the molecular assessment of many cyanobacteria was thus not available. However, several years ago, a method allowing to obtain reliably 16S rRNA sequence from just several cells or fragments of filaments of culture-resistant cyanobacteria was introduced (Mareš et al., 2015). This represents yet another important step in moving the taxonomic revisions forward and connecting the morphological and molecular data.

1.6 16S rRNA gene – great but not sufficient

As it follows from the previous paragraphs, the 16S rRNA gene plays an important role in modern cyanobacterial taxonomy. Clear numerical thresholds of the 16S rRNA gene sequence identity were proposed to distinguish among various taxonomic ranks (Yarza et al., 2014); however, it has been emphasized that only values well below the thresholds are informative by indicating presence of different taxa, whereas values above the thresholds are rather uninformative (Casamatta et al., 2005; Johansen and Casamatta, 2005; Yarza et al., 2014). Thus, the comparison of sequence identity is not used alone in cyanobacterial taxonomic studies, but it can add yet another piece of evidence to the polyphasic approach. The 16S rRNA gene has been used to study wide range of cyanobacterial taxa. Nevertheless, multiple studies have recommended to use this gene particularly at the generic level (Komárek et al., 2014; Mareš, 2018), as it does not provide enough resolution for species delimitation, and the resolution of higher taxonomic units based exclusively on the 16S does not correlate well with multilocus phylogenies (Mareš, 2018).

For the molecular delineation of species, the 16S-23S ITS region has been currently widely used (Erwin and Thacker, 2008; Mai et al., 2018; Osorio-Santos et al., 2014). This ITS region can be amplified together with the 16S rRNA gene and it exhibits more variability also among closely related organisms. At first, particularly the conserved hypothetical secondary structures were used to separate species, especially the D1-D1', Box B, and V3 helices (Johansen et al., 2011). Nowadays, also alignments of the ITS region allow phylogenetic comparison of studied taxa as well as assessment of their pairwise p-distances (Engene et al., 2013; Mai et al., 2018; Osorio-Santos et al., 2014). To provide better idea of present variability the identity thresholds have been suggested for the 16S-23S ITS region as well, with recent studies confirming their usefulness in species recognition (Bohunická et al., 2015; Erwin and Thacker, 2008; Mai et al., 2018; Osorio-Santos et al., 2014).

Despite the fact papers describing new cyanobacterial species or even genera are reported on the Web of Science nearly every month, the changes in higher level taxonomy have been much slower. Komárek et al. (2014) used multilocus phylogenies to update the system outlined previously by Hoffmann et al. (2005) and the authors recognized eight cyanobacterial orders: Gloeobacterales, Synechococcales, Spirulinales, Chroococcales, Pleurocapsales, Oscillatoriales, Chroococcidiopsales, and Nostocales. Since their work was

published, only the order Gloeomargaritales has been described (Moreira et al., 2017). Even though the 16S rRNA gene is the most widely represented marker, for delimitation of higher taxonomic levels multiple genes should be used (Mareš, 2018). And even though the phycological field is gradually shifting towards the multilocus analyses and more genomes have been sequenced, we are currently still far away from having data for all the groups that are currently known based on the 16S rRNA analyses (Komárek et al., 2014; Mareš, 2018; Mareš et al., 2019a; Mareš et al., 2019b; Shih et al., 2013). Moreover, data from reference strains are often the ones missing, plus names of many higher taxonomic units are tied to genera and species that have not been revised yet and thus their phylogenetic reference points have not been established so far (Komárek et al., 2014). An example of such complicated taxonomic unit is represented by the order Oscillatoriales of which several members were studied in this thesis.

1.7 *Oscillatoriales*

The order Oscillatoriales traditionally grouped simple filamentous cyanobacteria that do not possess the ability to create any specialized cells such as heterocytes or akinetes (Schaffner, 1922). In the current conception, the very narrow filamentous taxa with parietal thylakoids are characterized within other groups and the order Oscillatoriales includes mostly filamentous taxa with other than parietal thylakoid arrangement together with some coccoid members (Komárek et al., 2014; Mareš, 2018). With regard to the limited morphological characters available for taxa delineation within this order, many genera remain to be revised to obtain their monophyly.

During the last 20 years, multiple revisionary steps have been taken towards the clarification of the relationships in the order Oscillatoriales. One of the most important recognitions, based on the study of cell ultrastructure and limited amount of 16S rRNA sequences, was separation of thin filamentous forms with parietal thylakoids from the order Oscillatoriales to a different order (provisionally called Pseudanabaenales), second was acknowledgement of intermixed nature of filamentous and coccoid forms (Hoffmann et al., 2005). This was further supported by later analyses covering more cyanobacterial genera, even though the polyphyletic nature of Oscillatoriales still remained unresolved (Komárek et al., 2014). To the seven families of coccoid and filamentous types recognized by Komárek et al. (2014), families Desertifilaceae and Laspinemaceae represented by filamentous cyanobacteria have been

recently added (Hašler et al., 2017; Zimba et al., 2021). Thus, there are currently several well-defined monophyletic families within Oscillatoriales such as the newly established Laspinemaceae (Zimba et al., 2021), together with families for which either no molecular data exist or clearly indicate their polyphyly such as for instance Homoeotrichaceae or Oscillatoriaceae (Komárek et al., 2014). The ongoing trend of rather small and narrowly defined monophyletic taxa has resulted in description of numerous new genera and species within this order. On the other hand, it has been shown on members of this group that splitting taxa to smaller monophyletic units can go too far and three out of four *Desertifilum* species described in the past ten years have been recently recognized as a single species (Gonzalez-Resendiz et al., 2019). Nevertheless, in general the polyphyletic nature of present taxa is still the prevailing taxonomic problem within the order Oscillatoriales. This is the case also of the genus to which the name of the whole order is tight – *Oscillatoria*. This genus is among the ones that have been known to be polyphyletic for a long time (Litvaitis, 2002; Suda et al., 2002), and thus not surprisingly the monophyly of the whole order has not yet been attained.

The genus *Oscillatoria* is one of the oldest cyanobacterial genera, as it was described already at the very beginning of the 19th century (Vaucher, 1803). It has been traditionally characterized especially by isopolar growth of simple trichomes, cells always shorter than wide, together with the absence of sheaths, specialized cells, as well as branching (e.g. Komárek and Anagnostidis, 2005). These characteristics were met over the time by various organisms, and thus this genus served partially as a collective taxon reaching over 300 species names that have been accepted taxonomically according to the AlgaeBase in April 2021 (Guiry and Guiry, 2021). The long assumed polyphyly of the genus *Oscillatoria* (Anagnostidis and Komárek, 1988) was confirmed by the first phylogenetic analyses using the 16S rRNA gene (Suda et al., 2002). Suda et al. (2002) demonstrated that the sequence of type species *O. princeps* was on the phylogenetic tree clearly distant from the other *Oscillatoria* sequences used, and even with the limited sampling then available, the polyphyly of this genus was obvious.

As mentioned above, polyphyletic genera are not unusual within the order Oscillatoriales. *Lyngbya* can be used as an example of such genus that has been well-studied. *Lyngbya* bears many morphological similarities to *Oscillatoria* with the presence of the sheath surrounding its trichomes being the main

morphological difference delimiting these two genera. As well as *Oscillatoria*, *Lyngbya* has long been recognized as a polyphyletic with the phylogenetic position of its type species unclear (Caires et al., 2018b; Engene et al., 2010). Nevertheless, the revisions of *Lyngbya* have proceeded in much greater speed than in case of *Oscillatoria*. During the last decade multiple new genera such as *Moorena*, *Okeania*, *Limnoraphis*, *Microseira*, *Capilliphycus*, *Dapis*, and *Neolyngbya* have been separated from *Lyngbya* (Caires et al., 2018a; Caires et al., 2018b; Caires et al., 2019; Engene et al., 2013; Engene et al., 2012; Engene et al., 2018; Komárek et al., 2013; McGregor and Sendall, 2015; Tronholm and Engene, 2019). All these revisions proceed in a direction of splitting off groups of organisms that differ from the type species *L. confervoides*. Despite the availability of sequences named *L. confervoides* in the public depositories, neither of them originates from the holotype or the morphologically corresponding type from the type locality and was not officially declared as the reference sequence. Thus, the phylogenetic position of *Lyngbya*'s type species remains assumed rather than confirmed. Yet, the revisions keep moving forward and numerous clades unrelated to the available *L. confervoides* keep being described as new taxa.

Contrary to *Lyngbya*, the revisions of *Oscillatoria* have proceeded in much slower pace. The sequence of type species *O. princeps* (NIVA-CYA 150) has been available at the public depositories for nearly 20 years (Suda et al., 2002), despite its source locality is quite distant from the type locality described in original Vaucher's (1803) work. Consequently, similarly to *Lyngbya*, the phylogenetic position of the core *Oscillatoria* was rather assumed. The last complex revision of *Oscillatoria* was performed before the wide use of molecular data by Anagnostidis and Komárek (1988), who transferred multiple *Oscillatoria* species based on the combination of morphological, ecological, and ultrastructural features to different existing genera (such as *Phormidium*) or to the newly raised ones (such as *Planktothrix*). Later, Suda et al. (2002) described new genus *Planktothricoides* based on the phylogenetic distance of former *O. raciborskii* to other genera included in their study. Nevertheless, since then, no revisions leading to the description of new genera were connected directly to the genus *Oscillatoria*. Merely they could be connected to the former *Oscillatoria* species transferred previously to other genera such as *Phormidium* (e.g. *O. amphibia* to *P. amphibium* (Anagnostidis and Komárek, 1988)), later to *Geitlerinema* (*G. amphibium* (Anagnostidis, 1989)), and most recently to *Anagnostidinema* (e.g. *A. amphibium* (Strunecký et al., 2017)). Nevertheless,

from the 30 European *Oscillatoria* species listed in the Freshwater Flora of Central Europe (Komárek and Anagnostidis, 2005) at least 25 remain still included in *Oscillatoria*, despite the presence of morphological variability much larger than would be expected within single monophyletic genus (e.g. trichome width ranging from 5 μm to 50 μm).

On the other hand, within the order Oscillatoriales, there are numerous genera that have been carefully described (e.g. *Coleofasciculus* (Siegesmund et al., 2008)) or overall well-studied from various angles including biotechnological use (e.g. *Arthrospira* (Sili et al., 2012)). The nomenclature history of the genus *Arthrospira* is similarly long as *Oscillatoria*'s as it was described also before the Gomont's (1892) starting point (Stizenberger, 1854); however, the history of its use by humans is much longer (Ciferri, 1983). This genus is much smaller as it currently contains 20 taxonomically accepted species (Guiry and Guiry, 2021) and its helically coiled trichomes make it quite distinct from most of the other simple filamentous taxa, despite the spirality of trichomes is not unique solely for this genus - it is an important diacritical feature also for some other genera, for example *Spirulina* and *Halospirulina*. Whereas *Halospirulina* was described relatively recently (Nübel et al., 2000), both of the names *Arthrospira* and *Spirulina* appeared already in Gomont's (1892) work as separate genera. However, later various approaches were taken towards their recognition as Geitler (1932) recognized both of them under the name *Spirulina*, while Bourrelly (1970) united both *Arthrospira* and *Spirulina* with the genus *Oscillatoria* as he did not consider spirality of trichomes as sufficiently distinguishing feature. Surprisingly, Drouet (1968) recognized *Arthrospira* and *Spirulina* as separate genera despite his overall reduction of the cyanobacterial system to only few taxa. He considered the visibility of cross-walls and associated layers of granules as the main delimiting feature (Drouet, 1968). Finally, Anagnostidis and Komárek (1988) in their revisionary work recognized both *Arthrospira* and *Spirulina* separately. This separation was later confirmed also with the use of molecular data that led to description of the new cyanobacterial order Spirulinales, where *Spirulina* and *Halospirulina* currently belong (Guiry and Guiry, 2021; Komárek et al., 2014). The long and confused name history resulted in discrepancy between the commercial name used for the biotechnologically important taxa and their correct taxonomic classification, as most of them are simply called "Spirulina" for commercial purposes. At the same time the type species *A. jenneri* has long been studied only marginally and consequently the phylogenetic position of the whole genus was assumed based

only on the data available for the well-investigated species (Komárek et al., 2014). Thus, despite the impression of genus studied in detail, there were still important information missing from the phylogenetic point of view.

1.8 Aims of the study

The type species and data clearly connected to the type material play crucial role in the revisions of the cyanobacterial system and in linking the data available nowadays with old, traditional names. In my thesis I focused on the important representatives of the order Oscillatoriales and I contributed to their revisions by detail study of their type species resulting in connection of the taxa names to reliable molecular data.

First, I focused on the genus *Oscillatoria* that has long known to be polyphyletic, but the phylogenetic position of its type species remained unresolved, and thus the potential revisions were limited by this missing piece of information. My goal was to establish a firm reference point for future studies by specifying and clarifying the phylogenetic position of the type species of this genus and with the use of polyphasic approach contribute to solving its relationship with other morphologically similar taxa.

Secondly, I focused on the genus *Arthrospira* that has been well studied especially as food source and supplement, but whose type species has been overlooked for a long time as well. The aim was to establish reference phylogenetic point for the type species, *A. jenneri*, and thus contribute to the clarification of the name confusions long accompanying this genus.

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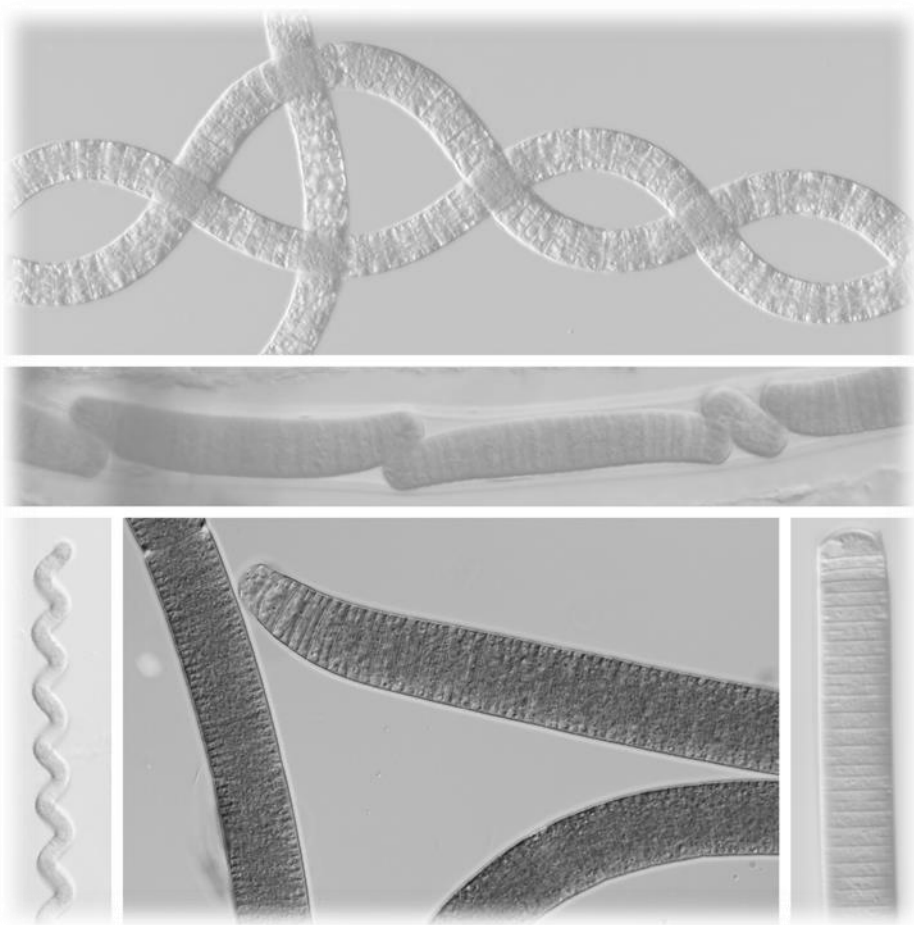
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Chapter 2

Seeking the true *Oscillatoria*: a quest for a reliable phylogenetic and taxonomic reference point

Mühlsteinová R., Hauer T., De Ley P. & Pietrasiak N. (2018), Preslia 90.



Seeking the true *Oscillatoria*: a quest for a reliable phylogenetic and taxonomic reference point

Hledání fylogenetického a taxonomického referenčního bodu pro rod *Oscillatoria*

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Reliable taxonomy of any group of organisms cannot be performed without phylogenetic reference points. In the historical “morphological era”, a designated type specimen was considered fully sufficient but nowadays this principle can prove to be problematic and challenging especially when studying microscopic organisms. However, within the last decades there has been tremendous advancement in microscopy imaging and molecular biology offering additional data to systematic studies in ways that are revolutionizing cyanobacterial taxonomy. Unfortunately, most of the existing herbarium specimens or even iconotypes of old established taxa often cannot be subjects of modern analytic methods. Such is the case for the widely known cyanobacterial genus *Oscillatoria* which was introduced by Vaucher in 1803. In this study, we establish an epitype of the type species *Oscillatoria princeps* and provide an emended generic description based on data obtained from light as well as electron microscopy. We also present phylogenetic relationships of the genus *Oscillatoria* sensu stricto, a well-supported monophyletic clade, to other related cyanobacterial taxa using DNA sequence data of the 16S rRNA gene region. Our epitypification of *Oscillatoria* and the establishment of a phylogenetic DNA benchmark will aid in improving the understanding of cyanobacterial diversity and deeper level taxonomy.

K e y w o r d s: 16S–23S ITS, 16S rRNA gene, cyanobacteria, epitype, *Oscillatoria*, *Oscillatoriales*, rRNA operon variability, taxonomy

Introduction

It has been shown previously for many cyanobacterial taxa that relying solely on morphology does not allow us to reliably represent and understand the actual diversity of these organisms. This is especially true for simple trichal types where morphology does not provide many diagnostic features. A very strong case is represented by the genus *Leptolyngbya*, into which nearly any thin simple trichal cyanobacterial species have been

placed in the past. This resulted not only in a very broad definition of the genus itself, but also yielded many sequences in the public repositories called *Leptolyngbya* despite representing lineages with very different evolutionary origins and fates (Perkerson III et al. 2011, Zammit et al. 2012). A similar story is true for thicker simple trichal cyanobacteria placed currently in the order *Oscillatoriales*. A good example is the genus *Lyngbya*, from which multiple new genera such as *Microseira*, *Okeania*, *Moorea*, and *Limnoraphis* were recently separated (Engene et al. 2012, 2013, Komárek et al. 2013, McGregor & Sendall 2015). A comparable fate awaits another important genus: *Oscillatoria*, which has yet to be considered for a thorough revision using integrative taxonomic approaches.

Oscillatoria represents a historical genus of simple trichal cyanobacteria. As one of very few cyanobacterial genera, *Oscillatoria* is known beyond the taxonomic community as it is familiar to water quality monitoring agencies as well as part of the general biology curriculum, often being represented as one of the typical freshwater cyanobacteria. According to the AlgaeBase database, there are 629 species names listed within this genus and 307 were marked as “currently accepted taxonomically” in February of 2018 (Guiry & Guiry 2018). This makes *Oscillatoria* not just one of the oldest valid names among cyanobacterial genera, but also one of the largest genera in described species richness.

Oscillatoria was first introduced at the very beginning of the 19th century by Vaucher (1803), when he described 12 *Oscillatoria* species and included their drawings, as part of a natural history of freshwater organisms. Seven of these species descriptions were adopted nearly 90 years later by Gomont (1892) and used in his landmark monographic work, later designated as a nomenclatoric starting point for simple trichal cyanobacteria. Whereas half of Vaucher’s original *Oscillatoria* species names are currently accepted from a taxonomic point of view, the second half includes species later recognized as bacterium, green alga or different cyanobacterial genera such as *Lyngbya*, *Microcoleus*, and *Phormidium* (Silva 2017, Guiry & Guiry 2018). The most important species from Vaucher’s (1803) work is *Oscillatoria princeps*, because over two centuries later Geitler (1942) formally designated *O. princeps* as the type species of the genus *Oscillatoria*. In fact, the designation of *O. princeps* as a type species occurred already in Gardner (1932), however, this designation is incomplete since it lacks full citation of the type.

Oscillatoria is currently diagnosed by the noticeable motility of the trichomes, which are composed of cells that are distinctly shorter than wide with a typical “ruler-like” cell division occurring in meristematic zones, and overall simple filaments that lack any branching or visibly specialized cells such as heterocytes or akinetes (Vaucher 1803, Gomont 1892, Anagnostidis & Komárek 1988, Komárek & Anagnostidis 2005). Different *Oscillatoria* species are traditionally distinguished from each other based on the trichome width and the shape of their apical cells (Komárek & Anagnostidis 2005). Because their relatively simple morphology lacks truly unique features, various organisms meet these few criteria and were therefore classified as *Oscillatoria*. This led to the very polyphyletic nature of the genus, which could no longer be overlooked after application of modern methods, specifically with the advent of electron microscopy. Following the detailed morphological and ultrastructural investigation of various species, many were transferred to different genera or gave rise to new taxa. Some examples include *Planktothrix* based on former *O. agardhii*, *Limnothrix* formed around former *O. redekei*, or *Geitlerinema* with former *O. splendida* as a type species (Anagnostidis & Komárek 1988, Meffert 1988, Anagnostidis 1989). All of the above-mentioned genera separated from *Oscillatoria* represent

fairly narrow cyanobacteria with trichome widths under 10 μm . Nevertheless, subsequent phylogenetic analyses based on DNA sequences further showed that at least *Geitlerinema* is still highly polyphyletic (Strunecký et al. 2017). All these revisions suggest that trichome widths of monophyletic cyanobacterial genera have much narrower ranges than traditionally believed, at least for the simple trichal types.

Oscillatoria, as currently defined, still includes taxa with trichome width ranging from 5 μm (*O. rupicola*) to over 70 μm (*O. kawamurae*) (Komárek & Anagnostidis 2005). Considering the findings of the above-mentioned revisions, such a broad range is highly unlikely to represent a single genus. These apprehensions indicated by morphology were fully supported by phylogenetic methods; sequences designated as *Oscillatoria* have been widely spread throughout various published phylogenetic trees. One of the first more thorough phylogenetic studies focusing on various *Oscillatoria* species was performed by Suda et al. (2002). This work led to the recognition that *O. princeps* represented in their study by strain NIVA-CYA 150 has a distant position from other sequences designated at that time as *Oscillatoria* (Suda et al. 2002). Even though numerous new taxa have been recognized by scientists and many of the former *Oscillatoria* sequences have been transferred to different taxa, there are still many sequences and organisms designated as *Oscillatoria* which scatter throughout *Oscillatoriales* and even beyond the order. Additionally, those taxa traditionally placed in the order *Oscillatoriales* for which molecular data are available, form several independent lineages in phylogenies based on multiple genes. Without knowing the position of the type species of *Oscillatoria* it is impossible to decide which one really represents *Oscillatoriales* (Mareš 2018).

To resolve this confusing situation and to reveal a credible phylogenetic position of the genus *Oscillatoria*, as well as update definitions of the higher taxonomic units around it (family *Oscillatoriaceae*, order *Oscillatoriales*, subclass *Oscillatoriophyceae*), the reliable phylogenetic position of the generitype has to be established first. Unfortunately, there is no herbarium specimen available for *O. princeps* and the type itself is represented solely by a drawing of a single filament in the starting point literature accompanied by the morphological description (Gomont 1892). Additionally, drawings and associated morphological observational records are available from the hand of the author of the original pre-starting point publication (Vaucher 1803). Nevertheless, it is impossible to expose these images to any modern methods such as electron microscopy or molecular analyses. Thus, we propose an organism whose morphology and ecology corresponds precisely with the description of *O. princeps* to serve as a reference strain of this species. Based on this strain, we propose an epitope of the genus *Oscillatoria*, which allows us to extend and specify its morphological and ultrastructural description, as well as to reveal its phylogenetic position.

Methods

Natural populations of various *Oscillatoria* morphotypes were collected in Europe and in the south-western U.S. (AZ, CA, NM). Detailed information about the sampling sites is listed in Table 1, along with information on strains obtained from culture collections and the sequences downloaded from public databases (GenBank, EMBL ENA, DDBJ) that were included in our phylogenetic analyses.

The morphology of fresh specimens was evaluated using light microscopy (Olympus BX53 and Zeiss Axioplan). For cultivation 1.6 $\times$ diluted Z-8 medium (Carmichael 1986)

Table 1. – Information about *Oscillatoria* organisms used in our study. Organisms listed above dashed line were studied during this project, for organisms listed below we have data only from literature/databases. Square symbols indicate that large pores in cell wall were observed in ■ electron microscope; □ light microscope; ▣ both electron and light microscope. Besides GenBank or NIVA culture collection information was taken from * <https://sites.google.com/site/centrostudiermalpietrodabano/lista-dei-microorganismi-list-of-microorganisms/ets-06/strain-ets-06>; Moro et al. 2007 (the original but invalid name is *O. duplisecta*). ** <http://www.kahaku.go.jp/research/db/botany/dam/107-1-0.html>; Ichise et al. 1999, Komárek & Anagnostidis 2005.

Organism	Strain / Designation	Trichome width Locality (in the middle)	GPS Coordinates	Collection date	Sample description
<i>O. princeps</i> ■	CCALA 1115	24–36 µm	Lunzer See, Austria	47°51'10.5" N 15° 2'23.6" E	6-7-2015 blue-green mat floating on water surface
<i>O. princeps</i> ■	NIVA-CYA 132 (19)	22–32 µm	Sundlaug, Hveragerdi area, Iceland	not found	April 1983 not found
<i>O. princeps</i> ■	NIVA-CYA 150	23–30 µm	Chao Phya River, Thailand	not found	28-11-1984 not found
<i>O. princeps</i> □	Socorro 3	27–30 µm	Socorro, NM, USA	34°04'05.6" N 106°54'33.4" W	13-7-2016 blue-green mat floating on water surface
<i>O. princeps</i> □	Rainbow Lake 1	30–37 µm	Rainbow Lake, AZ, USA	34°09'28.4" N 109°58'57.6" W	July 2016 bluegreen growth on the mud, submerged shallowly
<i>O. princeps</i> □	Rainbow Lake 2	32–38 µm	Rainbow Lake, AZ, USA	34°09'29.6" N 109°58'57.2" W	July 2016 bluegreen growth on the mud, submerged shallowly
<i>O. princeps</i> □	Verde River 1	42–50 µm	Verde River itself, AZ, USA	34°33'02.1" N 111°51'03.9" W	July 2016 mats floating on water surface
<i>O. princeps</i> ▣	Verde River 2	40–50 µm	small ditch along the river, AZ, USA	34°32'56.9" N 111°51'06.7" W	July 2016 big mat on water surface, held by a tree
<i>O. princeps</i> □	Parker Dam 2	41–51 µm	ditch close to Bluewater, CA, USA	34°10'27.6" N 114°16'31.6" W	3-7-2016 mat on decomposing cattail stems in deeper flowing water
<i>O. princeps</i> ▣	Strkovec	50–60 µm	Lake Strkovec, Bratislava, Slovakia	48°9'30.1" N 17°8'54.8" E	July 2016 blue-green mat floating on water surface
<i>O. princeps</i> □	Rainbow Lake 2 "big"	55–62 µm	Rainbow Lake, AZ, USA	34°09'29.6" N 109°58'57.2" W	July 2016 bluegreen growth on the mud, submerged shallowly
<i>O. princeps</i> *	ETS-06	40–50 µm	Montegrotto Terme, Italy	not found	May 2005 thermal mud
<i>O. kawamurae</i> **	iccl20060001	60–80 µm	Lake Biwa, Otsu, Shiga, Japan	not found	2006 field specimen collected from lake water
<i>O. princeps</i>	SERB 35	not found	India	not found	not found mangrove environment
<i>Oscillatoria</i> sp. not found		not found	Waiahole Valley taro field, HI, USA	30-07-2010	not found

or 5× diluted BG11 medium (Stanier et al. 1971) were used. Once unialgal cultures were successfully established, cultures were observed in different life cycle stages. Similar observations were obtained from culture collection strains. Observed morphology was compared with descriptions in literature (Vaucher 1803, Gomont 1892, Komárek & Anagnostidis 2005). The nomenclature used is according to Guiry & Guiry (2018).

Where possible, genomic DNA was isolated from dried biomass using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The 16S rRNA gene and 16S–23S ITS region were amplified using the primers 359F (Nübel et al. 1997) and 23S30R (Taton et al. 2003). The total volume of the reaction mix was 20 µl and contained 1 µl of gDNA, 10 µl of Plain PP Master Mix Combi (Top Bio, Prague, Czech Republic), 6.6 µl of PCR grade water and 1.2 µl of each primer (concentration 5 pmol · µl⁻¹). The cycling conditions were as follows: 5 min initial denaturation at 95 °C, continued with 40 cycles of 94 °C for 1 min, 52 °C for 45 s, and 72 °C for 2 min, and a final elongation step at 72 °C for 10 min.

For organisms for which culturing was not successful, single trichomes or trichome fragments were picked using a glass microcapillary following the method of Zapomělová et al. (2007) and Mareš et al. (2015). All trichomes were washed in at least 6 drops of sterile TE buffer, placed in sterile 0.2 ml PCR tube and kept in –20 °C prior to molecular analyses. For organisms where only single trichomes were available, direct PCR was applied using primers 16S27F/fD1 (Weisburg et al. 1991, Taton et al. 2003) and 23S30R (Taton et al. 2003) in 50 µl reaction volume. At least 4 PCR tubes per sample were amplified to confirm that at least three independent subsamples have the same 16S rRNA sequence. The reaction mix contained 25 µl of Plain PP Master Mix Combi (Top Bio, Prague, Czech Republic), 1.5 µl of each primer (concentration 5 pmol · µl⁻¹), and 21 µl of PCR grade water. The amplification started with 5 min denaturation at 95 °C, continued with 45 cycles of 94 °C for 1 min 30 s, 54 °C for 1 min 30 s, and 72 °C for 2 min, and final elongation at 72 °C for 10 min.

PCR products were stained with Sybr Green (Lonza, Rockland, ME, USA) and separated on 1.5 % low melting agarose gel (60 V, 1 hour). Bands of interest were excised from the gel and cloned with the standard pGEM®-T Easy vector system (Promega Corp., Madison, WI, USA). The plasmid containing insert was purified from at least three colonies per plate (1 plate ~1 PCR reaction), and then sequenced commercially in both directions using primers T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-TAT TTA GGT GAC ACT ATA G-3'), together with an internal primer CYA781F(a), when needed (Nübel et al. 1997). The sequences were submitted to the NCBI GenBank database (www.ncbi.nlm.nih.gov) under accession numbers MG250647–701 and MG255269–294.

Our 16S rRNA sequences were assembled in Geneious version 9 (<http://www.geneious.com>, Kearse et al. 2012) and subsequently aligned with sequences of other representatives of the order *Oscillatoriales* and other related groups according to the new taxonomic system (Komárek et al. 2014, Mareš 2018). Sequences of *Gloeobacter violaceus* and four members of *Pseudanabaenaceae* were used as outgroups. The 16S rRNA gene sequences were aligned using MAFFT v7 (Katoh & Standley 2013) with default settings. In the final dataset 1–3 16S rRNA sequences per morphotype were used, with the exception of the reference strain CCALA 1115, which was represented by six sequences covering different operon types as well as different sources of genomic DNA (isolation from biomass/direct PCR from washed trichomes isolated from environmental

samples prior to culture establishment). The alignment was checked by eye and minor adjustments were performed in BioEdit 7.2.5 (Hall 1999). Phylogenetic analyses of 1157 bp long alignment included Maximum Parsimony in Mega 7.0 (Kumar et al. 2016), Maximum Likelihood IqTree 1.5.5 (Trifinopoulos et al. 2016), and Bayesian inference in MrBayes 3.2.3 (Ronquist et al. 2012). In the Bayesian analysis, two runs of eight Markov chains were executed for four millions generations with default parameters, sampling every 100 generations (the final average standard deviation of split frequencies was lower than 0.01), while the first 25% of sampled trees were discarded as burn-in. The maximum likelihood analysis was performed using general time reversible model + invariant + gamma (GTR+I+ Γ) on the basis of Akaike Information Criterion values given by jModelTest2 (Darriba et al. 2012). In Maximum Parsimony the gaps were treated as missing data. The relative support of branches was determined in Maximum Parsimony and Maximum Likelihood by 1000 bootstrap pseudoreplicates. The confidence metrics produced with Maximum Parsimony and Maximum Likelihood phylogenies were mapped on the tree topology gained from Mr. Bayes.

For observations with the electron microscope selected cultured strains and specimens from natural collections were preserved with mixture of 2.5% glutaraldehyde and 0.1 M cacodylate buffer and subsequently postfixed with 2% osmium tetroxide. The material was then dehydrated in an acetone series (30, 50, 70, 80, 90, 95 and 100 %). For TEM the material was embedded in Spurr's resin (Spurr 1969). Ultrathin sections (70 nm) were placed on formvar-coated grids, contrast-treated with uranyl acetate and lead citrate, carbon coated, and subsequently observed in a JEOL TEM 1010 microscope. For the SEM procedure, the acetone from the dehydration series was replaced by carbon dioxide through critical point drying. The specimens were then mounted on stubs, sputter-coated with gold and observed in a JEOL JSM-7401F microscope.

Results

Vaucher (1803) described *O. princeps* from large flakes floating on the water surface in a pond located in Crevin, a village close to Geneva. He stated that this species was the largest he knew, with filaments recognizable by the naked eye and a diameter slightly thinner than half of a hair. He also pointed out that the ends of the filaments possessed a different morphology than its middle and distinguished a “head” and “tail” of the *Oscillatoria* “animal” (Vaucher 1803). Gomont (1892) did not consider *Oscillatoria* to be an animal to any further extent and provided a more detailed description contrary to the one mentioned above. The trichome width is indicated as (16) 25–50 (60) μm , the ends are described as slightly narrowed and curved with no calyptra. Granulation can be fine or coarse, but is never located on cross-walls. An important addition also was the ecological information that *O. princeps* can be found worldwide with the exception of cold areas (Gomont 1892).

The morphology and ecology of our strain collected from Lunzer See in Austria correspond closely with both of the descriptions above. The trichome width observed in the fresh sample (24–35 μm) falls well within Gomont's range and this trait does not change much under culture conditions. The overall morphological comparison is shown in Fig. 1. The emended description of the genus *Oscillatoria* and its type species are as follows:

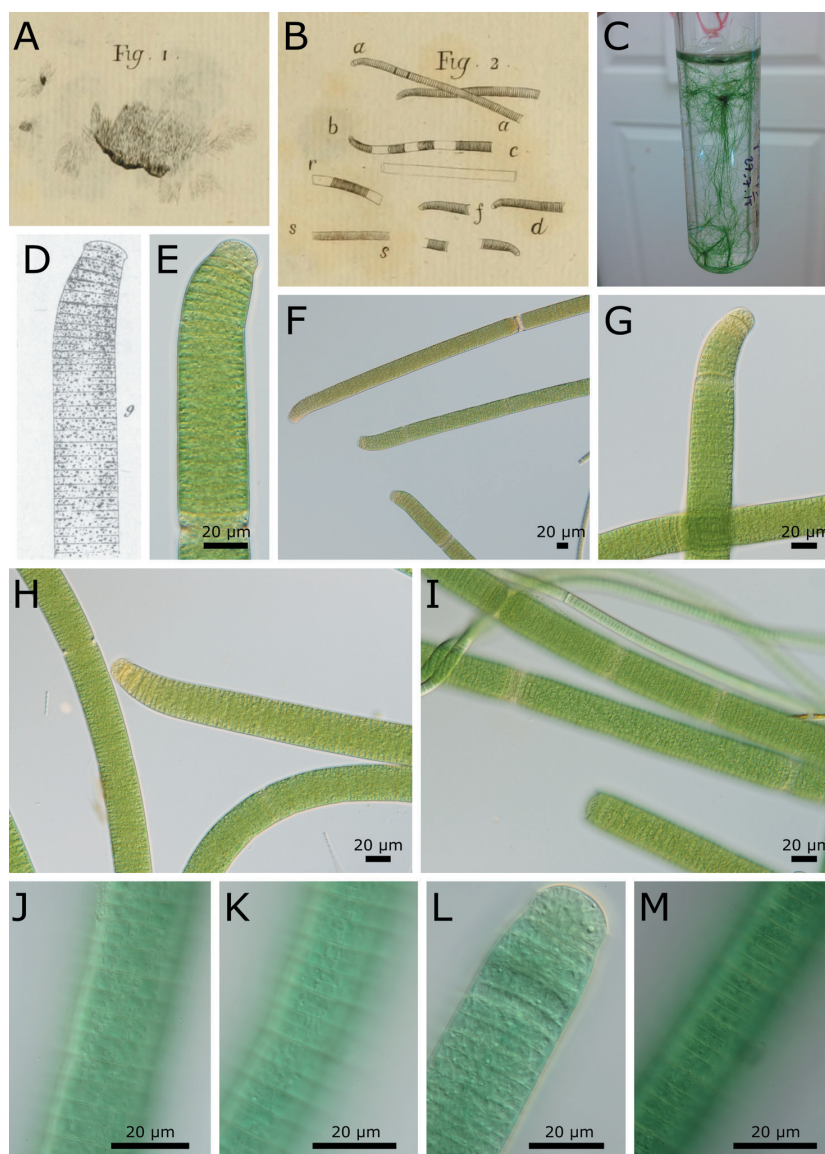


Fig. 1. – Morphology of the reference strain *Oscillatoria princeps* CCALA 1115 and its comparison with original drawings. (A) Macroscopic mat formed by *O. princeps* (Vaucher 1803). (B) Morphology of *O. princeps* as viewed in light microscope (LM) (Vaucher 1803). (C) Macroscopic view of filaments of strain CCALA 1115—single filaments are visible by naked eye. (D) Original drawing of *O. princeps* from starting point (Gomont 1892). (E–I) Morphology of *O. princeps* CCALA 1115 from nature, viewed in light microscope (LM). (J–M) Morphology of *O. princeps* CCALA 1115 in culture after (J) 8 weeks; (K–L) 2 weeks, and (M) 7 weeks in liquid medium as viewed in LM.

Class: *Cyanophyceae*

Order: *Oscillatoriales*

Family: *Oscillatoriaceae*

Genus: ***Oscillatoria* Vaucher ex Gomont (1892) emended description**

Morphology: The overall trichome morphology is shown on Fig. 2. Trichomes blue-green to brownish-green, occasionally purple when old, highly motile, not or slightly constricted at the cross-walls, sometimes narrowing toward the often bent ends, central section of trichomes (19) 22–80 µm wide. Apical cells rounded and sometimes yellowish (together with several adjacent cells). Granulation never located at the cross-walls, but fine to large granules may be present in the cells. Cells much shorter than wide, new cell walls forming perpendicularly to the trichome axis, often before the previous division is finished. Cell wall colourless and thick, necridic cells present, sheath observed only in culture under stress conditions. No calyptra observed.

Ecology: Blue-green mats form on the bottom of lakes, ponds, and maybe rivers, later in the season they release and float at the water surface. The genus is probably distributed worldwide outside of cold regions.

Ultrastructure: The whole genus seems to be well characterized by presence of large pores perforating its thick cell wall. These pores are conspicuous under both TEM and SEM (Fig. 3) and in some of the very large representatives with trichomes above 40 µm, their presence can be detected also by the means of light microscopy; when focused on the very surface of the filament, the cell wall has an appearance that is slightly reminiscent of a snakeskin (Fig. 4). We were not always able to observe the “snakeskin effect” in the light microscope on all the representatives that we had available, but even for the thinner specimen the pores were clearly visible in the electron microscope (Table 1).

Phylogeny: The phylogenetic position of the genus is based on the newly designated epitype CBFS A-89-1 originating from the strain *O. princeps* CCALA 1115. The phylogenetic placement of the genus *Oscillatoria* in the order *Oscillatoriales* and its relationship to other cyanobacteria included in its close proximity is shown in Fig. 5. Whereas the phylogenetic support for the genus itself is steadily high across all the analyses that were performed, its relationship to other simple trichal genera remains unclear. Within the analysed data that we have available, the genus *Oscillatoria* stands alone with no evident sister group.

Type species: *Oscillatoria princeps* Vaucher ex Gomont (1892) – emended description based on the reference strain

Morphology: Comparison of the reference strain’s appearance with the original publications is shown on Fig. 1. Trichomes blue-green, highly motile, not constricted at the cross-walls, narrowing toward the often bent ends, 24–36 µm wide in the central area of the trichome and 14–27 (30) µm wide at the ends. Apical cells nearly hemispherical and often yellowish (together with up to five adjacent cells). Granulation never located at the cross-walls, but fine to larger granules randomly dispersed throughout cells. Cells 2–9 µm long, new cell walls form from the outside of the trichome often before the previous division is finished. Cell wall colourless and thick, necridic cells present, no sheath or calyptra observed.

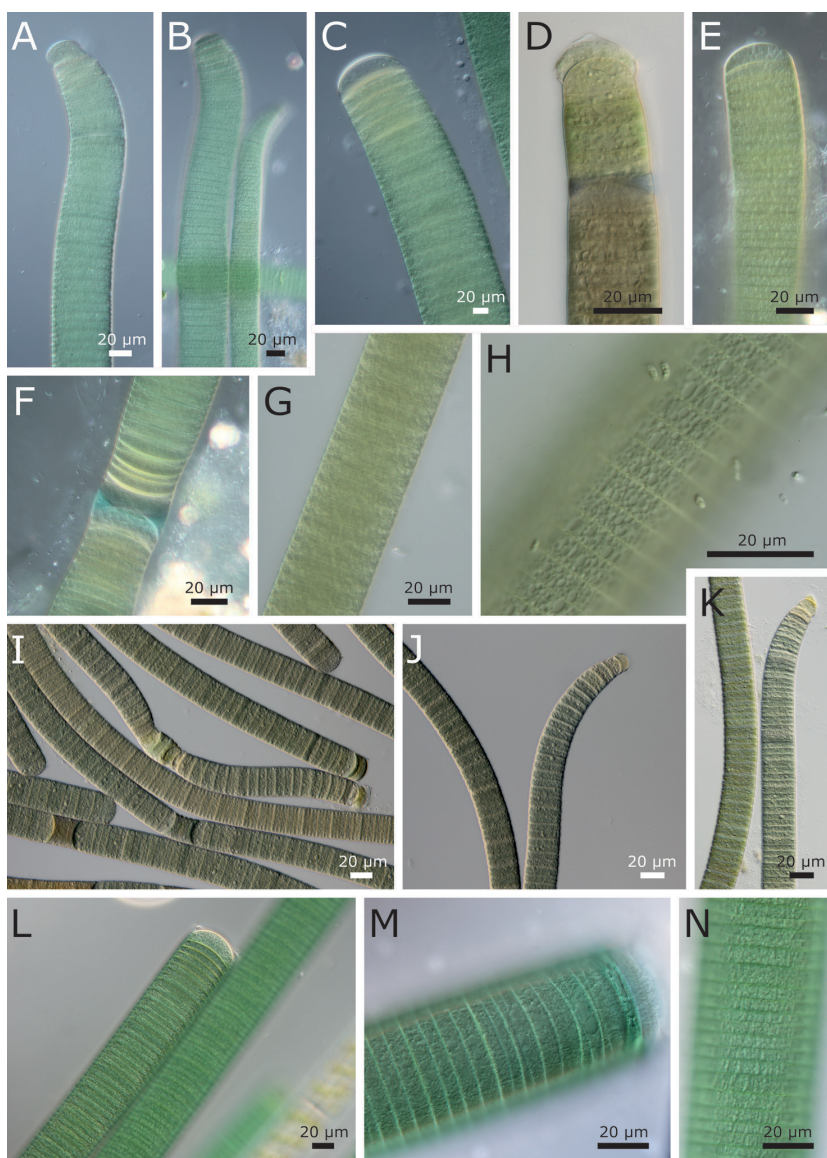


Fig. 2. – Morphology of different strains/fresh samples of *Oscillatoria* as viewed in light microscope. (A–C) *O. princeps* Rainbow Lake 2. (D) *O. princeps* NIVA-CYA 150. (E–H) *O. princeps* Parker Dam 2. (I–K) *O. princeps* NIVA-CYA 132. (L–N) *O. princeps* Strkovec.

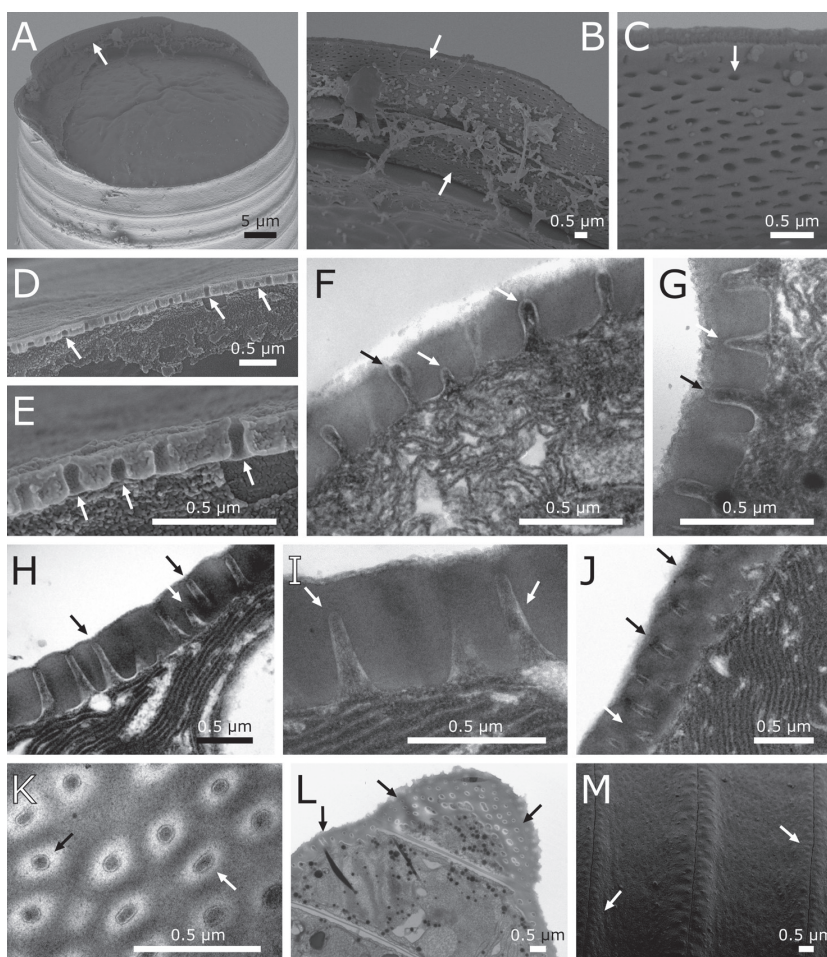


Fig. 3. – Large pores in thick cell wall of *Oscillatoria* visualized by the means of TEM and SEM. Arrows point out the position of the pores. (A–C) *O. princeps* Strkovec, SEM. (D–E) *O. princeps* CCALA 1115, SEM. (F–G) *O. princeps* CCALA 1115, TEM. (H–K) *O. princeps* Verde River 2, TEM. (L) *O. princeps* NIVA-CYA 150, TEM. (M) Surface of the trichome of *O. princeps* Strkovec, SEM.

Ecology: Blue-green mats first probably growing on the bottom of fresh-water reservoirs, later often released and floating at the water surface.

Holotype: Gomont 1892, Ann. Sci. Nat. Bot. ser. 7, 16, plate 6, fig. 9 (type illustration, reproduced in Fig. 1D in this paper).

Epitype here designated: CBFS A-89-1, desiccated strain CCALA 1115 deposited in Herbarium of the University of South Bohemia, České Budějovice, Czech Republic (CBFS).

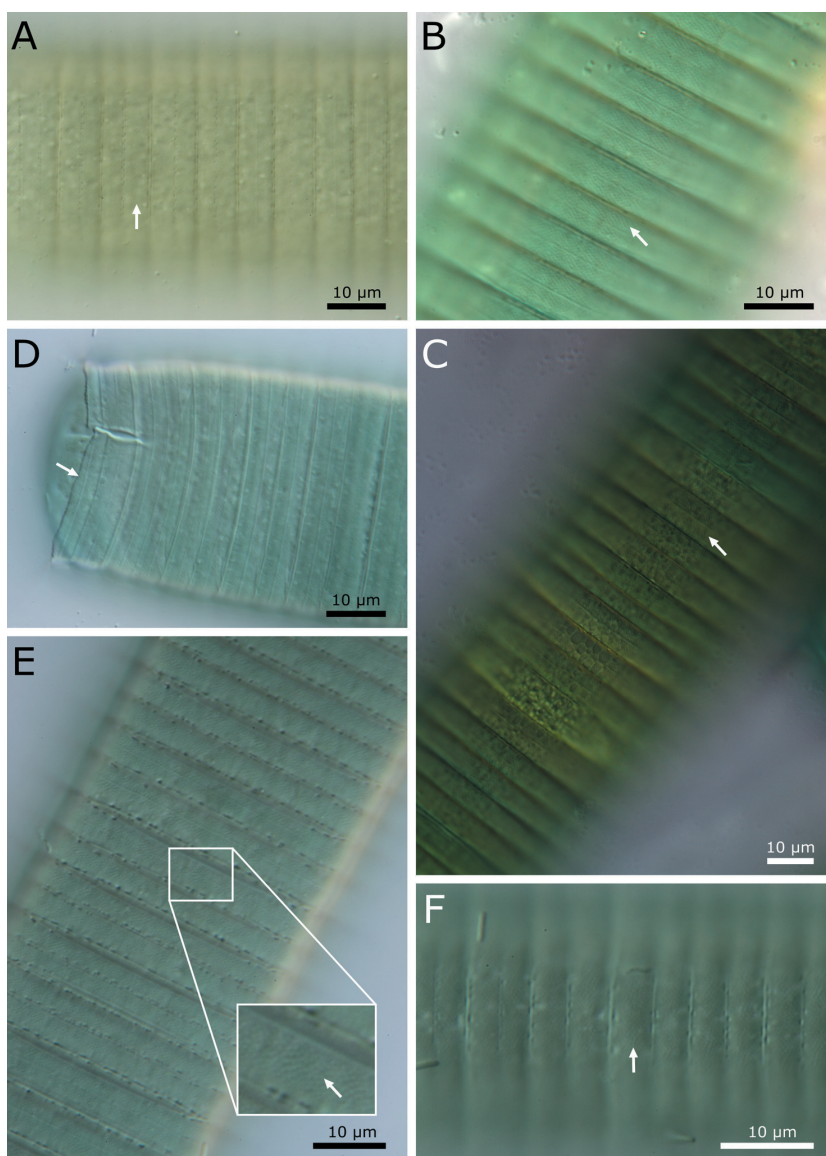


Fig. 4. – Detailed morphology of the surface of selected *Oscillatoria princeps* representatives visualized in light microscope. The presence of large pores in the thick cell wall can be noticed as roughly structured surface (arrows). (A) *O. princeps* Verde River 1. (B–C) *O. princeps* Strkovec. (D–F) *O. princeps* Verde River 2.

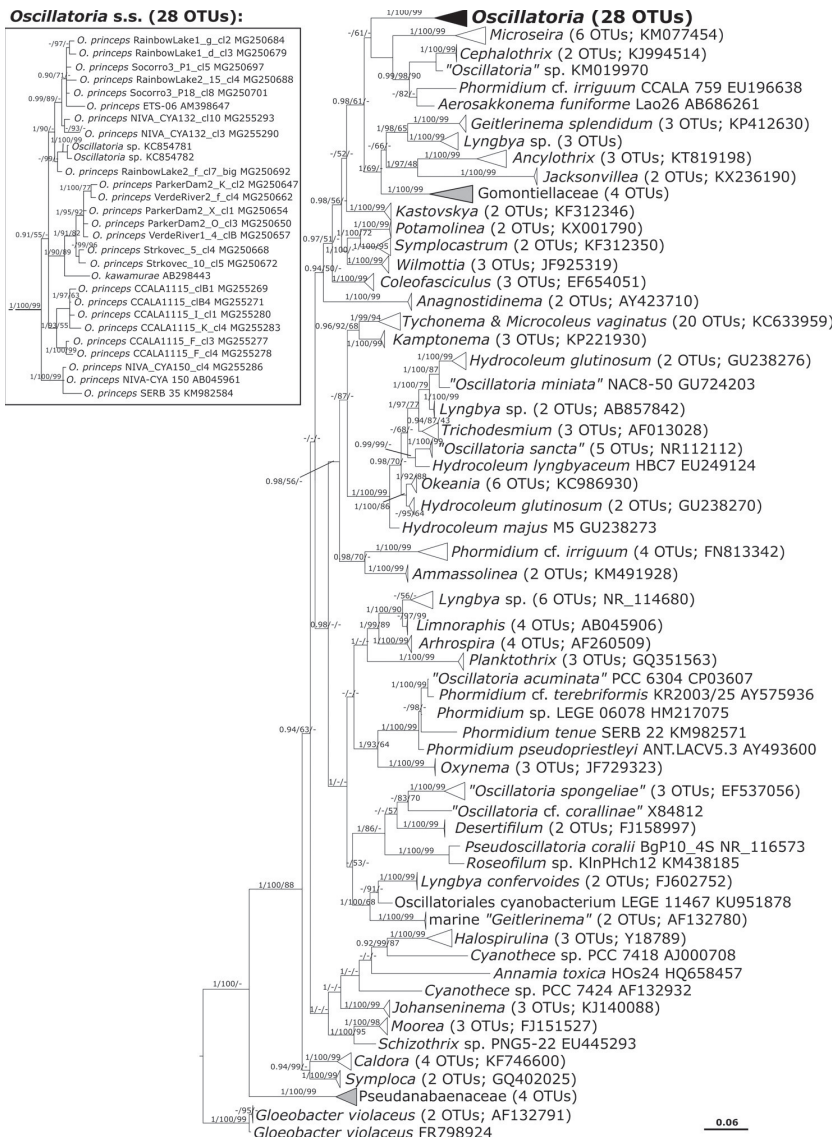


Fig. 5. – Phylogenetic analysis based on 184 sequences of the 1157 long fragment of the 16S rRNA gene. The position of the genus *Oscillatoria* is indicated by the black triangle. Branch support values are in following order: Bayesian posterior probability, Maximum Likelihood, and Maximum Parsimony bootstrap values (values smaller than 0.9 or 50 are not shown). For collapsed groups, usually representing genera, there are number of OTUs and a reference accession number indicated in brackets. For collapsed families only the number of OTUs is indicated. The scale bar represents number of nucleotide substitutions per site.

Reference strain: CCALA 1115, in culture collection CCALA, Třeboň, Czech Republic.

Based on the available data and molecular evidence, the genus *Oscillatoria* as redefined here contains only two species. Besides *O. princeps* the second species is *O. kawamurae*. The strains we had available for this study differed noticeably by their trichome widths and by the geographical and ecological origin (Table 1). However, the patterns in the molecular data were not congruent with the other data (morphology, ecology, etc.) we obtained, and thus, we use the name *O. princeps* for all of them. In total, we obtained 83 sequences containing the 16S rRNA gene with the subsequent 16S–23S ITS gene region. We sequenced 11 different morphotypes (Table 1) which yielded 81 sequences. Two additional sequences containing the ITS region were found on GenBank, representing *Oscillatoria* sp. collected in Hawaii (KC854781, KC854781). These 83 sequences originated from 12 cyanobacterial morphotypes and represented 14 different operons. Only operons with either both tRNAs (Isoleucine and Alanine) or without tRNAs were detected. For ITS regions lacking tRNA only three different operon types were revealed, while operons containing both tRNAs represented 11 different operon types (Electronic Appendix 1). Such high operon variability prevented us from thoroughly evaluating the intrageneric variability within the genus *Oscillatoria*. Specifically, in our study, only 1–3 morphotypes were represented within most of the 14 various operon types. The only exception was among the tRNA lacking operon types, where five morphotypes shared the same operon type (Electronic Appendix 1). Thus, more data is needed for credible conclusions towards unravelling the intrageneric variability; knowing the DNA sequence data of the same operon types for all morphotypes would aid in understanding the relationships of the likely species.

Discussion

The genus *Oscillatoria*, as defined here with the support of molecular sequence data, contains currently only two species – *O. kawamurae* from Japan, with prominent dark granules in its cells, and *O. princeps*. The latter species is despite our efforts still rather broadly defined. Even though we were able to establish a reference strain for the newly defined epitype, we were not able to clarify its relationship with all of the other strains and morphotypes we had available. One possibility is, that *O. princeps* is more or less cosmopolitan with the exception of cold areas, representing a fairly variable species with trichome widths expanding over a wide range; this would after all correspond with the original, rather broad definition by Gomont (1892). The second, in our opinion much more probable option, is that the organisms used in our study and so far named *O. princeps* (Table 1) represent multiple species, very likely new to science. Unfortunately, the high variability of ribosomal operons and incongruence with morphological and ecological data prevents us from satisfactorily addressing this question in the current paper.

Among the cyanobacteria currently ranked as *O. princeps* in our dataset, there are organisms derived from various areas of the world, including Europe, North America, Thailand, and Iceland (Table 1). In regards to their relatively simple morphology, the most profound feature is the trichome width. It spans from 22–62 µm for the entire group, but individual strains or natural populations seem to be defined by a much narrower span

of approximately 10 μm , which could suggest that multiple species are represented. Unfortunately, not all of the organisms could be cultivated and observed over a longer period of time. From previous experience and the comparison with the literature, we know that the morphology itself does not yield sufficient information for delimiting cyanobacterial species and other lines of evidence need to be taken into consideration. Recent works demonstrated that the 16S–23S ITS region can be used as a reliable tool for understanding the species variability (Osorio-Santos et al. 2014, Pietrasiak et al. 2014). Surprisingly, the ITS parts of rRNA operons we recovered during our study showed an unusually high variability. The 16S–23S ITS sequence reads of the 12 different morphotypes grouped into 14 different operon types (Electronic Appendix 1). Thus, it became virtually impossible to compare different morphotypes with each other, because most of the operon groups were represented by only one to three morphotypes.

Another taxon name, that appeared among the published sequences falling into the phylogenetic clade of *Oscillatoria*, referred to an organism described from thermal springs in Italy as *O. duplisecta*, a species rediscovered and sequenced by Moro et al. (2007). Gomont (1892) synonymized *O. duplisecta* with *O. princeps*, whereas Moro et al. (2007) proposed that this morphotype should be recognized as a valid species. However, the new species description was not presented correctly according to the International Code of Nomenclature (McNeill et al. 2012) due to the missing specification of a *typus*. Furthermore, the documentation of morphological features of the material analysed by Moro et al. (2007) is partially contradictory; Fig. 1 (light microscope) in their work shows constrictions at cross walls, on the other hand, no constriction is noticeable in Fig. 4 (TEM) in the cited paper. Further analyses of the material are needed in order to properly reestablish the species *O. duplisecta* as valid. Thus, in this study, we use the name *O. princeps* for their sequence data.

Even though the species delineation of *O. princeps* is not completely clear yet, we have clarified here the molecular delimitation and phylogenetic position of the genus. The morphological, ecological, and geographical data we were able to collect are congruent between our reference strain and Gomont's (1892) description. Suda et al. (2002) previously suggested that from all the taxa used in their study, only the strain NIVA-CYA 150 should be used as a reference point for the genus *Oscillatoria*. Since this strain is undoubtedly closely related to the reference strain CCALA 1115 here established as an *epitype*, it further supports our finding that this clade represents the trustworthy phylogenetic position of the genus *Oscillatoria*. We have not chosen the strain NIVA-CYA 150 as an *epitype* because we believe, that the *epitype* should have as near as possible geographical origin and habitat properties as those specified in the original description. We actually visited the locality close to Geneva, from which Vaucher (1803) described *O. princeps*. Unfortunately, a large part of the local landscape was turned into a golf course. The trophic level of the water bodies in that area may well have increased significantly since the 19th century, as we were not able to find any cyanobacterium corresponding morphologically with *O. princeps*. We came across large floating mats on the surface of Lunzer See in Austria within the same week. Even though Lunzer See is nearly 700 km far away from the type locality, it is connected by the same mountain range, similar climate, and limestone subsoil (www.geoportail.fr, www.geologie.ac.at). It is much closer to the place of the original occurrence than any other relevant strain currently available.

Besides the molecular data, the whole genus also seems to be well defined by the presence of remarkably large pores in the thick cell wall (Fig. 3). The unusual thickness and perforations of the cell wall do not constitute new information – *O. princeps* has long been known to possess this specific cell wall structure observable by the means of electron and atomic force microscopy (Ris & Singh 1961, Halfen & Castenholz 1970, 1971, Kurk et al. 2010). However, according to our knowledge, no one has reported that the presence of these pores also can be noticed, at least for sufficiently thick morphotypes, with the light microscope (with differential interference contrast). Of course, the detailed structure of the pores is not visible in the light microscope, but when focused on the very edge of the filament, the surface slightly resembles snakeskin due to the traces of the deeper structure (Fig. 4). Over the past 56 years these large pores have not been detected in any other cyanobacterium studied than the ones determined as *O. princeps* (e.g. Ris & Singh 1961, Hoiczky & Baumeister 1995, Kurk et al. 2010). It was hypothesized that the pores play a crucial role in the movement of this organism (Halfen & Castenholz 1971). Anagnostidis & Komárek (1988) considered pores in cell walls as a potential diacritical feature; however, they saw a discrepancy between the ultrastructural characterization of *O. princeps* and *O. limosa* Agardh ex Gomont which they considered to be close to each other. Nevertheless, from the molecular investigation (Fig. 5) we know that *O. princeps* stands alone and the morphotypes identified so far as *O. limosa* represent different clades not closely related to our proposed diagnosis of *Oscillatoria* sensu stricto. Thus, we believe that the large pores can serve as an important diacritical feature distinguishing the genus *Oscillatoria* from other simple trichal morphotypes.

Lyngbya, a genus similar to and traditionally considered as a close relative to *Oscillatoria*, has undergone many revisions over the past few years. Many new genera were separated and these newly cleaved taxa such as *Limnorphis*, *Microseira*, *Moorea*, or *Okeania* do not seem to be closely related to each other (Fig. 5). *Oscillatoria* has not gone through such revision work yet, but a similar fate is very probable. We approach the revision of *Oscillatoria* from a different but no less important direction by defining clearly what the core species of *Oscillatoria* sensu stricto is and how to recognize it with up-to-date diagnostic methods.

In this study, we propose an epitype of *Oscillatoria* and use the molecular data from the reference strain to introduce a well-defined phylogenetic anchor point for the truly monophyletic genus (Fig. 5). The clade representing the newly defined genus *Oscillatoria* was well supported in all the phylogenetic analyses we performed; however, its precise position on the 16S rRNA tree was dependent on the selected algorithms as well as on the selection of sequences. While certain groups of taxa (such as *Arthrospira*–*Limnorphis*, *Microcoleus vaginatus* group–*Kamptomena*, or *Okeania*–*Hydrocoleum/Trichodesmium* group) hold together regardless of the criteria mentioned above, positions of many other genera (including besides *Oscillatoria* also *Kastovskya*, *Anagnostidinema*, *Ammassolinea*, or *Desertifilum* for example) noticeably change among individual analyses. This suggests that there are still many missing taxa in the phylogenetic tree, for which we will need to fill in the information first. While 16S rRNA gene sequences are now available for a wide variety of cyanobacteria, many more species and genera are so far defined solely on morphology, and countless taxa remain yet to be discovered (De Clerck et al. 2013). Until we fill in these blanks, it is premature to define new families and other higher taxonomic units, especially when the analyses are based

solely on a single gene (Mareš 2018). Thus, we are reluctant to redefine the family *Oscillatoriaceae* in this study, as that would likely create even more confusion than there already is. For example, the recently established family *Desertifilaceae* (Hašler et al. 2017) seems to be very artificial. Hašler et al. (2017) based the family on genera *Desertifilum* and *Jacksonvillea*, whose sister taxon position is not well supported by the phylogenetic analyses even in the original work (page 288, Fig. 6). In our analyses these two taxa are placed phylogenetically very distant from each other – *Jacksonvillea* is in the vicinity of *Geitlerinema splendidum*, while *Desertifilum* is located in different clade close to *Roseofilum* (Fig. 5). We do not claim this position of these two genera to be final and definitive; nevertheless, we want to point out that the definition of family *Desertifilaceae* does not match the probable relationships of its included taxa.

The epitypification of the genus *Oscillatoria* is an important step allowing us to further understand diversity and variability of simple trichal cyanobacteria, providing a missing key element for deeper taxonomic revisions of the whole family and higher taxonomic units related to it. The next important step will be the sequencing of the *O. princeps*’ genome which will enable a better understanding of operon variability within this genus and also allow multilocus analyses, leading to the more reliable definition of the borders of the family *Oscillatoriaceae* and the order *Oscillatoriales*. Also, many species traditionally belonging to *Oscillatoria* will need to be transferred to different and potentially new genera. To which ones, it is going to be possible to say only after thorough study and revision of each of these species. That includes among others *O. limosa*, a taxon traditionally perceived as the most typical representative of the whole genus next to the type species (Anagnostidis & Komárek 1988). However, the misleading nature of morphological characterization in cyanobacteria has been shown many times previously.

See www.preslia.cz for Electronic Appendix 1

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Souhrn

Botanická nomenklatura vyžaduje pro platný popis taxonu stanovení typové položky jako referenčního materiálu, se kterým je svázáno jméno organismu. V případě sinic mnoho typů existuje pouze ve formě obrázků, v lepším případě jako herbariové položky. S rychlým rozvojem mikroskopických metod a pokroku v molekulární biologii dochází k velkému změně v taxonomii sinic a výlučně morfologická charakterizace je nedostačující. Toto je případ i jednoho z nejznámějších sinicových rodů *Oscillatoria*, který byl poprvé popsán Vaucherem již v roce 1803 a u něž je k dispozici pouze ikonotyp, na který není možné aplikovat moderní metody. Z dostupných dat vyplývá, že rod je v současném pojetí velmi polyfyletický a zahrnuje mnoho nepříbuzných fylogenetických linií. V této práci stanovujeme epityp a referenční kmen typového druhu *O. princeps* a představujeme

rozšířený popis rodu *Oscillatoria*, založený na datech ze světelného a elektronového mikroskopu. S využitím dat pro gen 16S rRNA, získaných z referenčního kmene, také ukazujeme fylogenetický vztah nově definovaného monofyletického rodu *Oscillatoria* sensu stricto k dalším sinicovým rodům tradičně řazeným do řádu *Oscillatoriales*. Stanovení epitypu, jeho zevrubná charakterizace a následné fylogenetické vymezení rodu pomůže lépe poznat diverzitu a zpřesnit systém sinic, ve kterém mnoho taxonů stále čeká na své revize.

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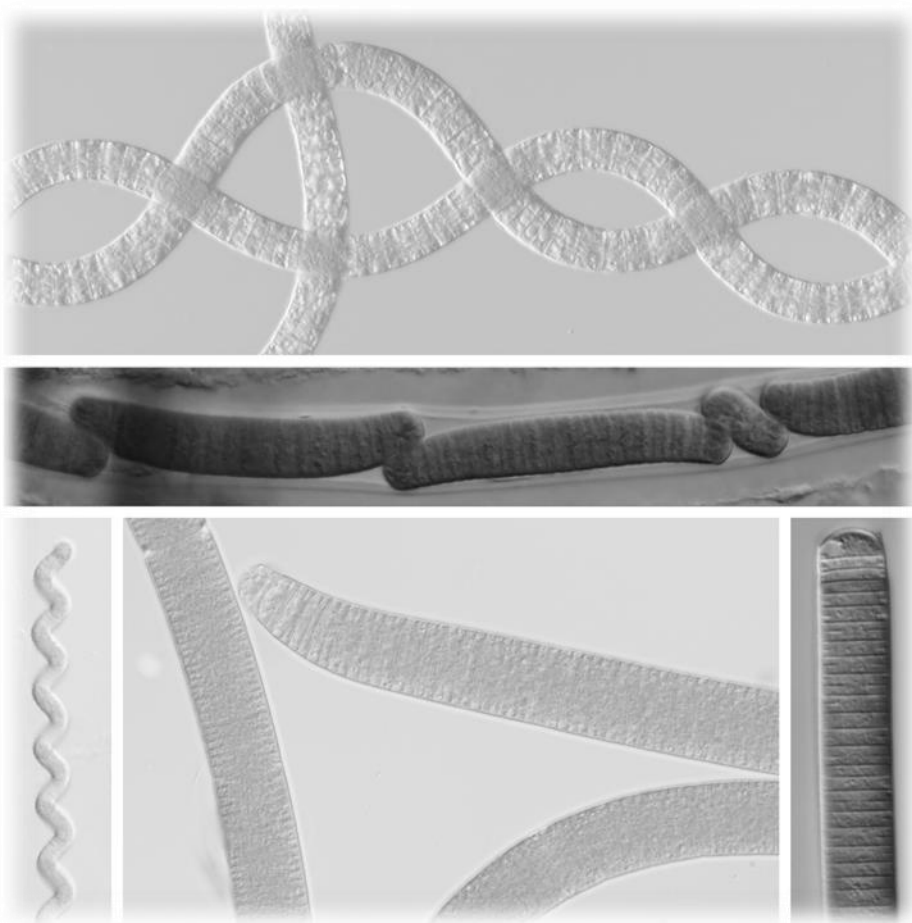
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Chapter 3

Tenebriella* gen. nov. – the dark twin of *Oscillatoria

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Tenebriella* gen. nov. – the dark twin of *Oscillatoria

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Abstract

In this paper we follow up with the revision of *Oscillatoria*, one of the oldest and most widespread cyanobacterial genera. *Oscillatoria* has long been known to be polyphyletic. After recent resequencing of the reference strain for this genus, many *Oscillatoria*-like groups phylogenetically distant from the type species *O. princeps* remained unresolved. Here we describe one of these groups as a new genus *Tenebriella*. Most of the studied strains originate from Central Europe, where they are able to form prominent microbial mats. Despite the overall *Oscillatoria*-like morphology, *Tenebriella* can be distinguished by darker trichomes and forms a separate monophyletic clade in phylogenies inferred from the 16S rRNA gene and two additional loci (*rpoC1*, *rbcLX*). Within *Tenebriella* we recognize two new species differing from each other by morphological and ecological characteristics. First species does not fit any known taxon description, and thus is described as a new species *T. amphibia*. The latter one corresponds with the information available for *Oscillatoria curviceps* Agardh ex Gomont, and thus new combination *T. curviceps* is proposed. The phylogenetic analyses of the 16S–23S ITS region together with the comparison of the hypothetical secondary structures confirmed recognition of these two species and additionally revealed presence of a morphologically cryptic species *Tenebriella* sp. The results corroborate frequent recurrence of convergent morphotypes in the evolution of cyanobacteria and justify further exploration of notoriously sampled European freshwaters using molecular phylogenetics to discover new and ecologically relevant taxa.

Keywords: cyanobacteria, phylogeny, multilocus analysis, ITS, polyphyly

1. Introduction

The 16S rRNA gene has been used in cyanobacterial phylogeny for more than 30 years (Giovannoni et al., 1988). During this time enormous numbers of the 16S rRNA gene sequences have accumulated in public databases allowing comparison of organisms from various environments and parts of the world, and cyanobacterial taxonomy has been quickly transforming. The focus shifted from solely morphological features to the polyphasic approach and the 16S rRNA gene sequence became one of the basic characteristics of revised and newly described cyanobacterial taxa (Dvořák et al., 2015; Johansen and Casamatta, 2005; Komárek et al., 2014; Osorio-Santos et al., 2014). Nevertheless, many of the broadly defined and ecologically widespread traditional genera were described more than a hundred years ago (e.g. *Calothrix*, *Chroococcus*, *Nostoc*, *Phormidium*, etc.), and thus their type species have not been linked to reliable molecular data yet. Their morphological and ecological descriptions together with authors' illustrations are often the only clues connecting the former knowledge with contemporary research. This complicates the decision making when describing new cyanobacterial taxa and it is often not entirely certain whether the studied organism really represents a genus or a species new to science or a taxon described earlier when molecular methods were not yet available. In some cases herbarium specimens are also available, although many of them were lost or destroyed over the time. Those that have been preserved are often too degraded to be used for DNA extraction and allow merely for another microscopy analysis (Bohunická et al., 2015a). However, in some instances, even old herbarium specimens were successfully subjected to 16S rRNA gene (Mareš et al., 2013; Palinska et al., 2006) or even whole metagenome (Dvořák et al., 2020) sequencing. Nevertheless, the attempts to evaluate the relationship of a newly discovered organism to taxa known from literature are generally more laborious and time consuming than description of newly sequenced cyanobacteria as new taxa based simply on the comparison with available 16S rRNA sequences. Yet, this step should not be omitted, otherwise the cyanobacterial taxonomy will not be able to move forward consistently and satisfying revisions of important taxa will never be accomplished.

The importance of various morphological diagnostic features has been re-evaluated multiple times and the use of morphology for cyanobacterial determination has been questioned as a whole. Drouet (1968) believed most of the observed variability can be attributed to the environmental conditions and suggested dramatic reduction of 2 400 cyanobacterial species to only 6 genera.

Despite appealing simplicity his proposed system was not generally accepted. Later on, different ways to understand and design the cyanobacterial taxonomy have been searched for with a common goal of establishing taxonomy congruent with the evolutionary relationships (Anagnostidis and Komárek, 1988; Fjordingstad, 1971; Komárek et al., 2014). With advance of the electron microscopy, molecular methods and gradual shift of cyanobacterial taxonomy from morphological to polyphasic approach, many new genera have been separated from big, often collective, simple filamentous taxa such as *Pseudanabaena*, *Lyngbya*, *Phormidium*, and *Oscillatoria* (Anagnostidis and Komárek, 1988; Chatchawan et al., 2012; Komárek et al., 2013; Meffert, 1988). Some of the morphological and ecological differences previously considered as features merely delimiting species, can gain more weight and importance after comparison to phylogenetic clustering, and subsequently serve as important diacritical features for the whole genera. For example the ability to form gas vesicles helped differentiating *Limnoraphis* from *Lyngbya* (Komárek et al., 2013), the morphology of terminal trichome parts contributed to separation of *Oxynema* from *Phormidium* (Chatchawan et al., 2012), and the shape of apical cell distinguished *Anagnostidinema* from *Geitlerinema* (Strunecký et al., 2017).

The genera newly raised by splitting from a traditional polyphyletic genus are thus usually more narrowly defined in terms of morphology and ecology, and frequently quite divergent in terms of phylogenetic position. Several taxa such as *Limnoraphis*, *Microseira*, *Moorena*, and *Okeania* separated from *Lyngbya* in the last decade can serve as examples (Engene et al., 2012; Komárek et al., 2013; McGregor and Sendall, 2015; Tronholm and Engene, 2019). According to AlgaeBase, all these taxa currently belong to the family Oscillatoriaceae (Guiry and Guiry, 2020), even though they are located in distant branches of the 16S rDNA phylogenetic tree of Oscillatoriales (Mühlsteinová et al., 2018).

The revisions of *Lyngbya* and other similar genera thus proceed in the direction of splitting off the taxa that do not fit perfectly to the core definition of the given genus. Nevertheless, the important question, where the type species of the revised genera are placed in the phylogenetic tree, remains often unresolved—in better cases estimated/assumed. The current state of *Oscillatoria* is improved by having its reference phylogenetic point recently established (Mühlsteinová et al., 2018). *Oscillatoria* has been long known for remarkably wide morphological and ecological variability of its species. In the nomenclatural starting point for simple filamentous cyanobacteria Gomont (1892) listed 38 *Oscillatoria* species with their trichome widths ranging from 2

to 60 µm. At present, only 12 of these species formally belong to *Oscillatoria*, others are classified within different genera such as *Phormidium*, *Planktothrix*, *Anagnostidinema*, *Kamptomonema*, etc. Nevertheless, the simple morphological criteria defining *Oscillatoria* such as isopolar growth, unbranched trichomes, cells shorter than wide, irregular arrangement of thylakoids, and the general absence of sheath and prominent cells such as heterocytes and akinetes, were met by many various organisms over time, leaving us currently with 307 taxonomically accepted species names within this genus (Guiry and Guiry, 2020). The polyphyletic nature of *Oscillatoria* has been long suspected – even before the 16S rRNA sequencing became commonly used, Anagnostidis and Komárek (1988) assumed that multiple narrower taxa will need to be transferred elsewhere. Suda et al. (2002) later found out that the phylogenetic position of the type species *O. princeps* seems to be isolated from other sequenced *Oscillatoria* taxa. This was confirmed recently by a detailed study of the newly established reference strain of *O. princeps* that was used for setting phylogenetic reference point of this taxon (Mühlsteinová et al., 2018). Thus the true *Oscillatoria* is currently defined rather narrowly containing particularly two species from the sequenced ones so far – *O. princeps* and *O. kawamurae*, leaving behind multiple groups morphologically corresponding to the generic description of *Oscillatoria*, but phylogenetically distant from the type species or even lacking any molecular data.

In this work, we would like to introduce one of these “general *Oscillatoria* groups” as a new genus *Tenebriella*. Most of its member strains were isolated from central Europe, where they are able to create prominent floating mats. *Tenebriella*, as currently defined, contains 2 species of which the first was described as new to science (*T. amphibia*) and the second was formed on the basis of *Oscillatoria curviceps* (*T. curviceps*). Nevertheless, more species are certainly going to be found in this genus as illustrated already in the current study by the presence of another putative cryptic species distinguished based on molecular data.

2. Materials and methods

2.1. Material collection and cultivation

Natural populations of *Oscillatoria*-like morphotypes often creating distinctive blue-green mats were collected mostly in the Czech Republic, with some samples originating also from the U.S., Sweden, and Italy. Detailed information about the origin of studied organisms together with the information on related

sequences harvested from the public database NCBI GenBank are summarized in Table 1.

Morphology of fresh and cultured specimens was studied by light microscopes (Olympus BX51, BX53 and Zeiss Axioplan). For cultivation either liquid or solidified media Z-8 (Carmichael, 1986) or BG11 (Stanier et al., 1971) were used. Observed morphological traits were compared with available descriptions in literature (Gomont, 1892; Komárek and Anagnostidis, 2005). The used nomenclature follows Guiry and Guiry (2020).

2.2. Molecular analyses

Genomic DNA was isolated from dried biomass with the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The partial 16S rRNA gene together with the complete adjacent 16S–23S ITS region were amplified using the primers 359F (Nübel et al. 1997) and 23S30R (Taton et al. 2003). The PCR reaction and cycling conditions were used as described in Mühlsteinová et al. (2018). The partial *rpoC1* gene and the partial *rbcLX* genomic region were amplified using the primer combination CW and CX (Rudi et al., 1998) and MF and CR-1 (Seo and Yokota, 2003), respectively, with PCR conditions as follows: initial denaturation at 94 °C for 5 min followed by 36 cycles of denaturation at 94 °C for 40 s, primer annealing at 54 °C for 1 min, and elongation at 72°C for 1 min 40 s; with final elongation at 72 °C for 7 min.

In case the cultivation of organisms of interest was unsuccessful or not providing enough biomass, single trichomes or trichome fragments were prepared for direct PCR by washing in drops of sterile TE buffer according to the method of Zapomělová et al. (2007) and Mareš et al. (2015). The direct PCR reaction was prepared with forward primers 16S27F/fD1 (Taton et al., 2003; Weisburg et al., 1991) or 359F (Nübel et al. 1997), and reverse primer 23S30R (Taton et al. 2003) in the same volume and cycling conditions as described in Mühlsteinová et al. (2018).

The 16S rRNA gene PCR products were stained with Sybr Green (Lonza, Rockland, ME, USA) and separated on 1.5 % low melting agarose gel. Bands of interest were subsequently excised and cloned with pGEM®-T Easy vector system (Promega Corp., Madison, WI, USA). Purified plasmids containing insert were commercially sequenced with the use of primers T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-TAT TTA GGT GAC ACT ATA G-3'), eventually using also an internal primer CYA781F(a) (Nübel et al., 1997). Obtained sequences were checked for chimeras using Decipher 2.17.1 (Wright et

al., 2012) and submitted to the NCBI GenBank database (www.ncbi.nlm.nih.gov) under following accession numbers: MT756298–343.

PCR products of *rbcLX* and *rpoC1* were visualized on 1.5 % agarose gel and either directly sequenced or excised from the gel and cloned with pGEM®-T Easy vector system (Promega Corp., Madison, WI, USA). Obtained sequences were submitted to the NCBI GenBank database with the following accession numbers: MT767410–26 (*rpoC1*) and MT792737–57 (*rbcLX*).

2.3. Phylogenetic analyses

Obtained sequences were assembled in Geneious Prime 2020.2.4 (<http://www.geneious.com>) and together with sequences of organisms either traditionally belonging to the order Oscillatoriales (Komárek et al., 2014) or recently described there, subsequently aligned using MAFFT v7 (Kato and Standley, 2013). Sequences of *Gloeobacter violaceus*, and selected members of Pseudanabaenaceae were used as outgroups.

The final 16S rRNA gene dataset was based on a 1492 bp long alignment of 233 sequences. Before running the phylogenetic analyses the alignment was manually checked and adjusted where needed in BioEdit 7.2.5 (Hall, 1999). The phylogenetic analyses included Bayesian inference in MrBayes 3.2.3 (Ronquist et al., 2012) and Maximum Likelihood in RaxML GUI (Edler et al., 2020) above RaxML 8 (Stamatakis, 2014). The program jModelTest 2 (Darriba et al., 2012) was used to select the best substitution model according to the Akaike Information Criterion. The proposed evolutionary model GTR+ Γ +I was used for both Bayesian and Maximum Likelihood analyses. The Bayesian analysis involved two runs of eight Markov chains for three million generations with sampling every 100th generation until the average standard deviation of split frequencies was <0.01 and the potential scale reduction factor (PSRF) for all parameters was between 1.00 and 1.01. The first 25 % of sampled trees were discarded as burn-in, posterior node probabilities were estimated based on the frequency of individual nodes in the remaining sampled trees. In Maximum Likelihood a thorough bootstrap analysis with 1000 pseudoreplicates was performed in order to obtain relative supports.

The 16S–23S ITS regions of comparable operons containing both tRNA^{Ile} and tRNA^{Ala} were manually aligned according to the positions of the conserved secondary structures. The alignment contained 55 sequences and was 468 bp long. The phylogenetic analyses were performed with the same software and settings as those of 16 rDNA.

For the multilocus tree strains with all three genomic regions (16S rRNA, *rbcLX*, *rpoC1*) available were preferably searched for on GenBank; however, for a few taxa only two of these genes were available. Before building the final concatenated alignment, separate tree was reconstructed for each of the genes to check for possible incongruences and contaminations. Subsequently the partial datasets were merged into the final one containing 58 sequences of a 3062 bp cumulative length. The phylogenetic analyses included Maximum Likelihood in RaxML GUI (Edler et al., 2020) above RaxML 8 (Stamatakis, 2014) in CIPRES Science Gateway (Miller et al., 2010), and Bayesian inference in MrBayes 3.2.3 (Ronquist et al., 2012). The Bayesian analysis was performed as previously but spanned twenty million generations. In Maximum Likelihood analysis 1000 bootstrap pseudoreplicates were performed in order to obtain relative supports. The best substitution model was searched for each of the partitions separately using the jModelTest 2 (Darriba et al., 2012) and Akaike Information Criterion. Model TIM2+I+G was selected for the 16S rRNA gene and the *rbcLX* genomic region, TIM3ef+I+G for *rpoC1* genomic region. As these particular models are not implemented in MrBayes nor RaxML, we selected the closest but more general model GTR+ Γ +I for both types of analyses. A separate GTR+ Γ +I substitution model was applied for each of the three partitions corresponding to the three aligned genomic loci.

The % identities of the 16S rRNA gene and the 16S–23S ITS region sequences of *Tenebriella* and representatives of its closely related taxa were calculated based on pairwise p-distances using Geneious Prime 2020.2.4 (<http://www.geneious.com>).

2.4. The 16S–23S ITS secondary structures

The conserved hypothetical secondary structures were looked up manually in the available sequences that included both tRNAs. The sequence parts were then folded in S-fold (<http://sfold.wadsworth.org>) and adjusted in Inkscape 1.0.1 (<https://inkscape.org/>).

2.5. Electron microscopy

Selected strains were preserved for observations in electron microscope with mixture of 2.5% glutaraldehyde and 0.1 M cacodylate buffer followed by post fixation with 2% osmium tetroxide. The strains were dehydrated in an acetone series (30, 50, 70, 80, 90, 95 and 100 %) and for transmission electron microscopy (TEM) embedded in Spurr's resin (Spurr, 1969). Afterwards the 70

nm thick sections were placed on formvar-coated grids, contrast-treated with uranyl acetate and lead citrate, coated with carbon, and viewed in a JEOL TEM 1010 microscope. For the observations in the scanning electron microscope (SEM), the dehydration series was followed by carbon dioxide critical point drying. Dried specimens were mounted on stubs, where they were sputter-coated with gold and observed in a JEOL JSM-7401F microscope.

3. Results

3.1. Morphological observations and taxonomic descriptions

The studied strains originally resembling to *Oscillatoria* were recognized based on the available data as a new genus described here as *Tenebriella*. Among the *Tenebriella* strains two distinct species were identified based both on the morphological and molecular data. The first species does not correspond to any known taxon and is described as a new species *T. amphibia* (Fig. 1). The second species with narrower trichomes corresponds morphologically and ecologically to the description of *Oscillatoria curviceps* Agardh ex Gomont. Thus we propose the combination of *O. curviceps* to the newly established genus as *T. curviceps* (Fig. 2).

***Tenebriella* Hauerová, Hauer et Kaštovský, gen. nov.**

Thallus in form of dark isopolar filaments with ability to form distinct mats. Sheaths facultatively present, colorless, sometimes lamellated, with only one trichome present in a single sheath, false branching not present. Trichomes gently tapering toward both ends; unbranched; not or only slightly constricted at the cross walls; color ranging from pale greyish green to golden brown and purple. Several last cells of the trichome often yellowish. Cells always distinctly shorter than wide, actual length depending on the phase of division. New cell walls often formed before the previous one is finished, making parts of the trichome reminiscent of a ruler. Small granules often located at the cross walls. Apical cells rounded, calyptra occasionally present. Necridia and hormogonia frequent. Trichomes movement by gliding. Division of cells exclusively cross-wise, reproduction by the disintegration of trichomes.

Ultrastructure: Radial arrangement of thylakoids (Fig. 1–1b in Lokmer (2007)). The cell wall is thin with no conspicuous pores (Fig. 1–1b in Lokmer (2007)); Fig. 3 here) contrary to the true *Oscillatoria* (Mühlsteinová et al. 2018).

Ecology: Genus members inhabiting benthic habitats of standing fresh waters but known also from greenhouse soils and stones, and mangroves in India.

Filaments creeping on or through substrate, eventually creating mats that subsequently detach and float on the water surface. When aquatic, it seems to prefer waters with elevated conductivity and pH.

Etymology: From the latin word *tenebris* – dark; referring to the conspicuously dark color of filaments when creating visible biomass.

Type species: *Tenebriella amphibia* Hauerová, Hauer et Kaštovský

***Tenebriella amphibia* Hauerová, Hauer et Kaštovský, sp. nov. (Figs 1, 3)**

Thallus in form of dark filaments creeping on substrate. Filaments up to 33 µm wide. Sheaths facultatively present, colorless, sometimes lamellated, only one trichome present in a sheath. Trichomes 14–24 µm wide, gently tapering toward the ends, (10) 12–20 µm wide at the end; unbranched; not or only slightly constricted at the cross walls; color ranging from golden brown to purple. Several last cells of the trichome often yellowish. Cells distinctly shorter than wide, 1.5–6 µm long depending on the phase of division. New cell wall often formed before the previous one finished, making parts of the trichome reminiscent of a ruler. Small granules often located at the cross walls. Apical cells rounded, calyptra occasionally present. Necridia and hormogonia frequent. Trichomes gliding.

Ultrastructural notes: The granulation is often located at the cross-walls and the ends of the trichomes can be terminated by a prominent calyptra (Fig. 3 B).

Ecology: Species forming mats in freshwater habitats that may be later floating detached on the water surface. It has been often found in waters with elevated conductivity and pH, but is also known from greenhouse soils and stones.

Epithet etymology: From latin word *amphibius* referring to both freshwater and terrestrial origin of studied strains.

Diagnosis: *T. amphibia* differs from morphologically similar *Oscillatoria* species by several morphological features including wider trichomes (cf. *O. curviceps*, *O. limosa*, *O. sancta*), trichome color (cf. *O. major*), and presence of calyptra (cf. *O. limosa*, *O. major*).

Holotype (here designated): CBFS A-123 exsiccated biomass of the reference strain CCALA 1132 (RMCB14) deposited in Herbarium of the University of South Bohemia (CBFS), České Budějovice, Czech Republic.

Reference strain: CCALA 1132 (originally RMCB14)

***Tenebriella curviceps* Hauerová, Hauer et Kaštovský, comb. nov. (Fig. 2)**

Basionym: *Oscillatoria curviceps* Agardh ex Gomont 1892, Monographie des Oscillariées (Nostocacées Homocystées). Deuxième partie. - Lyngbyées.

Annales des Sciences Naturelles, Botanique, Série 7 16: 213.

Emended description: Thallus in form of dark filaments creeping on substrate, eventually forming dark mats that can be released to float on water surface. Filaments 18–25 µm wide. Sheaths facultatively present, colorless, thin, only one trichome present in a sheath. Trichomes 10–17 µm wide, gently tapering toward the ends, 9–12 µm wide at the end; unbranched; not or only slightly constricted at the cross walls; color ranging from pale greyish green to brownish and purple. Several last cells of the trichome often yellowish. Cells distinctly shorter than wide, 2–5 µm long depending on the phase of division. New cell wall often formed before the previous one finished, making parts of the trichome reminiscent of a ruler. Small granules often located at the cross walls. Apical cells rounded, calyptra occasionally present. Necridia and hormogonia frequent. Trichomes gliding.

Ecology: Freshwater species creating mats in benthic habitats that may later detach and float on the water surface.

Lectotype: Sweden, vid Lund, Apr. 1824, in herb. Agardh, LD (Drouet 1968: 264).

Reference strain: CICALA 1133 (originally RMCB17); exsiccated biomass deposited in Herbarium of the University of South Bohemia (CBFS), České Budějovice, Czech Republic as CBFS A-124.

3.2. Phylogenetic analyses

The phylogenetic analyses of the 16S rRNA gene confirmed that the studied organisms create delimited and well supported clade among other oscillatoriacean taxa (Fig. 4). These analyses were based on 53 sequences originating from 17 organisms representing the new genus *Tenebriella* (Fig. 5) and 180 sequences representing mostly the genera belonging to the order Oscillatoriales based on Mareš (2018), together with *Gloeobacter* and *Pseudanabaenaceae* as outgroups. For most of the *Tenebriella* strains or populations sequenced during this study 3 or more copies of the 16S rRNA gene (and the following 16S–23S ITS region) were acquired by cloning the amplified PCR products to the plasmids of *E. coli* cells. The organism most closely related to *Tenebriella* is *Hydrocoleum lyngbyaceum* HBC7 (EU249124), followed by a cluster containing genera *Okeania*, *Trichodesmium*, *Lyngbya*, and *Dapis*.

Pairwise percent identity comparison of the 16S rRNA gene sequence showed that within the genus *Tenebriella* the sequences were mostly 98–100% identical. However, the sequence KM982580 (“*O. sancta*” SERB 31) shared only 94.7–96.4 % identity to the rest of the *Tenebriella* sequences, even though it clustered well within *Tenebriella* based on the phylogenetic tree. The outgroup taxa shared 95.6–97.9% identity to *Tenebriella* with less than 93.7% identity to the sequence SERB 31 KM982580 (Table 2). The sequence identity of the most closely related organism *Hydrocoleum lyngbyaceum* HBC7 EU249124 was less than 97.9% to the *Tenebriella* representatives. The full-scale table containing all the sequences and their percent identities to each other is part of the supplementary material (S1).

The *Tenebriella* clade maintained its separate position from the other known and sequenced Oscillatoriales also in the multilocus analyses combining 16S rRNA with *rpoC1* and *rbcLX* genes (Fig. 6). The latter two were not available for the strain *Hydrocoleum lyngbyaceum* HBC7, so it could not be included in this analysis. However, *Trichodesmium* and *Okeania* maintained their position close to *Tenebriella*. According to Komárek et al. (2014) *Okeania* belongs to the family Oscillatoriaceae, whereas all the closely related taxa such as *Trichocoleum*, *Dapis*, and *Hydrocoleum* belong to the family Microcoleaceae according to the cytomorphological markers. Based on the proximity of the clade containing *Microcoleus vaginatus* in both phylogenies (Figs 4, 6) to the clade containing *Tenebriella*, *Okeania*, and *Trichodesmium*, it is more probable that *Okeania* belongs to Microcoleaceae than to Oscillatoriaceae. The clade containing the type species of *Oscillatoria* maintains rather isolated position among Oscillatoriales and is not closely related to any of these taxa (Figs 4, 6). Thus, we suggest moving the genus *Okeania* to the family Microcoleaceae.

The 16S–23S ITS alignment contained 50 *Tenebriella* sequences representing 14 organisms. All the ITS operons contained both tRNA^{Ile} and tRNA^{Ala}. The phylogenetic analyses clearly separated two well supported clades within the genus *Tenebriella* (Fig. 7). In congruence with the observed morphological differences among these two clades, we recognized them as two species—*T. amphibia* and *T. curviceps*. However, within *T. amphibia* a well-supported sub-clade of two strains Fkv-3 and Fkv-4 was formed. With regard to their genetic difference from other studied strains we suspect these strains represent yet another species. Due to the lack of cultured material or herbarium specimen representing this clade, this species is tentatively recognized as *Tenebriella* sp.

Since the sequence of *Hydrocoleum lyngbyaceum* HBC7 does not contain the ITS part, only taxa from the *Okeania–Dapis* group were included as outgroups.

The sequences of the 16S–23S ITS regions shared 96.2–100% identity among the *T. amphibia* representatives and 98.5–100% identity among the *T. curviceps* sequences (Tab. 3). *T. amphibia* shared 94.7–96.7% identity to *Tenebriella* sp. (strains Fkv), and 86.1–89.9% to *T. curviceps*. *Tenebriella* sp. and *T. curviceps* were only 86.5–89.1% identical. The overall identity among *Tenebriella* sequences was not lower than 86.1%; outgroups were less than 81.8% identical to the *Tenebriella* sequences (Tab. 3). (Full version of the data is shown in S2).

3.3. ITS secondary structures and their lengths

The 16S–23S ITS secondary structures were compared among 20 different organisms. For half of them between 3 to 10 copies of the 16S–23S ITS region were sequenced and analyzed. Even though in most cases the given organisms shared the same secondary structure of the conserved ITS regions among their different operons sequenced; in several cases there were variations observed also among operons of the same strain (Fig. 8).

The lengths of various parts of the 16S–23S ITS region are summarized in Table 4. The outgroup taxa differed from *Tenebriella* in the lengths of their V3 and partially also the D1–D1' helices. Notable differences were observed also in the lengths of various spacers dividing the folded secondary structures. The length of the V2 spacer and the spacer dividing tRNA^{Ala} from BoxB was consistently a base longer in *T. curviceps* than in *T. amphibia*. *Tenebriella* sp. (strains Fkv) shared these spacer's lengths with *T. amphibia*; however, its D2–D3 spacer was a base shorter than both *T. amphibia* and *T. curviceps* (Tab. 4).

D1–D1' helix

The studied D1–D1' helices formed in total 10 different structures (Fig. 8). The length of this helix was 59 bases in all *Tenebriella* sequences as well as in *Trichodesmium erythraeum* and *Okeania hirsuta*. *Lyngbya* sp. MA2, MaedaD5 and *Dapis pleousa* shared the length of 57 bases.

All the folded structures shared the conserved basal clamp of 5 base pairs. In *Tenebriella* this basal clamp was followed by lateral loop consisting of 7 unpaired bases on the 3' strand, which were further followed by 2 unpaired bases on each side (Fig. 8 A–F). Almost all the operons of *Tenebriella* shared an unpaired A located in the central part of the helix placed on the 3' strand after

the 5th base pair following the lateral loop. Only in strain Fkv-4 (Fig. 8 C) the unpaired A was part of a bulge created by 3 unpaired bases. In the outgroup taxa *Lyngbya*, *Trichodesmium*, *Dapis*, and *Okeania* the lateral loop on the 3' strand was never followed by the 2 unpaired bases and the position of an unpaired A differed (Fig. 8 G–J).

For *T. amphibia* the terminal loop consisted of 12 unpaired bases (Fig. 8 A–B). *Tenebriella* sp. had the terminal loop consisting of only 3 unpaired bases (Fig. 8 C–D), whereas *T. curviceps* had the terminal loop formed by 4 unpaired bases (Fig. 8 E–F).

Box-B helix

The length of all but one Box-B helices was 35 bases; *O. hirsuta*'s helix was one base longer (Tab. 4). In total 6 different shapes were recovered (Fig. 8). *T. amphibia* formed two unique shapes differing from all the other helices (Fig. 8 K–L). One of these shapes (Fig. 8 K') was shared with *Tenebriella* sp. The shape recovered for *T. curviceps* was the same one as for *Trichodesmium* differing only slightly in sequence (Fig. 8 M, O). *Lyngbya*, *Dapis*, and *Okeania* shared the same secondary structure of Box-B differing from each other slightly in sequence and in a base larger terminal loop of *Okeania* (Fig. 8 N, P).

V3 helix

The length of V3 helix in *Tenebriella* was mostly conserved at 39 bases, but for clone 5 of Ute 3 it was 44 bases long. For *Lyngbya* the V3 region could not be recovered, however the V3 region length of *Dapis*, *Trichodesmium*, and *Okeania* was above 44 bases (Tab. 4).

Altogether 9 different V3 shapes were observed (Fig. 8). However, two operons of S23 (S23 clones 6 and 8), *Lyngbya* MA2, and *Lyngbya* MaedaD5 did not have sequences long enough to obtain this region.

The studied V3 helices were highly variable in the number and size of bulges present throughout the stem. Nevertheless, the upper part of the V3 stem seems to be conserved among the *Tenebriella* species. In *T. amphibia* the terminal loop of 5 bases together with 7 base pairs below forming undisturbed clamp before any next impairment was conserved throughout all studied operons (Fig. 8 Q–T). This shape was again shared with *Tenebriella* sp. differing only in a single base change (Fig. 8 T'). Whereas in *T. curviceps* the terminal loop consisted of 7 bases with 11 base pairs underneath between the terminal loop and the next bulge in the stem (Fig. 8 U, V). For the outgroup taxa the V3 helices were

available only for *Dapis*, *Trichodesmium*, and *Okeania*. The structures of *Dapis* and *Trichodesmium* looked very different from the other ones by containing 9–10 unpaired bases on the 3' strand forming large lateral bulge in the central part that bent the whole structure (Fig. 8 W, X). The V3 structure of *Okeania* resembled to the structure observed in several strains of *T. amphibia* and *Tenebriella* sp. Fkv, however differed in its proportions (Fig. 8 T, T', Y).

4. Discussion

4.1. The new genus delineation

Phylogenetic analyses based on the 16S rRNA gene and on the combination of 16S rRNA, *rpoC1*, and *rbcLX* loci confirmed separate position of *Tenebriella* from other genera characterized with the use of molecular data (Figs 4, 6). The identity of the 16S rRNA gene within the genus *Tenebriella* was above 98% with the exception of a sequence “*Oscillatoria sancta*” SERB31 KM982580 that was only 94.7–96.4% similar to the other *Tenebriella* sequences (Tab. 2). All these values are above the proposed 94.5% threshold sequence identity for the genus separation (Yarza et al., 2014). Lower quality of the SERB31 KM982580 sequence was probably the reason for the remarkable drop in the calculated identities. The most related taxon according to the 16S rRNA tree was *Hydrocoleum lyngbyaceum* HBC7 EU249124 (Fig. 4), which shared the sequence identity up to 97.9% with *Tenebriella* strains (Tab. 2). This identity was higher than the ones observed for SERB31 KM982580 and if the conclusions were based on the identities only, it would suggest that *H. lyngbyaceum* HBC7 EU249124 should be included within the genus *Tenebriella*. It would be the case also for the rest of the outgroup taxa (*Lyngbya* sp. MA2 AB857842, *Lyngbya* sp. MaedaD5 AB863124, *Dapis pleousa* FFP13-13 MF061800, *Trichodesmium erythraeum* IMS101 CP000393, *Okeania hirsuta* PAB-10-Feb-10-1 NR_118586) that shared sequence identities up to 97.4% with the *Tenebriella* strains (Tab. 2). Nevertheless, it has been demonstrated previously that the 16S rRNA sequence identities above the given threshold have rather limited informational potential, whereas the values below the threshold strongly indicate the presence of distinct taxa (Casamatta et al., 2005; Johansen and Casamatta, 2005; Yarza et al., 2014). Collapsing of all these well distinguished genera into a single taxon is therefore not advisable.

The status of *Tenebriella* as a separate genus is however well supported by the phylogenetic analyses, as well as the combination of ecological and morphological differences between *Tenebriella* and the outgroup taxa.

According to the available data it seems that the whole wider clade containing *Tenebriella* gathers cyanobacteria mostly originating from marine environments. *Hydrocoleum lyngbyaceum* HBC7, *Dapis*, and *Okeania* were described from tropical seas of Bahamas, Florida, and Panama; *Lyngbya* sp. MaedaD5 was described from coral reef in Japan, and *Lyngbya* sp. MA2 probably as well, since the location specified at GenBank is very similar to MaedaD5 (Tab. 1). Neither of the *Trichodesmium* sequences have their location of origin specified at GenBank, however, according to Komárek and Anagnostidis (2005) *T. erythraeum*, as well as *T. thiebautii*, and *T. radians* (= *tenue*) are marine taxa. *Tenebriella* strains analyzed thus far by us originate from non-marine (freshwater and terrestrial) habitats with a single exception of strain SERB31. This strain represented by an ambiguous GenBank sequence (KM982580) is supposed to have been isolated from mangrove environment, however detailed information is missing.

Morphologically *H. lyngbyaceum* HBC7 differs from *Tenebriella* by smaller trichome size and ability to host multiple trichomes in a thick sheath (Foster et al., 2009). *Dapis* shares similar trichome color with *Tenebriella* but its size is larger and gas vesicles can be present in the cells (Engene et al., 2018). Various *Okeania* species differ morphologically from *T. amphibia* and *T. curviceps* by combination of features including different trichome size, absence of calyptra or by distinct growth form of their thallus (Engene et al., 2013). For the *Lyngbya* MA2, MaedaD5 and the sequenced *Trichodesmium* species morphological data are not available.

4.2. The new species delineations

The 16S rRNA gene has been shown to work well for delimitation of genera, however, it fails to provide finer resolution on the intrageneric level (Boyer et al., 2002; Fox et al., 1992). There is a threshold value of 98.7% 16S rDNA sequence identity suggested and applied by bacteriologists for the species separation. However, as in case of genera separation, the threshold is mostly informative when the studied values are clearly below the threshold among the studied taxa (Johansen and Casamatta, 2005; Yarza et al., 2014). The identities of sequences within each *Tenebriella* species were much higher than between them (Tab. 2), nevertheless they were only partially below the 98.7% threshold value. In search for the ways allowing molecular characterization/separation of species, the 16S–23S ITS region has been suggested as a possible tool as its nature allows more variability among closely related organisms. The

hypothetical secondary structures of the 16S–23S ITS region has been successfully used to distinguish among studied species in multiple works (Caires et al., 2018; Johansen et al., 2011; Pietrasiak et al., 2014). Recently, also the alignment of this region allowed phylogenetic comparison and pairwise p-distance analysis to separate the studied taxa (Engene et al., 2013; Osorio-Santos et al., 2014).

The detailed comparison of the 16S–23S ITS region further supported the separation of *T. amphibia* and *T. curviceps* suggested by observed morphological differences in trichome color and size (Figs 1, 2), and revealed presence of a tentative cryptic species *Tenebriella* sp. consisting of strains Fkv-3 and Fkv-4, which unfortunately have been lost. Only operons including both tRNAs (isoleucine and alanine) were recovered for the studied *Tenebriella* strains and subsequently compared with available operons with similar structure of other taxa. It has been shown previously that comparison of the same operons is crucial for meaningful analyses (Boyer et al., 2001; Boyer et al., 2002). The operons of the outgroup taxa differed from *Tenebriella* in lengths of various ITS parts (Tab. 4), but were still similar enough allowing their alignment and subsequent phylogenetic analyses. However, the 16S–23S ITS sequences were not available for all the taxa that were suggested by the 16S rDNA phylogeny as closely related. From the recently described genera *Dapis* and *Okeania* only the type species were chosen for the ITS analyses. The ITS alignment clearly separated *T. amphibia* from *T. curviceps* and both of them from the outgroup taxa. Even though the strains Fkv-3 and Fkv-4 grouped with *T. amphibia*, their position was separated from the rest of the *T. amphibia* strains and they also differed by a base shorter spacer separating D2 and D3 regions. These differences were reflected also by the hypothetical secondary structures. The Fkv-3 and Fkv-4 formed D1-D1' helix different from other *T. amphibia* sequences (Fig. 8 C–D), their Box B and V3 helix varied in one or two base changes respectively (Fig. 8 K', T'). These differences lead us to the conclusion that these strains represent yet another cryptic species. However, the strains are not available anymore for any further studies or even formal description of this third species. Thus, we call it *Tenebriella* sp. until more data are available.

The ITS percent identity tends to be over 98% among members of the same species, whereas among members of different species it tends to be below 91% (Bohunická et al., 2015b; Erwin and Thacker, 2008; Mai et al., 2018). This was clearly true for the members of *T. curviceps*, whose ITS sequences were more

than 98.5% identical among its members as well as for *Tenebriella* sp. with identity of 99.8% between the two Fkv strains (Tab. 3). In case of *T. amphibia* the range of ITS identities was much wider and dropped to as low as 96.2% among its members. This may reflect higher variability among the *T. amphibia* strains caused by their different geographical origin (Supplement S2). Additionally, more operons of *T. amphibia* were sequenced and studied, and thus probably more variances were recovered than in *Tenebriella* sp. and *T. curviceps*. The drop in the ITS identity clearly separated *T. curviceps* from *Tenebriella* sp. and *T. amphibia* by falling below 90% when comparing their members. The drop between *T. amphibia* and *Tenebriella* sp. was less striking, but still obvious (Tab. 3).

Some of the GenBank sequences that clustered within *Tenebriella* were originally named *Oscillatoria sancta* (PCC7515, SAG 74.79) and *Oscillatoria* cf. *curviceps* (Fkv-3, Fkv-4). However, the detailed comparison of the 16S–23S ITS region placed these sequences close to *T. amphibia* strains (Fig. 7). According to Komárek and Anagnostidis (2005) both *Oscillatoria sancta* and *Oscillatoria curviceps* differ from the newly described *T. amphibia* by smaller trichome size. In the *Oscillatoria sancta* type specimen from Leiden herbarium (L0055443) only trichome fragments of width ranging from 9.5–12 µm were revealed, whereas the trichome width of strain SAG 74.79 (= CCALA 135) fitted well to the *T. amphibia* description with trichomes between 15–19 µm wide. Even though the age of the herbarium specimen very probably affected the shape of the trichomes, still it suggests that *O. sancta* is noticeably smaller than both *Tenebriella* species, and thus represents a separate taxon. Description of *Oscillatoria curviceps* corresponds well with the second *Tenebriella* species and thus the new combination *T. curviceps* was proposed. However, the sequences named “*Oscillatoria* cf. *curviceps*” Fkv-3 and Fkv-4 are phylogenetically closer to *T. amphibia* than to *T. curviceps* based on the 16S–23S ITS analyses (Fig. 7), and likely represent a separate cryptic species as argued earlier.

Based on the morphological descriptions only, other species similar to *T. amphibia* are *Oscillatoria major*, *Oscillatoria froelichii*, and *Oscillatoria limosa*. The trichome width of *Oscillatoria major* would correspond well with *T. amphibia*, but *Oscillatoria major* does not form calyptra, its trichomes are blue-green, cells possess distinctive granulation on both sides of the cell wall, and the cells are not as short as at *Tenebriella* (comp. Komárek and Anagnostidis, 2005). *Oscillatoria froelichii* would resemble significantly to *T. amphibia* according to description of Komárek and Anagnostidis (2005). Nevertheless, *Oscillatoria*

froelichii is not a valid taxon since it was synonymized by Gomont (1892) with *Oscillatoria limosa*. Also the original Kutzing's (1843) description differs remarkably from the one that later appeared in *Susswasserflora von Mitteleuropa* (Komárek and Anagnostidis, 2005). *Oscillatoria limosa* itself is a taxon defined widely enough that *Tenebriella* could more or less fit to its description. It differs from *T. amphibia* mainly by rather narrower trichomes, absence of calyptra, and potentially by its ecology as discussed below. Thus, we conclude that to the best of our current knowledge none of these *Oscillatoria* species represent *Tenebriella* and decided to describe *T. amphibia* as a distinct taxon.

4.3. Ecology

The strains originating from the south Moravian part of the Czech Republic (RMCB21, JM90/15, and JM72/15) were collected during the regular monitoring of freshwater bodies in this area. Here, *T. amphibia* has been found regularly since 2014 in pools with electric conductivity ranging from 1 110–3 100 $\mu\text{S}/\text{cm}$. Based on the monitoring data this organism prefers pools with advanced succession, where it thrives particularly during warmer seasons when water level decreases. *Tenebriella* dominates there primarily in the phytobenthos from July to September and creates massive mats on various submerged substrates including both dead and living plants. During this time the mats detach from the substrate and float on water surface in form of easily noticeable dark biomass of various diameter.

According to the monitoring data from the Moravian part of the Czech Republic it seems that *T. amphibia* profits from the warmer, dryer seasons and does not mind temporal drying out of its habitats. Other collected samples are mostly in accordance with this trend – they were either collected in shallow parts of the water bodies from benthos or as profound mats floating on the water surface during warm summer months (Tab. 1). All the *T. amphibia* strains collected and sequenced prior our study originate from greenhouses—either from pools or soil, suggesting inclination for warmer temperatures. The strain collected in Sicilian cemetery among damp mosses further support the potential thermophilic nature of this species. With the advancing climate change it can be expected that *T. amphibia* will be found more often in Central Europe.

In the Central Europe *T. curviceps*' range very probably overlaps with the range of *T. amphibia*, but it may be more cold-tolerant based on the strain S23 originating from the lake in Sweden (Tab. 1). However, with regard to the

limited amount of data for *T. curviceps*, any suggestions about its ecological preferences and distribution are so far preliminary.

Remarkably, *Tenebriella* represents an almost exclusively non-marine clade that evolved within a larger group of typically marine cyanobacteria. While the deep branching within this clade is currently not fully resolved, the position of *Hydrocoleum lyngbyaceum* and the presence of *Tenebriella* sp. strain SERB 31 isolated from mangrove environment within the genus suggests that it may represent a rare example of secondary colonization of freshwater and terrestrial environment by originally marine cyanobacteria. While cyanobacteria as a whole very probably appeared in non-marine settings (Ponce-Toledo et al., 2017), and repeatedly colonized the oceans (Sánchez-Baracaldo, 2015), it is uncommon to see an opposite transition back to freshwater. This hypothesis however needs to be verified when better taxon sampling is available.

The ability to form mats that float conspicuously on various water surfaces is common among different *Oscillatoria*-like taxa. Even though these “Oscillatorieta” associate various algae, they are dominated by simple filamentous cyanobacteria traditionally assigned to the genus *Oscillatoria* (Komárek, 1989; Prát and Cyrus, 1948). Due to this ability and their generally *Oscillatoria*-like morphology they have been probably recognized as *Oscillatoria limosa* and similar species until now. This ability was well studied and documented for example for *Oscillatoria limosa*. For this taxon, however, the formation of floating mat was observed mainly at the beginning of the growing season, starting as early as in February in central Europe (Rott 1976). This may suggest that the ability to form profound “Oscillatorieta” in specific biotopes and parts of the season could be another feature adding in determination. Currently it seems that *Oscillatoria limosa* is connected with rather eutrophic water bodies and the peak of floating mats is early in the season, *Oscillatoria princeps* seems to prefer less eutrophic water bodies and later season, whereas *Tenebriella* forms profound “Oscillatorieta” in habitats with rather higher conductivity in the warm part of the season (Komárek, 1989; Mühlsteinová et al., 2018; Rott, 1976). Nevertheless, *Oscillatoria limosa*, has been long known as a collective taxon (Fjordingstad, 1971; Geitler, 1932; Komárek and Anagnostidis, 2005), which needs to be revised before more data can assess how much the ability to form floating mats in different parts of the season is taxon specific. The characteristic *Oscillatoria*-like habitus and formation of floating mats seem to be another example of serially convergent evolution, previously well-described on the example of simple coccoid

cyanobacteria (Dvořák et al., 2014). The phenotypic convergence has however occurred very frequently in cyanobacteria, probably due to their extremely long evolutionary history. It affects even seemingly unique traits such as the radial arrangement of thylakoids, typical also for *Tenebriella* (Mareš et al., 2019).

5. Conclusions

Even though Central Europe is thoroughly explored in terms of cyanobacterial diversity, there is still significant potential for discovery of new taxa, even on the genus level. In this study we found a novel taxon of simple trichal cyanobacterium resembling to the genus *Oscillatoria* in its appearance but clustering in a distant phylogenetic position, providing evidence for convergent evolution. Due to its morphology, it has been probably classified by other authors as well as water quality monitoring professionals as *Oscillatoria* so far. We have analyzed data on 18 strains or available GenBank sequences representing the new genus *Tenebriella* including 11 original isolates. Our analyses suggested the presence of at least 3 species; however for only two of them we had all the data and material necessary for formal treatment. Most of our strains originated from Central Europe, but members of the genus were recorded from various regions in northern hemisphere including also Sicily, SW USA or India. Based on this distribution we expect *Tenebriella*'s occurrence will be confirmed in other areas worldwide. The taxon also deserves attention in terms of potential toxicity, since according to our observations it may form extensive mats in various aquatic habitats.

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Attached figures and tables

Figure 1: The morphology of *T. amphibia* visualized by light microscope.

Figure 2: The morphology of *T. curviceps* visualized by light microscope.

Figure 3: The cell ultrastructure of *T. amphibia* visualized by transmission electron microscope.

Figure 4: Phylogenetic position of the new genus *Tenebriella* within the order Oscillatoriales based on the Bayesian analyses of the 16S rRNA gene.

Figure 5: Detail of the *Tenebriella* clade based on the Bayesian analyses of the 16S rRNA gene.

Figure 6: Phylogenetic position of the new genus *Tenebriella* within the order Oscillatoriales based on the concatenated alignment of the 16S rRNA, *rpoC1*, and *rbcLX* genes.

Figure 7: Phylogenetic relationships of the *Tenebriella* species based on the Bayesian analyses of the 16S–23S ITS region.

Figure 8: The secondary structures of the 16S–23S ITS region.

Table 1: Information about studied organisms.

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Table 4: The lengths of different parts of the 16S–23S ITS regions of studied strains and sequences.

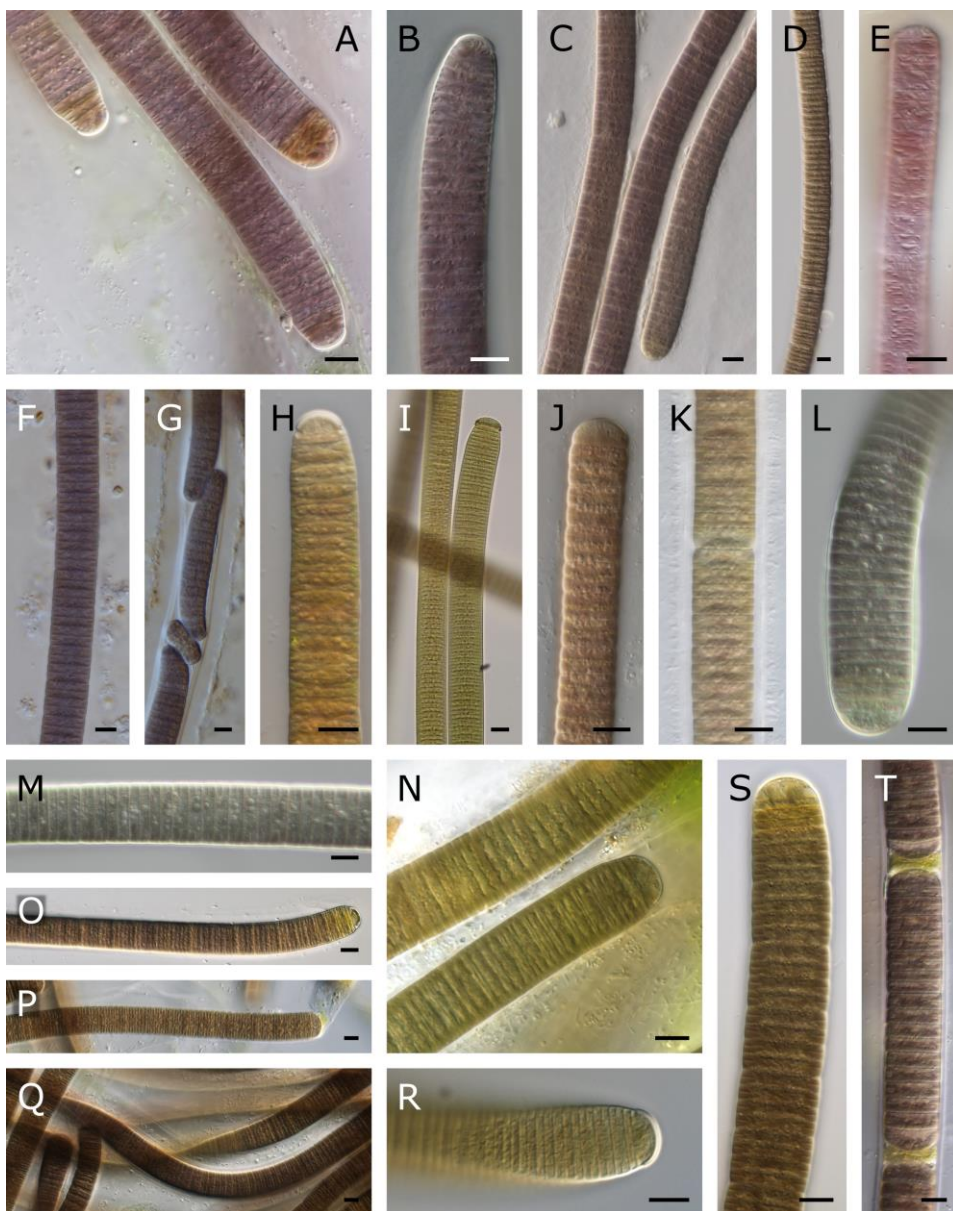


Figure 1: The morphology of *T. amphibia* visualized by light microscope. A–E strain CCALA 1132; F–G Sicily Cemetery; H – RMCB18; I–K RMCB21, L–M Ute Lake; N – JM72/15; O–Q RMCB19; R – JM90-15; S–T RMCB16. The length of the scale bar is 10 μ m.

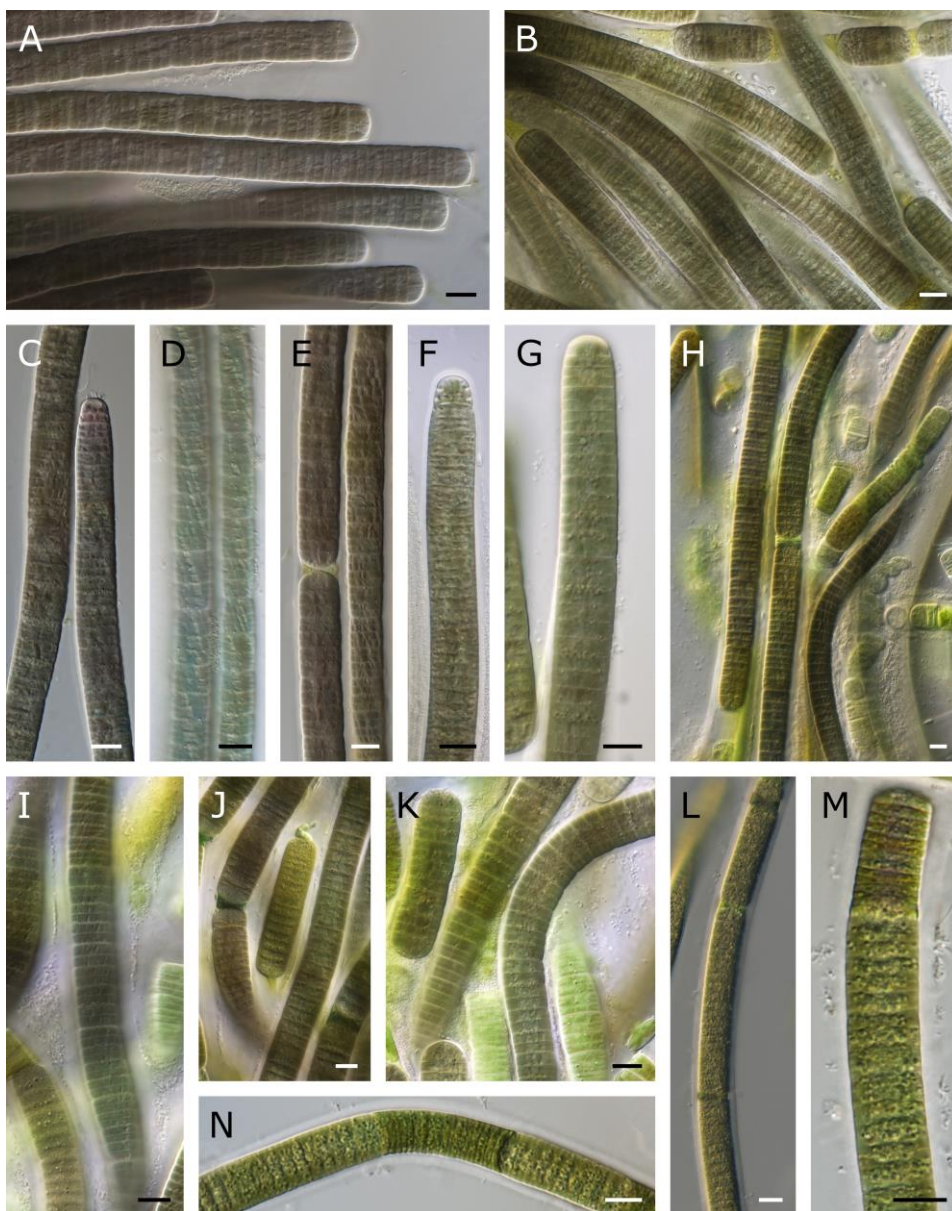


Figure 2: The morphology of *T. curviceps* visualized by light microscope. A–K strain CCALA 1133; L–N strain S23. The length of the scale bar is 10 μm .

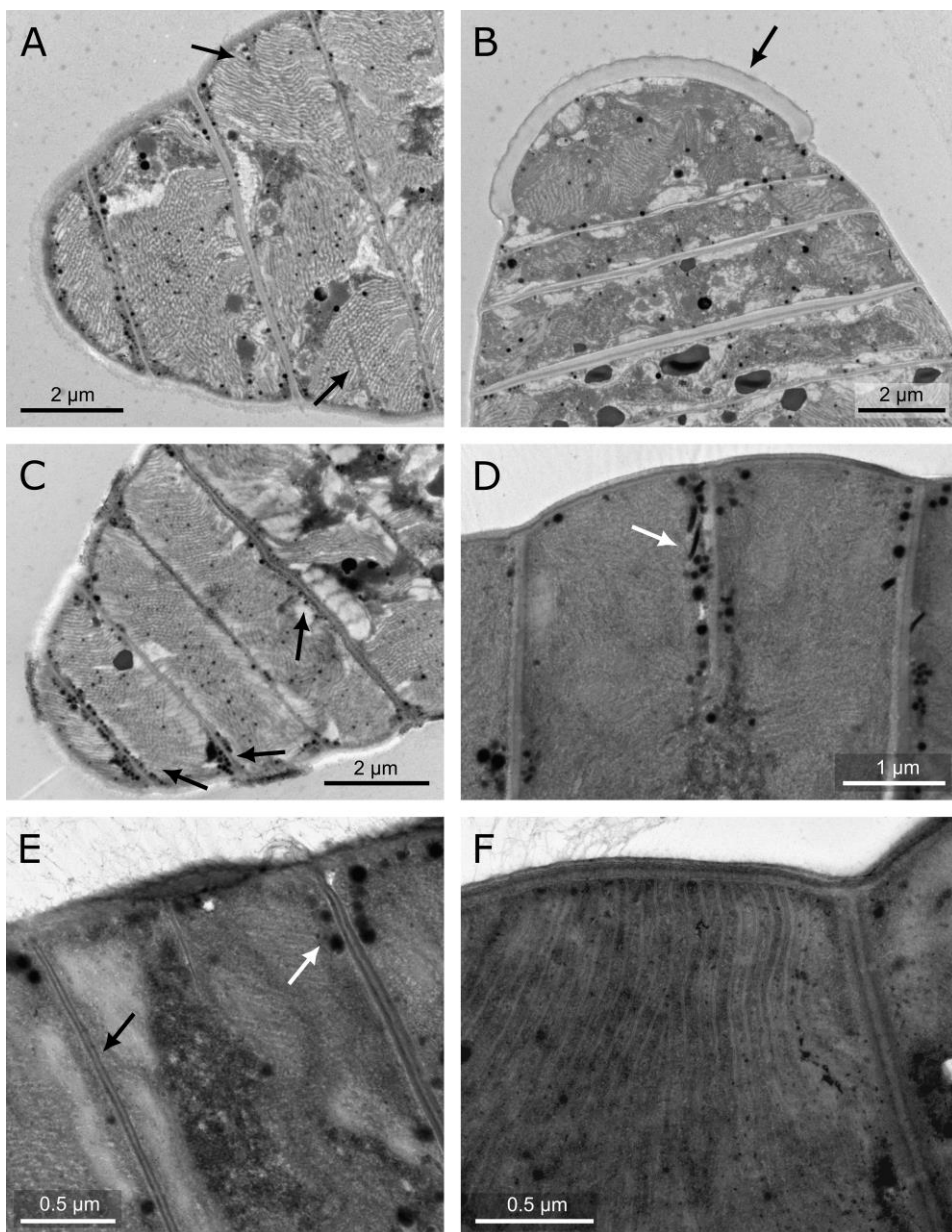


Figure 3: The cell ultrastructure of *T. amphibia* visualized by transmission electron microscope. A–C strain RMCB21: A – the thylakoid pattern and formation of the new cell wall (arrows); B – the end of the trichome with calyptra (arrow); C – the accumulation of granules at the cell wall (arrow). D–F strain Sicily Cemetery: D – formation of the new cell wall with granule accumulation; E – completed cell walls with and without granules; F – the thylakoid pattern and structure.

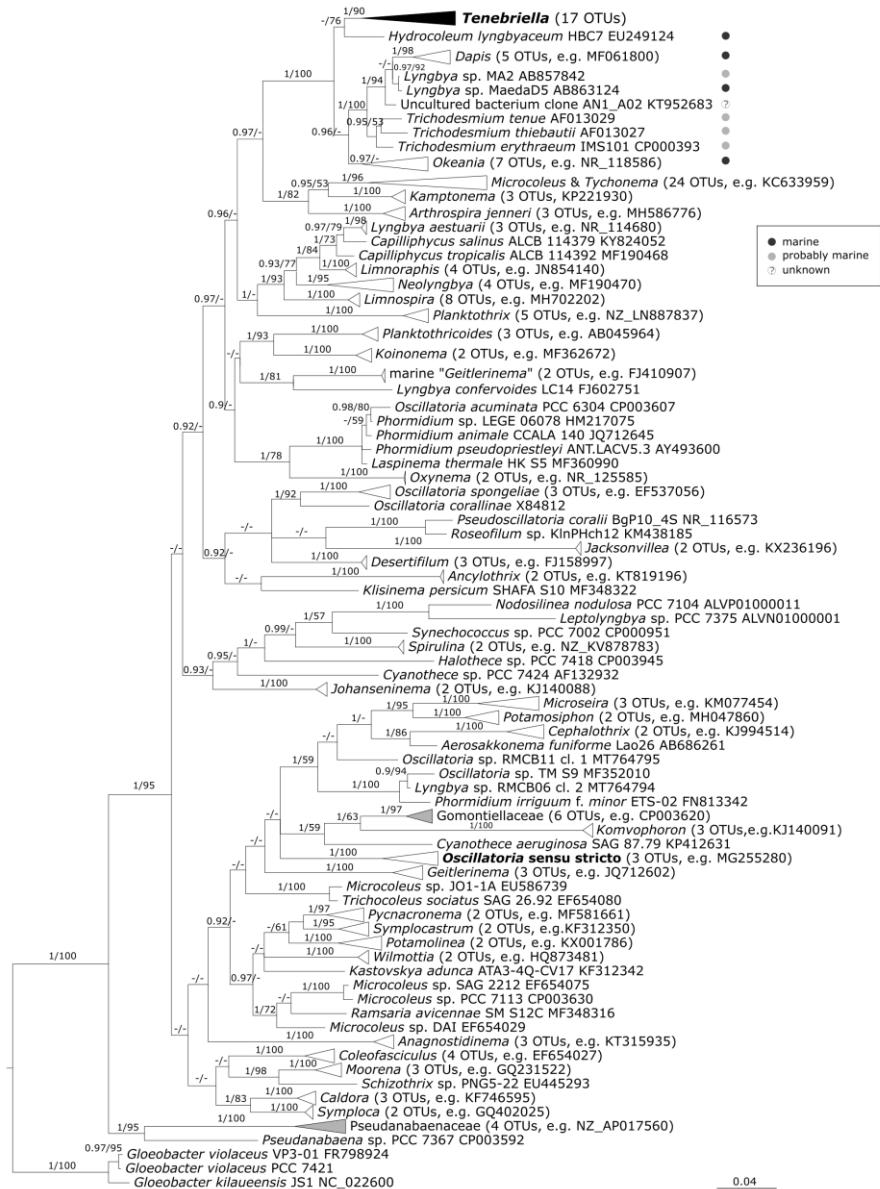


Figure 4: Phylogenetic position of the new genus *Tenebriella* within the order Oscillatoriales based on the Bayesian analyses of the 16S rRNA gene. The position of *Tenebriella* and *Oscillatoria sensu stricto* is highlighted by bold text. Above nodes Bayesian posterior probabilities are followed by Maximum Likelihood bootstrap values (values smaller than 0.9 and 50 are not shown). For collapsed groups representing genera the number of included OTUs is indicated in brackets together with a reference accession number of the given group. The scale bar indicates number of nucleotide substitutions per site.

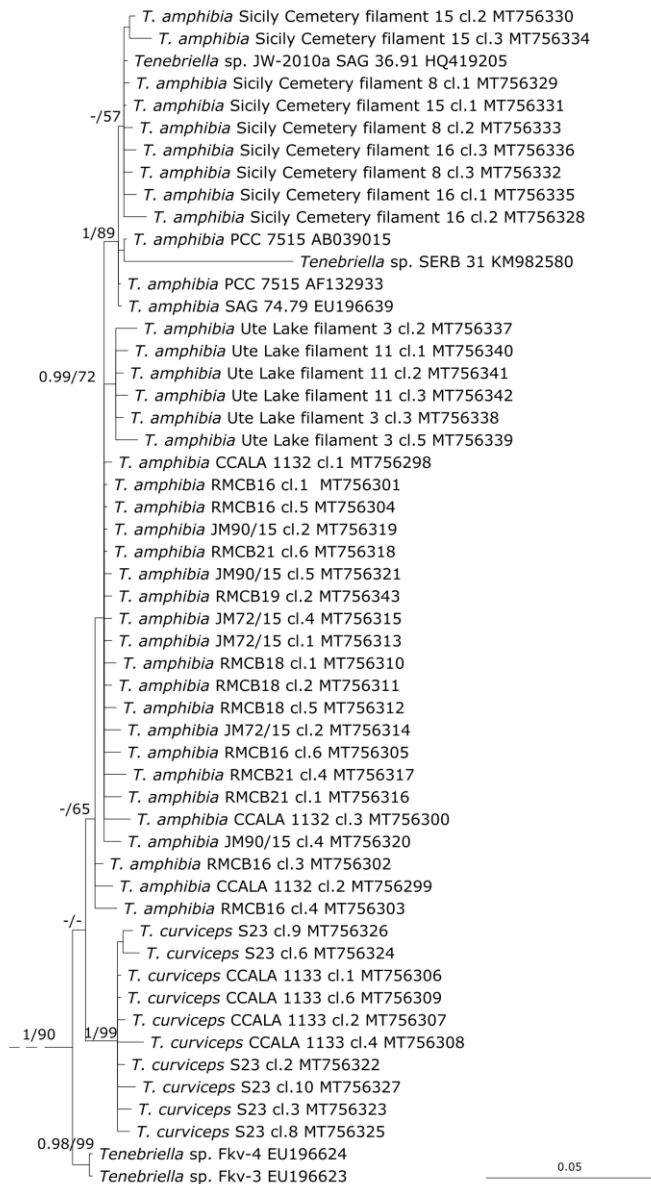


Figure 5: Detail of the *Tenebriella* clade based on the Bayesian analyses of the 16S rRNA gene. Above nodes Bayesian posterior probabilities are followed by Maximum Likelihood bootstrap values (values smaller than 0.9 and 50 are not shown). The scale bar indicates number of nucleotide substitutions per site.

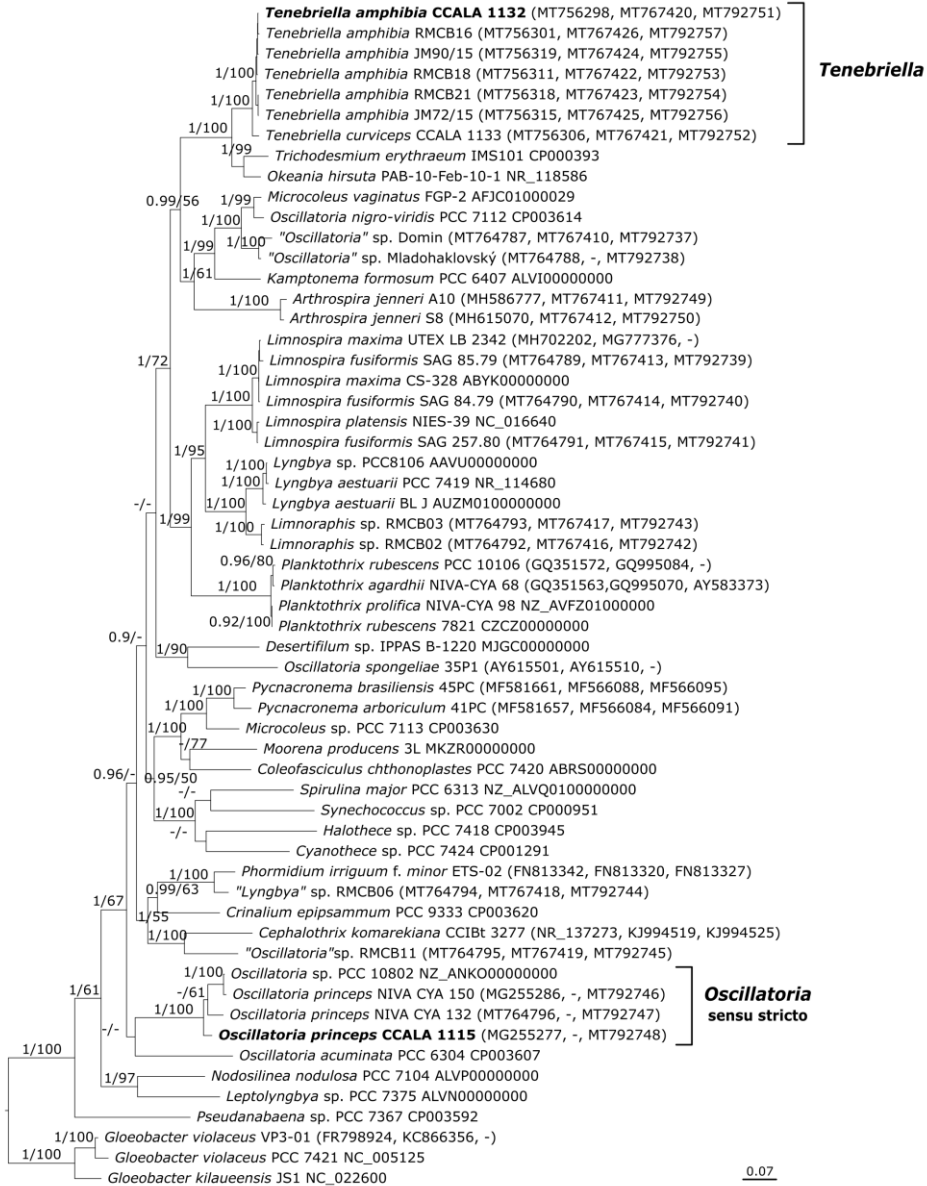
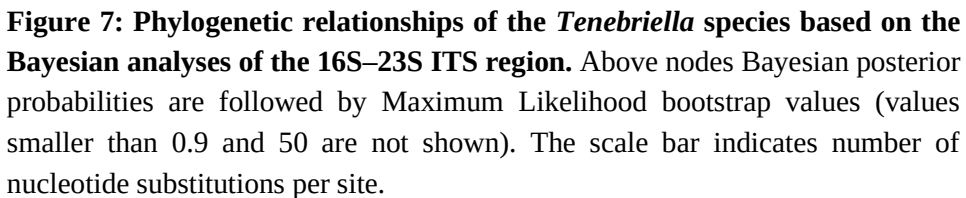


Figure 6: Phylogenetic position of the new genus *Tenebriella* within the order Oscillatoriales based on the concatenated alignment of the 16S rRNA, *rpoC1*, and *rbcLX* genes. The accession numbers in brackets are in the respective order. The position of *Tenebriella*'s and *Oscillatoria*'s type species based on the reference strains is highlighted by bold text. Above nodes Bayesian posterior probabilities are followed by Maximum Likelihood bootstrap values (values smaller than 0.9 and 50 are not shown). The tree topology is based on the Bayesian analyses. The scale bar indicates number of nucleotide substitutions per site.



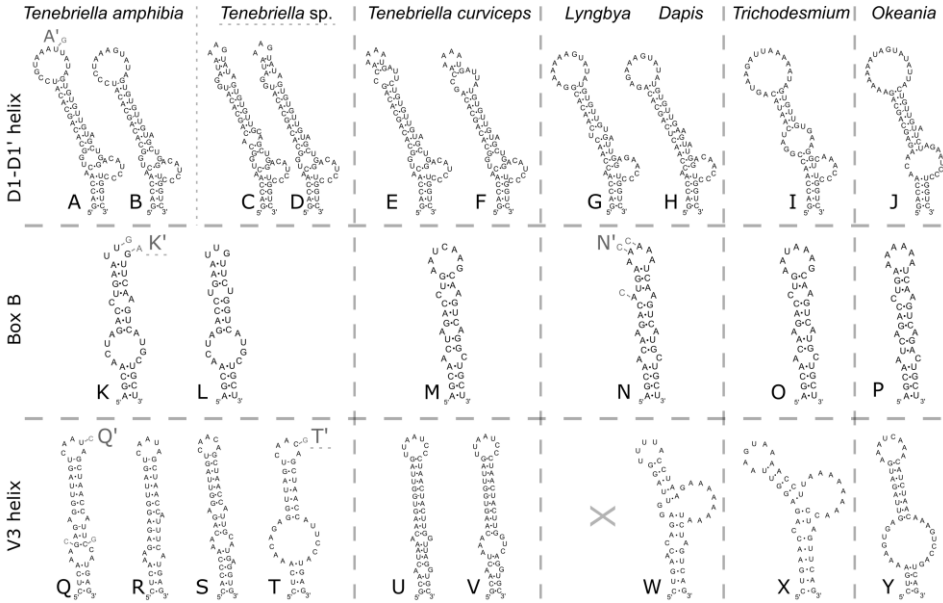


Figure 8: The secondary structures of the 16S–23S ITS region. A–C represent D1-D1' helix of *T. amphibia*: A–CCALA 1132, RMCB16, RMCB19, RMCB18, with minor modification A' observed at strains RMCB21, JM72/15, Ute Lake, SAG 74.79, and PCC 7515; B–JM90/15, Sicily Cemetery; C–D represent D1-D1' helix of *Tenebriella* sp.: C–Fkv-4; D–Fkv-3. E–F represent D1-D1' helix of *T. curviceps*: E–CCALA 1133; F–S23. G–D1-D1' helix of *Lyngbya* sp. MA2 & MaedaD5. H–*D. pleousa* FFP13-13. I–*T. erythraeum* IMS101. J–*O. hirsuta* PAB-10-Feb-10-1. K–L represent Box B of *T. amphibia* and *Tenebriella* sp.: K–CCALA 1132, RMCB16, RMCB18, RMCB19, RMCB21, JM90/15, JM72/15, Ute Lake, SAG 74.79, PCC 7515, and Sicily Cemetery with minor modifications K' observed at *Tenebriella* sp. Fkv-3 and Fkv-4; L–Sicily Cemetery 16 clone 2. M–box B of *T. curviceps* (CCALA 1133, S23). N–structure observed at *Lyngbya* sp. MA2 & MaedaD5 with slight modification of *D. pleousa* FFP13-3 sequence (N'). O–*T. erythraeum* IMS101. P–*O. hirsuta* PAB-10-Feb-10-1. Q–T represent V3 stem of *T. amphibia* and *Tenebriella* sp.: Q–CCALA 1132, RMCB16 clones 1, 3, 4, 5, RMCB18, RMCB19, Sicily Cemetery, and JM90/15 with several substitutions observed in Q'–representing Ute Lake 3 clones 2, 3, and Ute Lake 11 clones 1, 2, 3; R–SAG 74.79 and PCC 7515, RMCB 16 cl. 6; S – Ute Lake 3 cl. 5; T – RMCB21 and JM72/15 with a single substitution observed in T'–*Tenebriella* sp. Fkv-3 and Fkv-4. U–V represent V3 stem of *T. curviceps*: U–CCALA 1133 and S23 cl. 10; V–S23 clones 2, 3, 9. W–V3 stem of *D. pleousa* FFP13-13. X–V3 stem of *T. erythraeum* IMS101. Y–V3 stem of *O. hirsuta* PAB-10-Feb-10-1. V3 stem is not available for *Lyngbya* sp. MA2 and MaedaD5, and for S23 clones 6 and 8. If individual clones are not listed, consensus sequence of all available sequences for the given strain was assembled and used to build the structure.

Table 1: Information about studied organisms. Organisms studied and sequenced in this work are marked by asterisk. For sequences downloaded from GenBank the information was looked up there or in associated papers.

	Strain / Designation	16S rRNA	16S-23S ITS	Location	GPS Coordinates	Collection Date	Habitat
<i>T. amphibia</i> *	CCALA 1132 (RMCB14)	MT756298–300		flooded sand pit Baraba, Mělník, Czech Republic	50°22'22.2" N; 14°26'01.8" E	August 2014	bentos + brown- greyish mats floating on water surface
<i>T. amphibia</i> *	RMCB16	MT756301–305					
<i>T. amphibia</i> *	RMCB18	MT756310–312		Darkovské moře, Karviná, Czech Republic	49°50'07.9" N 18°32'53.1" E	Sept. 2014	bentos + floating mats
<i>T. amphibia</i> *	RMCB19	MT756343		2 nd pool before Úvalský rybník, Úvaly, Czech Republic	48°44'43.5" N; 16°42'40.6" E	June 2015	massive epipellic mats (cond. 1520 uS/cm)
<i>T. amphibia</i> *	RMCB21	MT756316–318					massive epipellic mats (cond. 1520 uS/cm)
<i>T. amphibia</i> *	JM90/15 (RMCB29)	MT756319–321		Kutnar pool, Rakvice, Czech Republic	48°50'09.6" N; 16°47'32.7" E	Sept. 17, 2015	massive epipellic along bank, on mud and plants (conductivity 1110 uS/cm, pH=7.7)
<i>T. amphibia</i> *	JM72/15	MT756313–315		4 th pool before Úvalský rybník, Úvaly, Czech Republic	48°44'41.3" N; 16°42'37.5" E	August 31, 2015	small epipellic mats among sparse reeds (cond. 2022 uS/cm, pH=8.2)
<i>T. amphibia</i> *	Sicily cemetery	MT756328–336		Scillato cemetery, Sicily, Italy	x	summer 2016	growing among damp mosses
<i>T. amphibia</i> *	U - Ute Lake	MT756337–342		Ute Lake, NM, USA	35°20'32.4" N; 103°29'38.5" W	June 11, 2016	sediment with Zygnematophyceae
<i>T. amphibia</i>	PCC 7515 (<i>O. sancta</i>)	AF132933	EF178272, AY768378				
<i>T. amphibia</i>	PCC 7515 (<i>O. sancta</i>)	AB039015	x	Stockholm, Sweden	x	Jan. 1, 1972	water, Greenhouse tank
<i>T. amphibia</i>	SAG 74.79; CCALA 135	EU196639	EU196675	Botanical Garden, Göttingen University, Germany	51°32'15.4" N; 9°56'08.9" E	1970	soil in Old Victoria greenhouse
<i>Tenebriella</i> sp.	Flkv-3 (<i>O. cf.</i> <i>curviceps</i>)	EU196623	EU196673	Botanical Garden, Prague, Czech Republic	x	probably 2006-2007	flowerpot in a tropical greenhouse

<i>Tenebriella</i> sp.	Flv-4 (<i>O. cf. curviceps</i>)	EU196624	EU196674					
<i>T. curviceps</i> *	CCALA 1133 (RMCB17)	MT756306–309		flooded sand pit Baraba, Mělník, Czech Republic	50°22'22.2" N; 14°26'01.8" E	August 2014	bentos + brown-greyish mats floating on water surface	
<i>T. curviceps</i> *	S23	MT756322–327		Förhultsjön lake, Vaxjö N, Sweden	57°07'51" N; 14°30'54" E	June 7, 2015	ferrous crusts on rocks of the bottom	
<i>Tenebriella</i> sp.	SAG 36.91 (<i>Lyngbya</i> sp.)	HQ41920 5	<i>no ITS</i>	Botanical Garden, Göttingen University, Germany	51°32'16.1" N; 9°56'09.4" E	1990	moist stone in greenhouse	
<i>Tenebriella</i> sp.	SERB 31 (<i>O. sancta</i>)	KM98258 0	<i>no ITS</i>	India	x	x	mangrove environment	
<i>H. lyngbyaceum</i>	HBC7	EU249124	<i>no ITS</i>	Highborne Cay, Exuma, Bahamas	24°43' N; 76°49' W	July 2006	subtidal stromatolite	
<i>Lyngbya</i> sp.	MA2	AB857842	yes	Okinawa, Onna, Yamada, Japan	x	Sept. 6, 2013	x	
<i>Lyngbya</i> sp.	MaedaD5	AB863124	yes	Okinawa, Onna, Maeda, Japan	26°26'24.0" N; 127°46'37.2" E	Sept. 4, 2013	macroscopic colony from coral reef	
<i>D. pleousa</i>	FFP13-13	MF06180 0	MF167701	Harbor Branch, Florida, USA	27°32'11.0" N; 80°20'55.9" W	x	sandy beach	
<i>T. erythraeum</i>	IMS101	CP000393	yes	x	x	x	x	
<i>O. hirsuta</i>	PAB-10-Feb-10-1 ^T	NR_11858 6	KC992978	Bocas del Toro, Panama	9°17' N; 82°11' W	Feb. 10, 2010	mangrove, subtidal seagrass	

*AY768378 not used in the analyses due to its short length - the available fragment identical to EF178272

Table 2: Comparison of the 16S rRNA gene sequence identities among *Tenebriella* species and the outgroup taxa.

	<i>T. amphibia</i>	<i>Tenebriella</i> Fkv	<i>T. curviceps</i>	<i>Tenebriella</i> HQ419205	<i>Tenebriella</i> KM982580	<i>H. lynghyac.</i> EU249124	Outgroups
<i>T. amphibia</i>	98.6–100%						
<i>Tenebriella</i> sp. Fkv-3, Fkv-4	98.5–99.3%	99.8%					
<i>T. curviceps</i>	98.0–99.2%	98.5–98.9%	99.1–100%				
<i>Tenebriella</i> sp. HQ419205	99.2–100%	99.2%	98.8–99.3%	100%			
<i>Tenebriella</i> sp. KM982580	95.4–96.4%	95.4%	94.7–95.2%	96%	100%		
<i>H. lynghyaceum</i> EU249124	96.9–97.5%	97.8–97.9%	96.9–97.3%	97.5%	93.7%	100%	
Outgroups	95.6–97.3%	96.0–97.1%	95.6–97.4%	96.2–97.3%	92.3–93.3%	96.4–96.8%	96.3–100%

Table 3: Comparison of the 16S–23S rRNA gene sequence identities among *Tenebriella* species and the outgroup taxa.

	<i>T. amphibia</i>	<i>Tenebriella</i> sp. (Fkv)	<i>T. curviceps</i>	Outgroups
<i>T. amphibia</i>	96.2–100%			
<i>Tenebriella</i> sp. (Fkv)	94.7–96.7%	99.8%		
<i>T. curviceps</i>	86.1–89.9%	86.5–89.1%	98.5–100%	
Outgroups	74.9%–80.4%	76.4–80.4%	75.2–81.8%	76.8–99.8%

Table 4: The lengths of different parts of the 16S–23S ITS regions of studied strains and sequences. The lengths differing from the major observed trend in given ITS part are highlighted in grey.

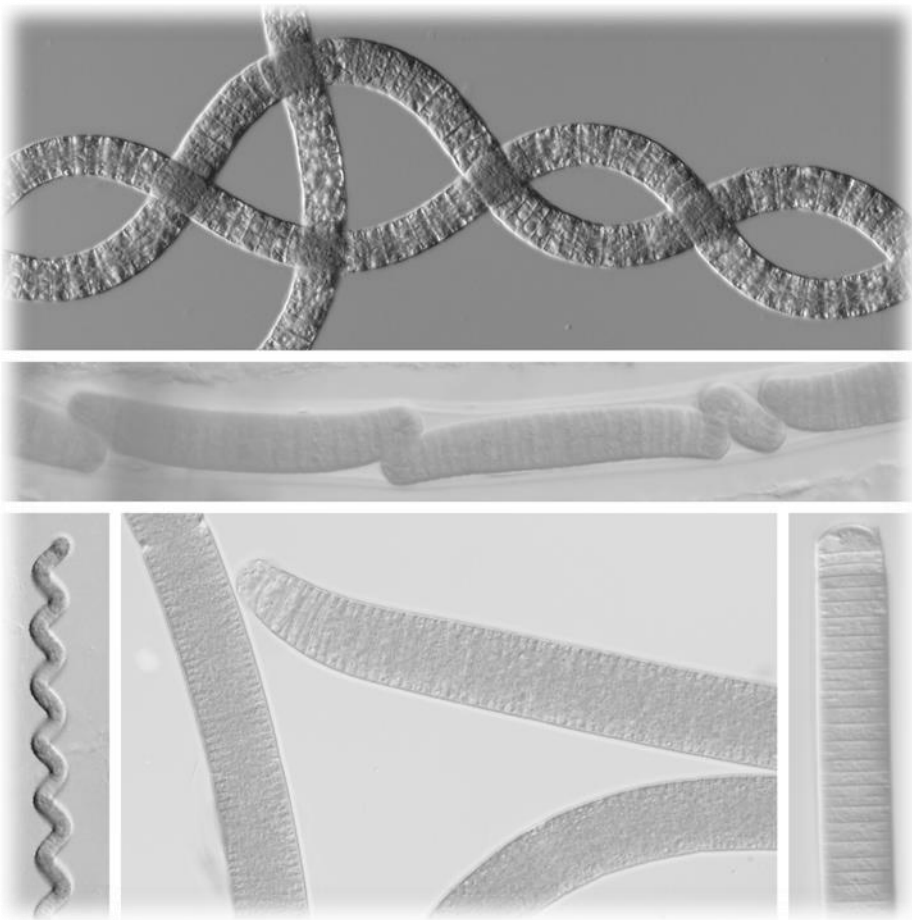
16S–23S ITS parts in order:	D1-D1' helix	spacer	D2	spacer	D3	spacer	tRNA Ile	V2 spacer	tRNA Ala	spacer	Box B	spacer	Box A	D4	spacer	V3 helix	pre-23S rRNA spacer
<i>T. amphibia</i> CCALA 1132 cl. 1	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> CCALA 1132 cl. 2	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> CCALA 1132 cl. 3	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB16 cl. 1	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB16 cl. 3	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB16 cl. 4	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB16 cl. 5	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB16 cl. 6	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB18 cl. 1	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB18 cl. 2	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB18 cl. 5	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB19 cl. 2	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB21 cl. 1	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB21 cl. 4	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> RMCB21 cl. 6	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> JM72/15 cl. 1	59	29	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> JM72/15 cl. 2	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> JM72/15 cl. 4	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> JM90/15 cl. 2	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> JM90/15 cl. 4	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> JM90/15 cl. 5	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Sicily Cem. 8 cl. 1	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Sicily Cem. 8 cl. 2	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Sicily Cem. 8 cl. 3	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Sicily Cem. 15 cl. 1	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11

<i>T. amphibia</i> Sicily Cem. 15 cl. 2	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Sicily Cem. 15 cl. 3	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Sicily Cem. 16 cl. 1	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Sicily Cem. 16 cl. 2	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Sicily Cem. 16 cl. 3	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Ute 3 cl. 2	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Ute 3 cl. 3	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Ute 3 cl. 5	59	30	12	6	4	8	74	13	73	22	35	20	11	6	18	44	8
<i>T. amphibia</i> Ute 11 cl. 1	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Ute 11 cl. 2	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> Ute 11 cl. 3	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> PCC 7515 EF178272	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. amphibia</i> SAG 74.79 EU196675	59	30	12	6	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>Tenebriella</i> sp. Fkv-3 EU196673	59	30	12	5	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>Tenebriella</i> sp. Fkv-4 EU196674	59	30	12	5	4	8	74	13	73	22	35	20	11	6	20	39	11
<i>T. curviceps</i> CCALA 1133 cl. 1	59	30	12	6	4	8	74	14	73	23	35	20	11	6	20	39	11
<i>T. curviceps</i> CCALA 1133 cl. 2	59	30	12	6	4	8	74	14	73	23	35	20	11	6	20	39	11
<i>T. curviceps</i> CCALA 1133 cl. 4	59	30	12	6	4	8	74	14	73	23	35	20	11	6	20	39	11
<i>T. curviceps</i> CCALA 1133 cl. 6	59	30	12	6	4	8	74	14	73	23	35	20	11	6	20	39	11
<i>T. curviceps</i> S23 cl. 2	59	30	12	6	4	8	74	14	73	23	35	20	11	6	20	39	11
<i>T. curviceps</i> S23 cl. 3	59	30	12	6	4	8	74	14	73	23	35	20	11	6	20	39	11
<i>T. curviceps</i> S23 cl. 6	59	30	12	6	4	8	74	14	73	23	35	20	11	6	NA	NA	NA
<i>T. curviceps</i> S23 cl. 8	59	30	12	6	4	8	74	14	73	23	35	20	11	6	20	NA	NA
<i>T. curviceps</i> S23 cl. 9	59	30	12	6	4	8	74	14	73	23	35	20	11	6	20	39	11
<i>T. curviceps</i> S23 cl. 10	59	30	12	6	4	8	74	14	73	23	35	20	11	6	20	39	11
<i>Lyngbya</i> sp. MA2 AB857842	57	27	12	6	4	8	74	15	73	24	35	21	11	6	22	NA	NA
<i>Lyngbya</i> sp. MaedaD5 AB863124	57	27	12	6	4	8	74	15	73	24	35	21	11	6	22	NA	NA
<i>D. pleousa</i> FFPI3-13 MF167701	57	30	12	6	4	8	74	16	73	22	35	25	11	6	22	45	14
<i>T. erythraeum</i> IMS 101 CP000393	59	34	12	6	4	8	74	14	73	21	35	21	11	6	22	44	18
<i>O. hirsuta</i> PAB-10-Feb-10-1 KC992978	59	26	12	6	4	7	74	13	73	19	36	20	11	6	22	48	13

Chapter 4

Detailed characterization of the *Arthrospira* type species separating commercially grown taxa into the new genus *Limnospira* (Cyanobacteria)

Nowicka-Krawczyk P., Mühlsteinová R. & Hauer T. (2019): Scientific Reports 9.



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Detailed characterization of the *Arthrospira* type species separating commercially grown taxa into the new genus *Limnospira* (Cyanobacteria)

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The genus *Arthrospira* has a long history of being used as a food source in different parts of the world. Its mass cultivation for production of food supplements and additives has contributed to a more detailed study of several species of this genus. In contrast, the type species of the genus (*A. jenneri*), has scarcely been studied. This work adopts a polyphasic approach to thoroughly investigate environmental samples of *A. jenneri*, whose persistent bloom was noticed in an urban reservoir in Poland, Central Europe. The obtained results were compared with strains designated as *A. platensis*, *A. maxima*, and *A. fusiformis* from several culture collections and other *Arthrospira* records from GenBank. The comparison has shown that *A. jenneri* differs from popular species that are massively utilized commercially with regard to its cell morphology, ultrastructure and ecology, as well as its 16S rRNA gene sequence. Based on our findings, we propose the establishment of a new genus, *Limnospira*, which currently encompasses three species including the massively produced *L. (A.) fusiformis* and *L. (A.) maxima* with the type species *Limnospira fusiformis*.

Among the simple trichal cyanobacteria, three genera possess helically-coiled trichomes as a prominent diacritical feature: *Spirulina* Turpin ex Gomont 1892, *Halospirulina* Nübel, Garcia-Pichel et Muyzer 2000, and *Arthrospira* Stizenberger ex Gomont 1892. The latter is a widely-known taxon with long history of use as a food source, for example as dihé in Africa or tecuitlatl in Mexico^{1–3}. Presently, the members of this genus are still used as food, but a substantial part of *Arthrospira* production is sold in a form of food supplements or food additives^{4,5}; furthermore, under the commercial name of *Spirulina*, it is the most widely-produced microalgae in large-scale production⁶. Latest reports even mention the value of *Arthrospira* in bio-nanotechnology - the coiled shape of its trichomes and their gliding motility with rotation have been used as a biological matrix for creating biohybrid microbots for imaging-guided therapy⁷.

In most industrial applications, *Arthrospira* is used under the common name “Spirulina”, which may be easier to pronounce and remember, and thus appear more suitable from a marketing point of view. However, it is only remotely related to the true *Spirulina*, and despite great efforts, it was not possible to identify any facility producing any species of this genus anywhere in the world. Besides its marketing value, the name is used for historical reasons. In the nomenclatoric starting point for simple trichal cyanobacteria, Gomont⁸ mentions both the genera *Arthrospira* and *Spirulina*. However, *Arthrospira* was not accepted as a separate genus by Geitler⁹ and was hence merged with *Spirulina*. The issue of these two genera was well described by Komárek and Lund¹⁰, and by Sili and others⁶. Nowadays, a wide array of studies have confirmed that *Arthrospira* and *Spirulina* represent two independent genera, each classified in different orders, and apart from possessing helically-coiled trichomes, the two share a minimal morphological resemblance¹¹.

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One of the most prominent morphological features of the genus *Arthrospira* is the spiral coiling of multicellular trichomes with easily visible cross-walls^{6,12}. The distance between spirals and width of individual coils, both often mentioned in literature as a typical attribute of particular taxa, cannot be considered a permanent morphological feature, since it may change in each trichome. Some authors even describe the ability of this genus to form completely straight and linear trichomes^{13,14}. Twenty-three species, either forming mats or living as solitary trichomes, are currently taxonomically accepted within *Arthrospira*^{12,15}. Three species are marine, three come from waters with elevated pH and/or electric conductivity, and the rest are freshwater types. The species have been recorded in all continents except Antarctica. In warmer regions, the planktic types often form heavy blooms and are traditionally harvested by local people for direct consumption⁷.

According to the current taxonomic classification, *Arthrospira* is one of the nineteen genera in the Microcoleaceae family within the order Oscillatoriales¹¹. Even though several recent studies have dealt with the phylogenetic relationship of *Arthrospira* to other taxa^{11,16}, none of these works include molecular data for the type species *A. jenneri* Stizenberger ex Gomont. Unlike more popular species cultivated commercially, *A. jenneri* originates from freshwater ecosystems with low salinity¹⁷. Strains/organisms determined as *A. maxima* Setchell & Gardner, *A. fusiformis* (Voronichin) Komárek & Lund, and *A. platensis* Gomont have often been isolated from highly alkaline and saline inland habitats in tropical and subtropical climate zones^{18,19}; this knowledge, combined with the lack of information on the type species incorrectly suggests that the preference for high pH and high salinity and/or electrical conductivity is a general feature shared by the entire genus⁸. To better understand not only the ecological requirements of the whole genus *Arthrospira* but also its relationships with other taxa within the order Oscillatoriales, it is necessary to study the type species *A. jenneri* to which the genus name is connected in more detail.

In June 2015, a phytoplankton biodiversity survey of an urban reservoir in Poland (Central Europe) revealed the presence of small mats of *A. jenneri* floating in the water column. Further observations found a thick layer of the organism to be covering the whole bottom of the reservoir. Since then, the presence of *A. jenneri* has been constant: in winter, it creates a visible benthic mat under the ice cover, while in summer, when the air temperature increases to 28 °C, it detaches from the bottom of the reservoir and large lobes of its mat float to the surface.

The present study investigated the relationship of the type species of the genus *Arthrospira*, i.e. *A. jenneri*, to the massively-produced species *A. fusiformis* and *A. maxima*, using traditional and molecular methods. Based upon our findings, we amend the description of the genus *Arthrospira* and delimit the commercially produced species to a new genus, *Limnospira*.

Results

Arthrospira jenneri was first mentioned under the name *Spirillum jenneri* by Hassall in 1845 from Tunbridge, UK. The genus itself was later established by Stizenberger²⁰, who compared his own collections from a pond in vicinity of Konstanz, Germany with Hassall's¹⁷ description. Gomont⁶, in the literature designated as a starting point for the nomenclature of simple filamentous cyanobacteria, accepts the genus and indicates that this species can be found in various pools and garden lakes in different parts of Europe. Gardner²¹ designated *A. jenneri* as a type species of the whole genus *Arthrospira*, and thus connected the generic name with this organism. None of these authors suggest that *A. jenneri* should inhabit highly saline or alkaline waters. The morphology, ecology and to an extent, the geographical origin of the population of *A. jenneri* collected in Tomaszowska Reservoir, Poland, studied in this paper, correspond well with those given in the original descriptions in previous papers. It can hence be regarded as a reliable candidate for type species of *Arthrospira* in investigating its relationship to other species currently included in this genus.

Amended description of *A. jenneri* based on the studied population. *Morphology.* Trichomes are isopolar, cylindrical, spirally coiled or straight (Fig. 1, Fig. 2a–c). Trichomes of both types (spirally coiled and straight) are motile, unsheathed or with a thin, inconspicuous sheath, not or only slightly constricted at the cross walls and with rounded apical cells lacking calyptra or thickened cell wall (Fig. 1e–i; Fig. 2a,b). Cells of both trichome types are shorter than they are wide; 6–8 µm wide, 2.6–5.6 µm long. Both types of trichomes possess granulation at cross walls (Fig. 1g–i).

Ultrastructure. Cells possess radially-arranged thylakoids (Fig. 2e) and facultative gas vesicles forming aerotopes near the cross-walls (Fig. 2f–h).

Ecology. Benthic mats in standing waters in temperate zones with optimum growth between pH 6.8 and 7.5, and electrical conductivity between 250 and 445 µS.cm⁻¹.

Phylogenetic analyses. Phylogenetic analyses based on the 16S rRNA gene placed all sequences of *A. jenneri* from Tomaszowska Reservoir, Poland, within a well-supported clade well distant from all other sequenced genera (Fig. 3). With regard to the congruence of morphological and ecological data of the sequenced population with the original and starting point descriptions of the type species, this clade represents the genus *Arthrospira*. Maximum Likelihood and Bayesian inference placed the genus *Planktothrix* within the close vicinity of *Arthrospira*. All sequences of widely-consumed taxa in public sequence repositories determined as *A. platensis*, *A. maxima*, or *A. fusiformis* were grouped together with *A. erdosensis*, *A. indica* and *A. jenneri*, in a well-supported clade, distant from *Arthrospira*. The similarity between the sequences included in these two clades is below 91.21%.

Based on this information, we propose the establishment of a new genus, *Limnospira*, containing, *inter alia*, the mass-produced species of former "*Arthrospira*". The sequence of *A. jenneri* (GQ184185) located in the *Limnospira* clade originates from organism inhabiting alkaline lakes, and thus cannot represent true *A. jenneri*. All performed analyses suggest that the new genus *Limnospira* is related to two recently-described genera, *Limnoraphis*

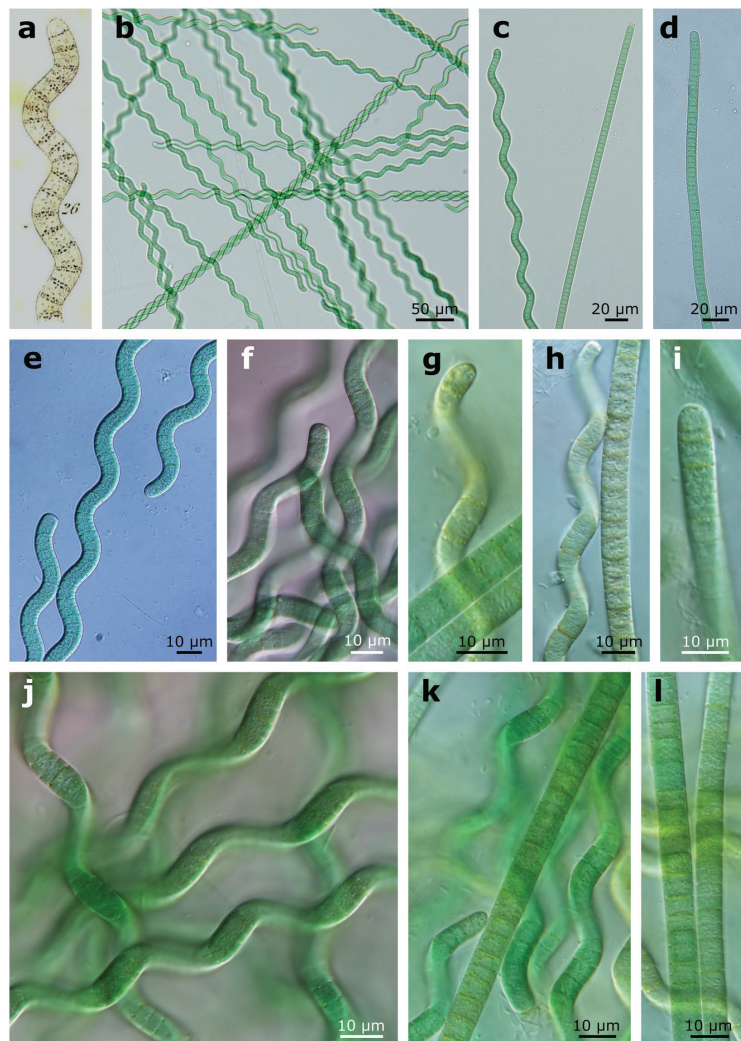


Figure 1. The morphology of *Arthrospira jenneri*, (a) original drawing of *A. jenneri* by Gomont⁸, (b–l) morphology of straight and spiral trichomes of *A. jenneri*'s population from Tomaszowska Reservoir in LM.

(freshwater) and *Neolyngbya* (marine), together with the *Lyngbya aestuarii* PCC 7419 strain originating from a salt marsh in the USA (Fig. 3).

***Limnospira* gen. nov.**
 Class: Cyanophyceae
 Order: Oscillatoriales
 Family: Microcoleaceae

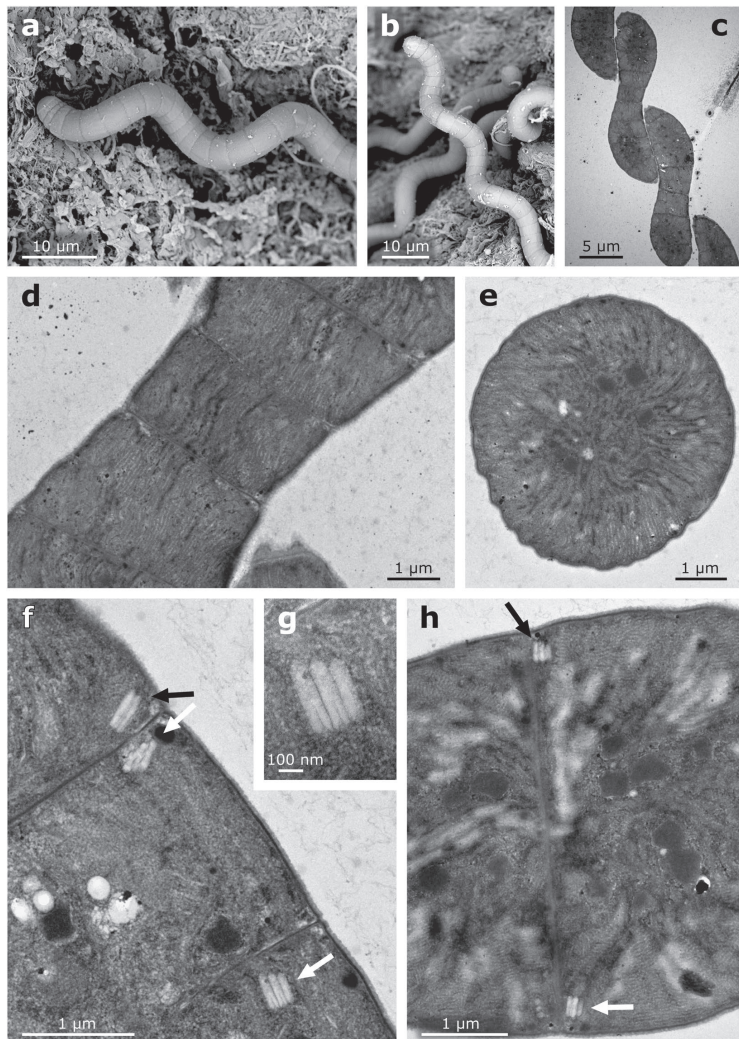


Figure 2. The morphology and ultrastructure of *Arthrospira jenneri* under electron microscope, (a,b) morphology of the trichomes in SEM, (c–h) ultrastructure of the trichomes in TEM, aerotopes indicated by arrows, (g) detail of an aerotope.

Morphology. Trichomes isopolar, cylindrical, regularly spirally coiled with a tendency to loosening the coils, unbranched, more or less tapering towards the ends, not or slightly constricted at cross-walls, blue green or dark green. Cells always shorter than wide. Terminal cells rounded or subcapitate with thickened outer cell wall or calyptra (Figs 4d,e,h,i,m,n,p–r and 5e,f).

Ultrastructure. Thylakoids have an irregular arrangement (Fig. 5). Aerotopes are facultatively present.

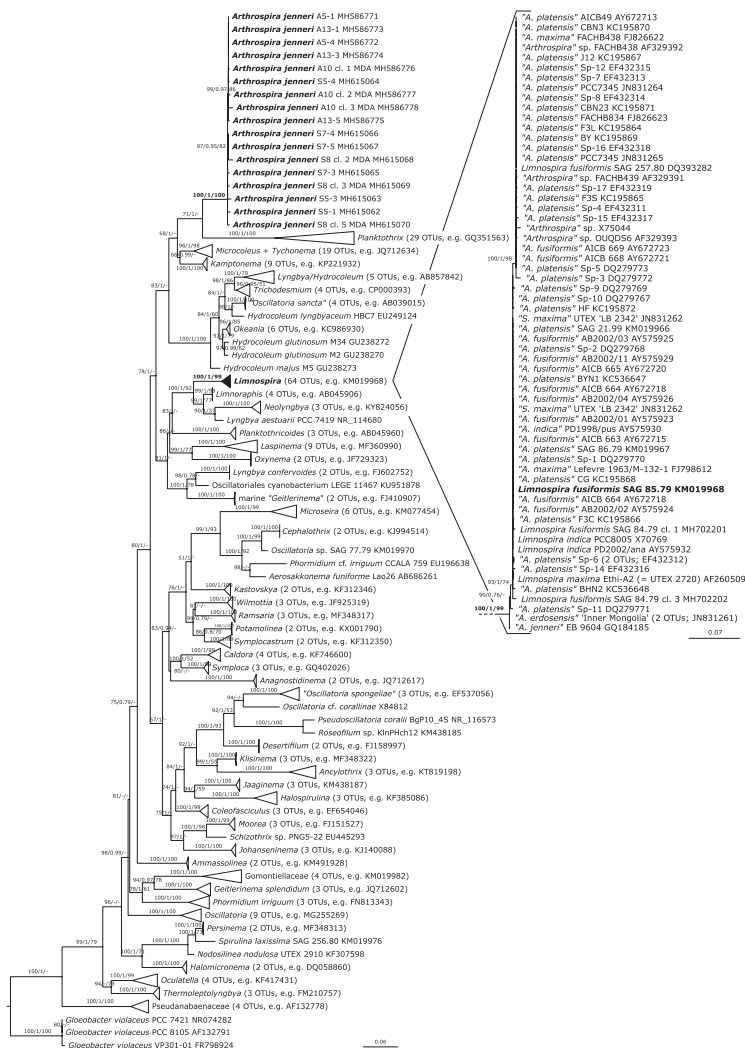


Figure 3. Phylogenetic tree based on 1800 bp fragment of the gene for 16S rRNA, including 291 sequences and showing the position of the genera *Arthrospira* and *Limnospira*. Branch support values (above 50 or 0.75) are shown as Maximum Likelihood, Bayesian posterior probability, and Maximum Parsimony bootstrap values, respectively. Note to Fig. 3: Quotation marks indicate the uncertainty of the real name of the sequenced organism.

Ecology. Planktic in freshwater as well as in water with higher salinity, most of the species known so far prefer habitats with elevated pH and electrical conductivity.

Type species. *Limnospira fusiformis* (Voronichin) comb. nov.

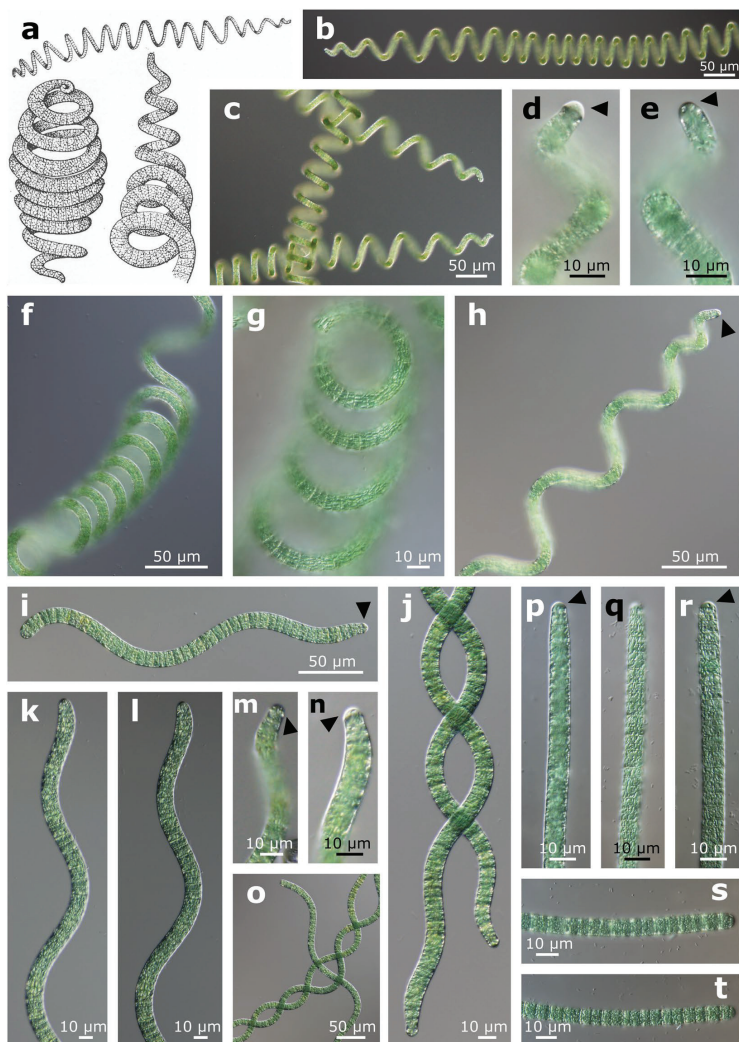


Figure 4. The morphology of *Limnospira*, (a) original drawing of *L. fusiformis* (*Spirulina fusiformis*) by Voronichin²², (b–h) morphology of the *L. fusiformis* reference strain SAG 85.79 in LM (i–o) morphology of the *L. fusiformis* strain SAG 84.79 in LM, (p–t) morphology of the *L. fusiformis* strain SAG 257.80 in LM; black triangles point out calyptra/thickened cell wall.

Etymology. Limni- [Gr., λίμνη], lake, lagoon; -speira [Gr., σπείρα] - spiral; the generic name refers to the morphology of trichomes and typical habitat, i.e. lakes.

***Limnospira fusiformis* (Voronichin) comb. nov.** Basionym. *Spirulina fusiformis* Voronichin 1934, Trudy Soveta Izucheni Prirodnich Resursov, Ser. Sibirsk. 8: 182²² Reference strain: SAG 85.79 = CCALA 026 = UTEX 2340 (Fig. 4b–h, Fig. 5a–c)

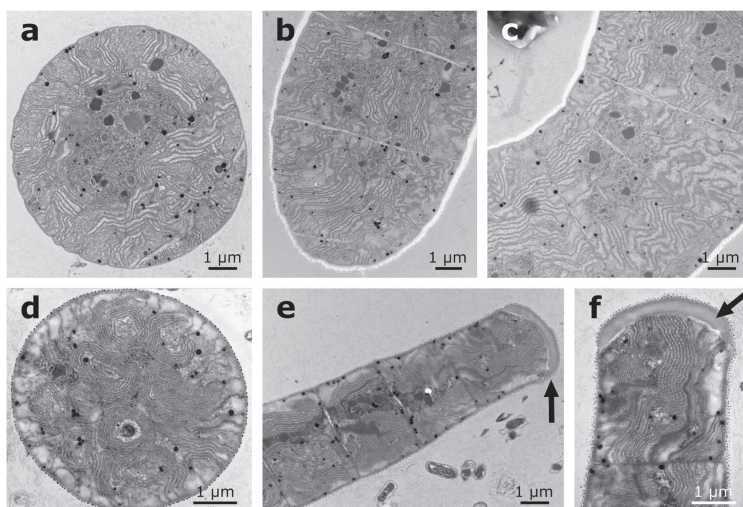


Figure 5. The ultrastructure of *Limnospira* in TEM, (a–c) the reference strain of *L. fusiformis* SAG 85.79, (d–f) the strain of *L. fusiformis* SAG 257.80, black arrows point out the calyptra/thickened cell wall at the end of the trichome.

***Limnospira maxima* (Setchell et Gardner) comb. nov.** Basionym. *Arthrospira maxima* Setchell et Gardner 1917, Univ. Calif. Publ. Bot. 6: 377²³ Reference strain: UTEX 2720 (sub. “*A. fusiformis*” Ethi-A2).

***Limnospira indica* (Desikachary et Jeeji Bai) comb. nov.** Basionym. *Arthrospira indica* Desikachary et Jeeji Bai 1992, ETNA Nat. Symp., Madras: 15²⁴ Reference strain: PCC 8005.

Discussion

Our phylogenetic analyses allowed us to group former *Arthrospira* sequences from main public repositories (64 OTUs) into a single homogenous cluster, closely related to marine *Neolyngbya*, halophilic *Lyngbya aestuarii* PCC 7419 and freshwater cyanobacteria from the genus *Limnoraphis*; while the sequences obtained from both the spiral and straight trichomes of *A. jenneri* (17 OTUs) formed a coherent cluster related to the freshwater genus *Plankothrix* which were distant from the newly-established *Limnospira* (Fig. 3).

Most strains/species forming the genus *Limnospira* are well known for their preference of alkaline habitats (e.g. *L. fusiformis*). This is also one of the characteristics that makes the mass production of the varieties widely used in the food industry easier and less prone to contamination by other organisms^{5,6,25,26}. Nevertheless, it seems that the ecological preferences of alkaline waters cannot be generalized for the whole genus. Based on our phylogenetic analyses, sequences representing *A. indica* also belong to the *Limnospira* clade (Fig. 3), and the authors of this taxon and Ballot and others²⁷ report that this species comes from a freshwater environment. As it was impossible to track the origin of all of the other sequences grouped in *Limnospira* in the present study, there may be more representatives of this genus that do not require elevated pH for their growth.

The arrangement of thylakoids within the cells and the presence of calyptra/thickened cell wall in apical cells represent more stable and reliable features that can be used to distinguish *Arthrospira* from *Limnospira*. The ultrastructural investigations revealed that *Arthrospira* contains radially-arranged thylakoids (Fig. 2), while *Limnospira* possesses many irregular, whirl-like sections across the central part of the cell (Fig. 5). Whereas the number or density of thylakoids may be modified by environmental factors, the arrangement is very stable in genetic clusters within the 16S rRNA gene^{28,29}. Furthermore, the presence of a thickened cell wall or calyptra seems to be present only in the genus *Limnospira* and was not observed in any of the *Arthrospira* trichomes, nor mentioned by the author of the original description of *A. jenneri*.

The current work proposes that only three former *Arthrospira* species be transferred to the newly-established genus *Limnospira*: *L. fusiformis*, *L. maxima* and *L. indica*. All three possess credible sets of morphological, molecular and ecological data on which their transfer can be based. Unfortunately, many other taxa currently classified as *Arthrospira* lack such necessary data. Despite the fact that many of the sequences forming the *Limnospira* clade are labelled as “*A. platensis*” (Fig. 3), this determination could not be verified by our results. According to the literature, *A. platensis* Gomont represents a benthic organism described from Uruguay with rare, relatively small and inconspicuous broadly rounded calyptras and probably no aerotopes^{10,30}. Hence, based only on the presence

of calyptra, this species should also be transferred to *Limnospira*. On the other hand, the benthic habitat and apparent absence of aerotopes would suggest a closer proximity to *Arthrospira*, as re-defined here. Regrettably, the available sequences designated as “*A. platensis*” do not seem to originate in the benthic habitats of Uruguay; on the contrary, all seem to represent planktic forms. Therefore, it is not certain whether the available molecular data really represents *A. platensis* and hence, whether the whole species should remain in *Arthrospira* or be transferred elsewhere.

Another example of unclear taxon requiring revision based on more detailed information is the species “*A. erdosensis*”, a type isolated from alkaline ponds in Erdos Plateau, Inner Mongolia, China. “*A. erdosensis*” has not been formally established and insufficient information is available to conclusively place it in a taxon. Hence, it was decided to present most of the names listed in the *Limnospira* clade, as currently named in GenBank/EML ENA/DDJB, with quotation marks to indicate the uncertainty of their proper name (Fig. 3).

Despite the fact that such high morphological variability occurs in cyanobacteria, and certain morphological features can be used for the identification of taxa, the use of trichome spirality as a diacritical feature on its own may be misleading. As reported by several authors¹⁴ *Limnospira* is capable of reversible straightening and our results have shown the same for *Arthrospira* sensu stricto. This raises the question whether Hassall’s¹⁷ note in the very first description of *A. jenneri* “...mixed up with different species of *Oscillatoria*” may apply to straight trichomes of the same species of *Arthrospira*. Non-heterocytous cyanobacteria exhibiting spiral trichomes were divided into three distinct genera outside of the order Synecococcales: the true genus *Arthrospira*, the closely-related *Planktothrix* (Fig. 3), which also contains species characterized by both straight (e.g. *P. agardhii*) and spiral (e.g. *P. spiroides*) trichomes, and the designated genus *Limnospira*. However, none of these genera seem to possess exclusively spiral trichomes, all of them either contain taxa with both coiled and straight trichomes, or the organisms themselves are able to change from one form to another. The only genus where the presence of straight and spiral trichomes has not yet been reported are *Halospirulina* and *Spirulina*, classified to the Synecococcales; however, partial straightening of trichomes has been observed in this case (Komárek pers. comm.). We therefore hypothesize, that some described species of the genus *Oscillatoria* Vaucher ex Gomont may represent the straight trichomes of *Arthrospira* or *Limnospira*, e.g. *Oscillatoria curvipes* Agardh ex Gomont.

The unsuitability of employing trichome spirality as a diacritical feature has also been demonstrated by strain SAG 31.96 maintained in the culture collection under the name *Arthrospira massartii*. Molecular and detailed morphological analyses place this strain within the genus *Planktothrix* Anagnostidis et Komárek and it probably represents species *P. spiroides* originally described from pond in China³¹. Since the strain SAG 31.96 was isolated from a pond with *Microcystis* spp. bloom (<http://sagdb.uni-goettingen.de>), the ecology does not seem to correspond with that described for *A. massartii* (i.e. clear springs). Hence, based on morphological and ecological data given in previous literature, *A. massartii* may truly belong to the genus *Arthrospira*^{23,32}. However, to ultimately confirm whether the organism corresponds closely with the original description, with regard to its morphological and ecological characteristics, it needs to be sequenced.

The long history of the utilization of *Limnospira* under the commercial name “Spirulina” has resulted in many incorrect taxonomic designations. Despite the substantial number of scientific papers highlighting the clear separation of *Arthrospira* from *Spirulina*, *Arthrospira/Limnospira platensis* still occurs in many scientific studies under the invalid designation of “*Spirulina platensis*”^{33–35}. Despite its value as a marketing tool, being easier to sell dietary products as “Spirulina” than “Arthrospira”, which calls to mind arthrosis, there is no scientific justification for using this invalid designation. Komárek²⁹ argues that such common practice is a serious problem in modern science, and not using the formal nomenclatoric rules undermines the validity of research papers. Unfortunately, in some cases, culture collections also contribute to the confusion surrounding the species level nomenclature by offering strains with incorrect species designations: for example, UTEX LB 2720 is not *L. (A.) fusiformis* but *L. (A.) maxima* (according to Li and others³⁶) or SAG 85.79 is not *A. platensis* according to its morphology or ecology.

Such nomenclature is also often incorrectly used in the case of commercial products. Biomass producers as well as retail products manufacturers cannot adopt nomenclatoric changes as easily as the scientific community due to several reasons, such as legislation and the need to avoid confusion by the consumer. On the other hand, it would be reasonable to expect them to follow long term trends in taxonomy which may take place over several decades. The polyphasic approach applied recently in cyanobacterial diversity research has resulted in many changes among the genera, families and orders, revealing more authentic phylogenetic relationships. For environmental scientists and specialists in monitoring laboratories who rely almost solely on traditional morphological traits, the molecular approach allows the creation of new taxonomic units which would be impossible to recognize without at least detailed ultrastructural studies. Fortunately, no such analysis is required for the newly-established genus *Limnospira* described herein, since some of its diacritical features are recognizable under the light microscope. Nevertheless, since modern criteria have been accepted within the classification system and have been applied to cyanobacterial taxonomy, it is essential that scientific papers and reports adopt the modern approach and use the names consistent with formal nomenclature.

In conclusion, our detailed investigations of the type species of the genus *Arthrospira* based on a polyphasic approach revealed clear differences between *A. jenneri* and organisms produced under the common name “Spirulina”, hence the need for the establishment of a new genus. We therefore propose the name *Limnospira* for the new genus that encompasses species with high industrial utilization and potential.

Materials and Methods

Study material and environmental background. Tomaszowska Reservoir (51°43′48″N; 19°31′47″E), where a massive occurrence of *A. jenneri* was noted, is located in the margins of the city of Łódź (Central Poland). It is a flow-through man-made reservoir located on the Olechówka River, with a surface area of 1 ha, average depth of 1.75 m and capacity of 17500 square m³⁷. Hydrochemical data is presented in Table S1.

The *A. jenneri* samples were collected from the benthos of the reservoir once every two months for a year, beginning December 2016. Samples for analyses were taken from 25 cm² of the bottom sediment using a plastic frame and small shovel.

Fresh samples of *A. jenneri* were examined by light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). In addition, strains of “*A. platensis*” SAG 257.80, SAG 85.79 and “*A. maxima*” SAG 84.79 were analysed in TEM. The LM observations were performed using an Olympus BX51 light microscope equipped with Nomarski DIC optics and an Olympus DP71 digital camera. The surface of trichomes was visualized using a Phenom Pro-X Scanning Electron Microscope. For ultrastructural observations in TEM fresh sample was fixed in 6% glutaraldehyde, left for several hours at room temperature and washed with 0.05 M phosphate buffer (pH 7.2). Then the sample was postfixed with 2% osmium tetroxide in the same buffer and temperature for two hours and repeatedly washed. Trichomes were dehydrated in isopropanol at gradually increasing concentration series and embedded in Spurr's resin. Ultrathin sections were cut and contrasted with 2.5% uranyl acetate and analysed using a digital JEOL JEM-1010 Transmission Electron Microscope.

16S rRNA gene sequencing. Individual trichomes or trichome fragments were isolated from natural material using glass microcapillary as described in Zapomělová and others³⁸, and Mareš and others³⁹. Every trichome was washed in 6 drops of sterile TE buffer, subsequently placed to sterile 0.2 ml PCR tube and kept frozen (−20 °C) until further use. Three PCR tubes per morphotype were used for the molecular analyses to confirm that the same 16S rRNA is amplified from all of them. Direct PCR was applied to 2 of the PCR tubes using primers 359F⁴⁰ and 23S30R⁴¹ in 50 µl reaction volume. The PCR mix contained 25 µl of Plain PP Master Mix (Top Bio, Prague, Czech Republic), 1.5 µl of each primer (concentration 5 pmol·µl^{−1}), and 21 µl of PCR grade water. The cycling conditions were: 5 min denaturation at 95 °C, continued with 45 cycles of 94 °C for 1 min 30 s, 54 °C for 1 min 30 s, and 72 °C for 2 min, and final elongation at 72 °C for 10 min. The genomic DNA from the third trichome was amplified by the MDA method⁴² using Repli-g Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, followed by PCR using primers 16S27F/1D1^{41,43} and 23S30R⁴¹ in 20 µl reaction volume. The PCR mix contained 10 µl of Plain PP Master Mix (Top Bio, Prague, Czech Republic), 1.2 µl of each primer (concentration 5 pmol·µl^{−1}), and 6.6 µl of PCR grade water. The cycling conditions were: 5 min initial denaturation at 95 °C, continued with 40 cycles of 94 °C for 1 min, 57 °C for 45 s, and 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. After staining by Sybr Green (Lonza, Rockland, ME, USA) the PCR products were cut out of low melting agarose gel (1.5%, 60 V, 1 hour). The bands of interest were cloned into plasmids with pGEM[®]-T Easy vector system (Promega Corp., Madison, WI, USA). Three bacterial colonies containing the PCR product were purified from each plate and then sequenced commercially using following primers: T7 (5'-TAA TAC GAC TCA CTA TAG GG-3'), SP6 (5'-TAT TTA GGT GAC ACT ATA G-3'), and when needed also internal primer CYA781F(a)⁴⁰. The obtained sequences were submitted to the NCBI GenBank database (www.ncbi.nlm.nih.gov): MH586771-78 and MH615062-70. The sequences were assembled in Geneious version 9⁴⁴ (<http://www.geneious.com>).

Phylogenetic Analyses. For phylogenetic analysis, the 16S rRNA sequences of *A. jenneri* obtained by us together with other *Arthrospira* records and representatives from simple trichal groups available in GenBank, and the outgroup taxa - *Gloeobacter violaceus* (NR_074282; AF132791; FR798924) were aligned using MAFFT v. 7⁴⁵ (<http://mafft.cbrc.jp/alignment/server/>). A total of 291 sequences were included in the analysis. Phylogenetic calculations were performed using maximum likelihood analysis (ML) in IQ-TREE web server⁴⁶ (<http://iqtree.cibiv.univie.ac.at/>), Bayesian inference (BI) in MrBayes 3.2.2⁴⁷ and maximum parsimony analysis (MP) in MEGA 7⁴⁸. The ML tree was constructed applying the GTR + I + Γ model chosen according to Akaike Information Criterion provided by jModelTest 2 software⁴⁹ and 1000 bootstrap replicate searches were performed to evaluate the relative support of branches. The MP involved 1000 replicate searches using the tree bisection-reconnection (TBR) branch-swapping algorithm. A total of 1000 replications were run to evaluate the relative branch support.

Data Availability

The sequence data generated during the current study are available in the NCBI GenBank database (www.ncbi.nlm.nih.gov). Other data analysed during this study are included in this article and its Supplementary Information file.

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Author Contributions

P.N.K. - collected the material, performed environmental measurements, contributed to L.M. analyses, performed SEM observations, contributed to processing of natural material in molecular lab, contributed to manuscript preparations; R.M. - contributed to L.M. analyses, processed natural material in molecular lab, contributed to manuscript preparations, prepared figures and plates in manuscript; T.H. - performed TEM observations, performed phylogenetic analyses, contributed to manuscript preparations.

Additional Information

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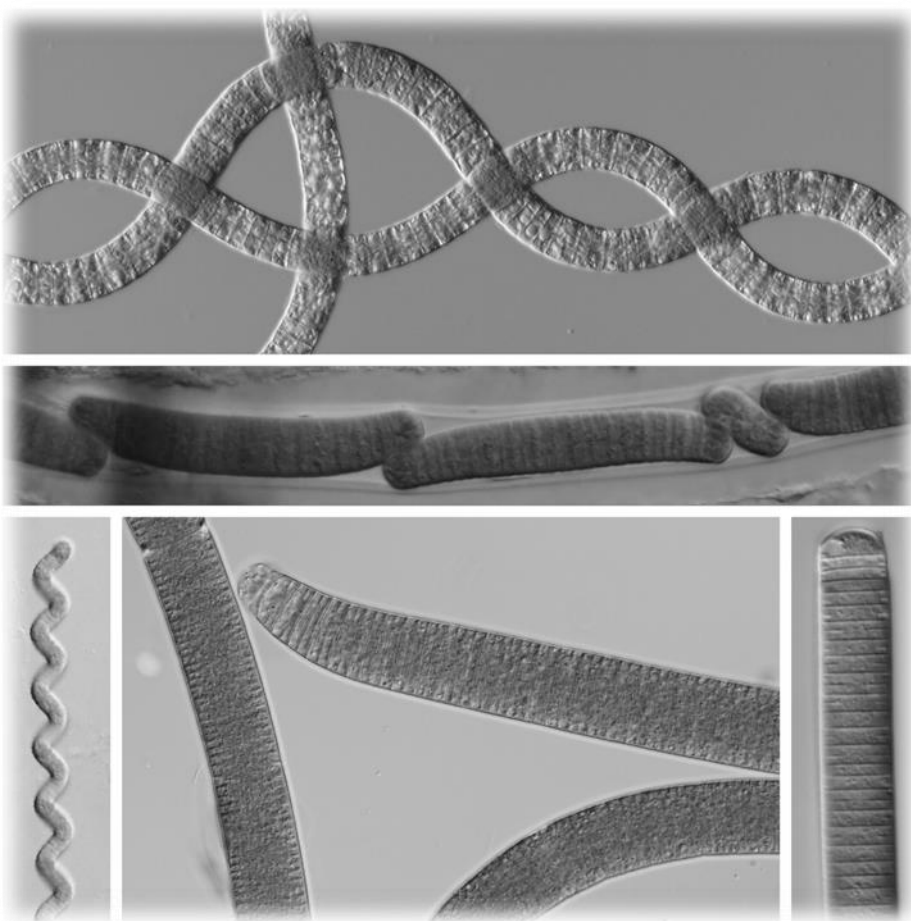
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Results and Conclusions



5. Results and Conclusions

Despite deepening our understanding of the cyanobacterial diversity in various parts of the world, our knowledge on the old, traditional taxa, and their placement in the current taxonomic system is often rather poor. To solve this issue, detail study of the type species, including the acquisition of the molecular data, is necessary. Unfortunately, in multiple genera the type species is either unclearly defined (e.g. *Phormidium* (Komárek and Anagnostidis, 2005)), or among the taxa that are not commonly found or are difficult to grow in cultures (e.g. *Petalonema* (Mareš et al., 2015)).

Lack of cultivable strains and certain scarcity of the type species prevented scientists from any more thorough revisions of the genus *Oscillatoria* for a long time. In **Chapter Two** we were able to connect the studied material based on the available information with the description of the type species *O. princeps*, and thus we outlined its phylogenetic position (Mühlsteinová et al., 2018). Subsequently, the long-debated position of the *Oscillatoria* sensu stricto could be established. This study was based on the 16S rRNA gene due to the widest availability of reference sequences for other cyanobacterial genera of the Oscillatoriales. However, in the following study in **Chapter Three** the multilocus analyses based on the concatenated alignment of 16S rRNA, *rpoC1*, and *rbcLX* loci proved the secluded position of the genus *Oscillatoria* from the other available data as well, even though fewer genera could be included in the dataset due to the limited availability of the molecular data (Hauerová et al., in rev.). Since the reference strain of *O. princeps* was designated and is available in the public culture collection (CCALA), it will be possible to gain additional molecular data as they will be probably necessary with the gradual shift towards the multilocus analyses (Komárek et al., 2014; Mareš, 2018; Mareš et al., 2019). Establishment of the *Oscillatoria*'s type species reference strain and availability of the molecular data will speed up not only the revisions of the genus *Oscillatoria* but represents the first step and important reference point in subsequent revisions also of the family Oscillatoriaceae and the whole order Oscillatoriales.

The phylogenetic position of *O. princeps* has been outlined, however, there are still many strains or sequences in public repositories that are called “*Oscillatoria*” despite their phylogenetic distance from the type species. On the other hand, there are many *Oscillatoria* species described, which have not been connected to any molecular data yet. Thus, only the species *O. princeps* and *O.*

kawamurae have been confirmed to belong to revised genus *Oscillatoria* based on the available 16S rRNA gene sequences (Mühlsteinová et al., 2018), suggesting that only those species possessing rather thick trichomes (at least 20 µm) may belong to this genus. Nevertheless, to confirm the presence of other species within this genus, molecular data will be necessary.

In **Chapter Three** one of the “general *Oscillatoria* groups” phylogenetically distant from the *Oscillatoria* sensu stricto was investigated in detail and subsequently described as a new genus *Tenebriella*. *Tenebriella*’s morphology corresponds overall to *Oscillatoria*, as well as its ability to create floating mats (Hauerová et al., in rev.). Nevertheless, the phylogenetic position of this group was distant from the core *Oscillatoria* based both on the 16S rRNA gene dataset that included wide range of the taxa currently classified within the order Oscillatoriales, as well as on the combination of the 16S rRNA, *rpoC1*, and *rbcLX* loci. Despite containing almost exclusively organisms from non-marine environment, it seems to be located among typically marine cyanobacteria such as *Hydrocoleum lyngbyaceum*, *Dapis* or *Okeania*.

Tenebriella currently contains two species – the type species *T. amphibia* that is newly described in this study, and *T. curviceps* that is based on the former *O. curviceps* regarding the available data. These two species differ from each other morphologically and their difference was supported also by analyses of the 16S-23S ITS region. The comparison of the secondary structures further revealed tentative presence of yet another species *Tenebriella* sp., whose presence was supported also by its separated position in the ITS alignment. Unfortunately, the strains representing this putative new species were lost, so their morphology could not be investigated, nor the new species could be formally described (Hauerová et al., in rev.).

Central Europe is among the regions where algae and cyanobacteria have been studied thoroughly for a long time. Yet, there is potential for findings of new taxa even in notoriously sampled freshwaters. Although *Tenebriella* was probably previously determined, based on its overall morphology, as *O. limosa*, it is a genus that is distinguishable from other described taxa when morphological and ecological information are combined and carefully examined (Hauerová et al., in rev.).

The detail research of this area thoroughly studied for several centuries can, however, not only broaden our knowledge of the present cyanobacterial

diversity and contribute to description of new taxa, but it can also considerably contribute to clarification of the taxonomic situation of the old traditional genera. This was shown in **Chapter Two** on the example of the genus *Oscillatoria* and in **Chapter Four** on the genus *Arthrospira*. The type species of each of these genera was described in pre-starting point times from Europe, but for long remained among the less studied representatives of these genera. *Arthrospira*'s type species *A. jenneri* was described in 1845 based on the specimen collected from Tunbridge in England (Drouet, 1968). Gomont (1892) later generalized that *A. jenneri* can be found in pools and lakes in various parts of Europe. The organism we studied corresponded well with all the information available from the literature, and thus could be used as a trustworthy representative of the type species to investigate its phylogenetic position and relationship to other taxa (Nowicka-Krawczyk et al., 2019). Similarly, as in case of *Oscillatoria*, the phylogenetic position of the type species seems to be distant from the other well-studied species. This distance resulted in proposal of the new genus *Limnospira*, where the commercially produced and so far studied species belong. The difference between *Arthrospira* and *Limnospira* was further supported by the divergent ecological preference of each of them, as well as minor morphological distinctions. Unlike the type species, most of the *Limnospira* species prefer saline or high pH habitats, create calyptas at the ends of their trichomes, and possess different thylakoid pattern. Each of such difference separately could be regarded as unimportant, but when taken into account together and in alignment with the phylogenetic information, they considerably support the delineation of these two genera.

Despite the morphology is often misleading when used on its own, it would be inaccurate to throw it off the table completely. First, it represents irreplaceable link when trying to connect the old descriptions with organisms of interest as was shown in previous chapters (Mühlsteinová et al., 2018; Nowicka-Krawczyk et al., 2019; Hauerová et al., in rev.). Second, in many cases the minor morphological or ultrastructural differences considered previously merely as a diacritical features of species have been proven to indicate different genera in the revised cyanobacterial system. In addition to the examples presented in the previous chapters, several other taxa can be further named such as: *Ocellatella* that was differentiated from *Leptolyngbya* based on the presence of red granules in the trichome tips (Zammit et al., 2012), *Nodosilinea* based on the presence of filament nodules under certain conditions (Perkerson III et al., 2011), *Limnoraphis* was distinguished from *Lyngbya* by the presence of aerotopes

(Komárek et al., 2013), and *Anagnostidinema* from *Geitlerinema* by the shape of the apical cells (Strunecký et al., 2017) for example.

To establish the phylogenetic position of the type species is crucial not only in terms of solving the phylogenetic placement of many traditional genera, it is important also with regard to delimitation of higher taxonomic units such as families and orders. The establishment of reliable phylogenetic reference points for species and genera is crucial also for reliable navigation through the quickly accumulating molecular data. With the growing importance of the genome sequences, it is essential to know where to look for the missing data when building phylogenies answering phylum-wide questions. Whereas the currently available cyanobacterial genomes allow insight to the cyanobacterial relationships (Mareš, 2018; Mareš et al., 2019), they still do not provide comparison as detail as the 16S rRNA data with regard to many genera missing in the genome-based phylogenies. Nevertheless, clarified reference points for type species, at least with the 16S rRNA characterization, allow to direct efficiently the sampling efforts filling the missing genomic data, especially if the reference strains of the species are established and available.

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Curriculum Vitae

Curriculum vitae

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Education

since 2013 **Doctoral study in botany (Ph.D.)**

Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

Thesis: Contribution to the taxonomic revision of the order Oscillatoriales.

Advisor: Doc. RNDr. Jan Kaštovský, Ph.D.

2017 **Rigorous exam (RNDr.)**

Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

Thesis: A peek into diversity of terrestrial cyanobacteria collected in San Gerardo de Rivas region, Costa Rica.

2011–2013 **Master study in biology (MSc.)**

Department of Biology, John Carroll University, Cleveland, OH, USA

Thesis: Microcoleus-like morphotypes from the soils of the Atacama desert, Chile.

Advisor: Prof. Jeffrey R. Johansen, Ph.D.

2008–2011 **Bachelor study in biology (Bc.)**

Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

Thesis: Aerophytic cyanobacteria from San Gerardo region, Costa Rica. [in Czech]

Advisor: RNDr. Tomáš Hauer, Ph.D.

Scientific employment

since 2019 Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

since 2017 Institute of Soil Biology, Biology Centre CAS, České Budějovice, Czech Republic

2015–2017 Institute of Botany CAS, Třeboň, Czech Republic

2014–2017 Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

2011–2013 Department of Biology, John Carroll University, Cleveland, USA

Teaching experience

- 2018 Weekend workshop for high school teachers: Determination course of cyanobacteria and algae. (organized by Culture collection of autotrophic organisms, Institute of Botany CAS, Třeboň and Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic – *sampling demonstration and microscopy; in Czech*)
- 2015–2017 Weekend workshop for high school teachers: “How to teach about algae.” (organized by Culture collection of autotrophic organisms, Institute of Botany CAS, Třeboň, Czech Republic – *lecture, field part, algae games; in Czech*)
- 2016 Weekend workshop for high school teachers: “The ecology of algae and cyanobacteria.” (organized by Culture collection of autotrophic organisms, Institute of Botany CAS, Třeboň, Czech Republic – *lecture, preparation of permanent diatom slides; in Czech*)
- 2015 Phycology (KBO/134 – *lab part, in Czech*)
Field work I. (FBI/002 - *two days of phycology, in Czech*)
- 2014 Introductory botany, phycology, and mycology (KBO/137 – *labs for the Phycology part, in Czech*)
Field Work I. (FBI/002 – *three days of phycology, in Czech*)
- 2012–2013 Graduate Teaching Assistant in Principles of Biology I (BL 157) and Microbiology (BL 310), *in English*

Grants and Awards

- 2018 BC Grant Program (IBERA), support of the international internship
- 2016 Grants-in-Aid of Research Program, Phycological Society of America
Title: Where to find the true Oscillatoria? Understanding the diversity and distribution of wider morphotypes in the genus Oscillatoria.
- 2013 Desert Legacy Fund, California Desert Research Program
Title: Sequencing and identification of cyanobacterial species from biological soil crusts in California wilderness areas.
- 2011–2013 Graduate Assistantship Scholarship, Graduate School, John Carroll University

Internships

- 2018 7 weeks at New Mexico State University, NM, USA
(Dr. Nicole Pietrasiak, Ph.D.)
- 2016 3 months at New Mexico State University, NM, USA
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Conferences

- 2016 The 20th Symposium of the International Society for Cyanophyte Research, University of Innsbruck, Innsbruck, Austria.
Mühlsteinová R., Hauer T.: Seeking the true Oscillatoria. [talk]
Hauer T., Mareš J., Mühlsteinová R. & Vondrášková A.: Phylogenetic position of the genus Kyrtothrix Ercegovic within the order Nostocales. [poster]
- 2013 The 19th Symposium of the International Society for Cyanophyte Research, John Carroll University, Cleveland, Ohio, USA.
Mühlsteinová R., Johansen J.R., Pietrasiak N., Martin M.P., Osorio-Santos K. & Warren S.D.: Polyphasic characterization of Trichocoleus desertorum sp. nov. (Pseudanabaenales, Cyanobacteria) from desert soils and phylogenetic placement of the genus Trichocoleus. [talk]
Mühlsteinová R., Johansen J.R., Pietrasiak N. & Martin M.P.: Polyphasic characterization of Kastovskya adunca gen. nov. et comb. nov. (Oscillatoriales, Cyanobacteria) from desert soils of the Atacama Desert, Chile. [poster]
- Midwest Ecology and Evolution Conference 2013, University of Notre Dame, South Bend, Indiana, USA.
Minerovic A., Mühlsteinová R., Johansen J. & Sgro J.: Diatoms as water quality indicators in Tinker's Creek, Ohio. [poster]

Publications

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