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

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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 *Oscillatoriophycideae*), 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 epitype 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× 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/centrostuditermalipietrodabano/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 (in the middle)	Locality	GPS Coordinates		Collection date	Sample description
O. princeps ■	**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**
O. princeps ■	NIVA-CYA 132 (19)	22–32 µm	Sundlaug, Hveragerdi area, Iceland	not found		April 1983	not found
O. princeps ■	NIVA-CYA 150	23–30 µm	Chao Phya River, Thailand	not found		28-11-1984	not found
O. princeps	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
O. princeps	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
O. princeps	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
O. princeps □	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
O. princeps ▣	Verde River 2	40–50 um	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
O. princeps □	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
O. princeps ▣	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
O. princeps □	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
*O. princeps**	ETS-06	40–50 µm	Montegrotto Terme, Italy	not found		May 2005	thermal mud
*O. kawamurae***	icclb20060001	60–80 µm	Lake Biwa, Otsu, Shiga, Japan	not found		2006	field specimen collected from lake water
O. princeps	SERB 35	not found	India	not found		not found	mangrove environment
Oscillatoria sp.	not found	not found	Waiahole Valley taro field, HI, USA	not found		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+Ă) 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:

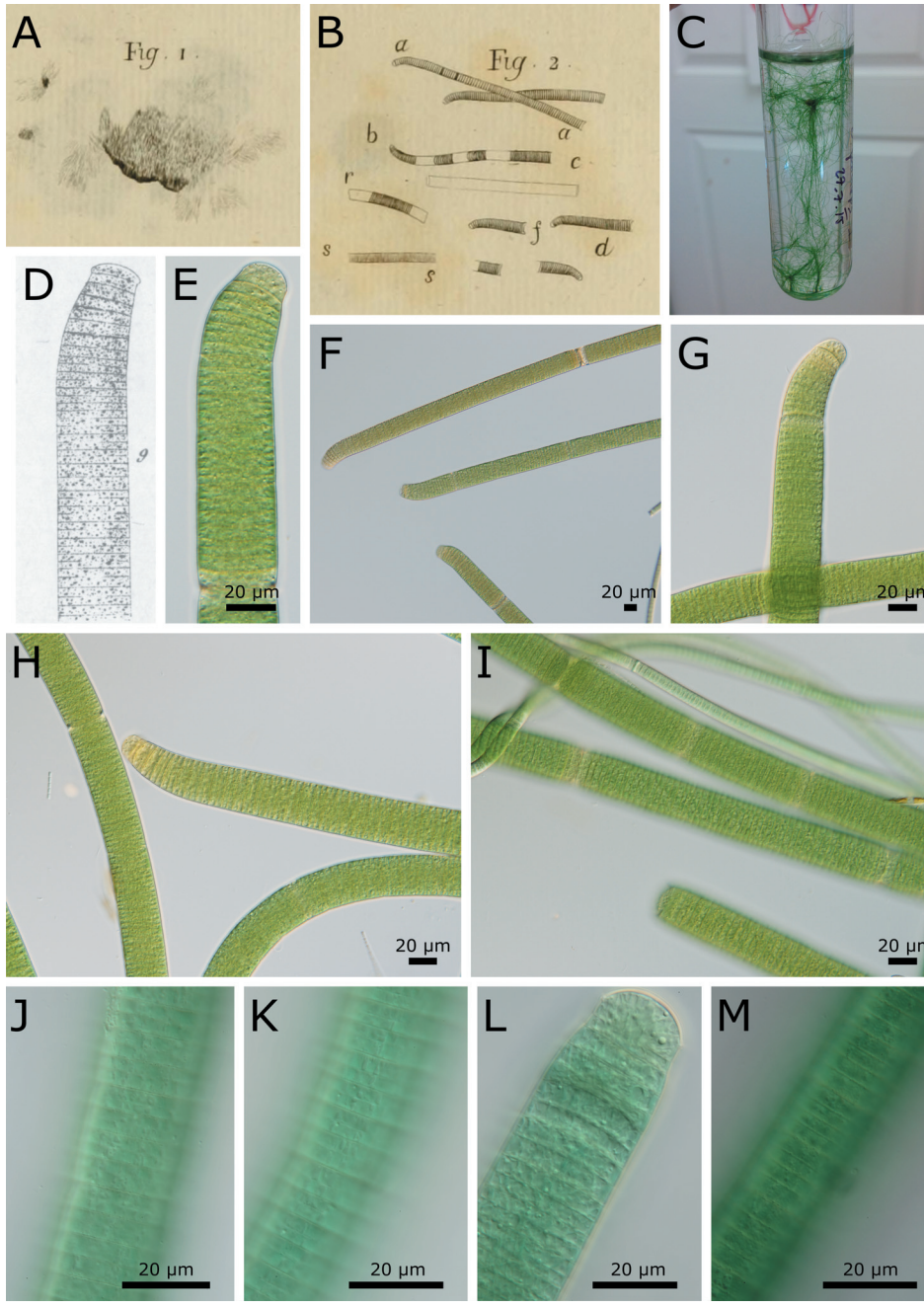

Fig. 1. – Morphology of the reference strain *Oscillatoria princeps* CCALA 1115 and its comparison with original drawings. (A) Macroscopic mat formed by *O. princeps* (Vaucher 1803). (B) Morphology of *O. princeps* as viewed in light microscope (LM) (Vaucher 1803). (C) Macroscopic view of filaments of 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**

M o r p h o l o g y: 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.

E c o l o g y: 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.

U l t r a s t r u c t u r e: 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 reminescent 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).

P h y l o g e n y: 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

M o r p h o l o g y: 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.

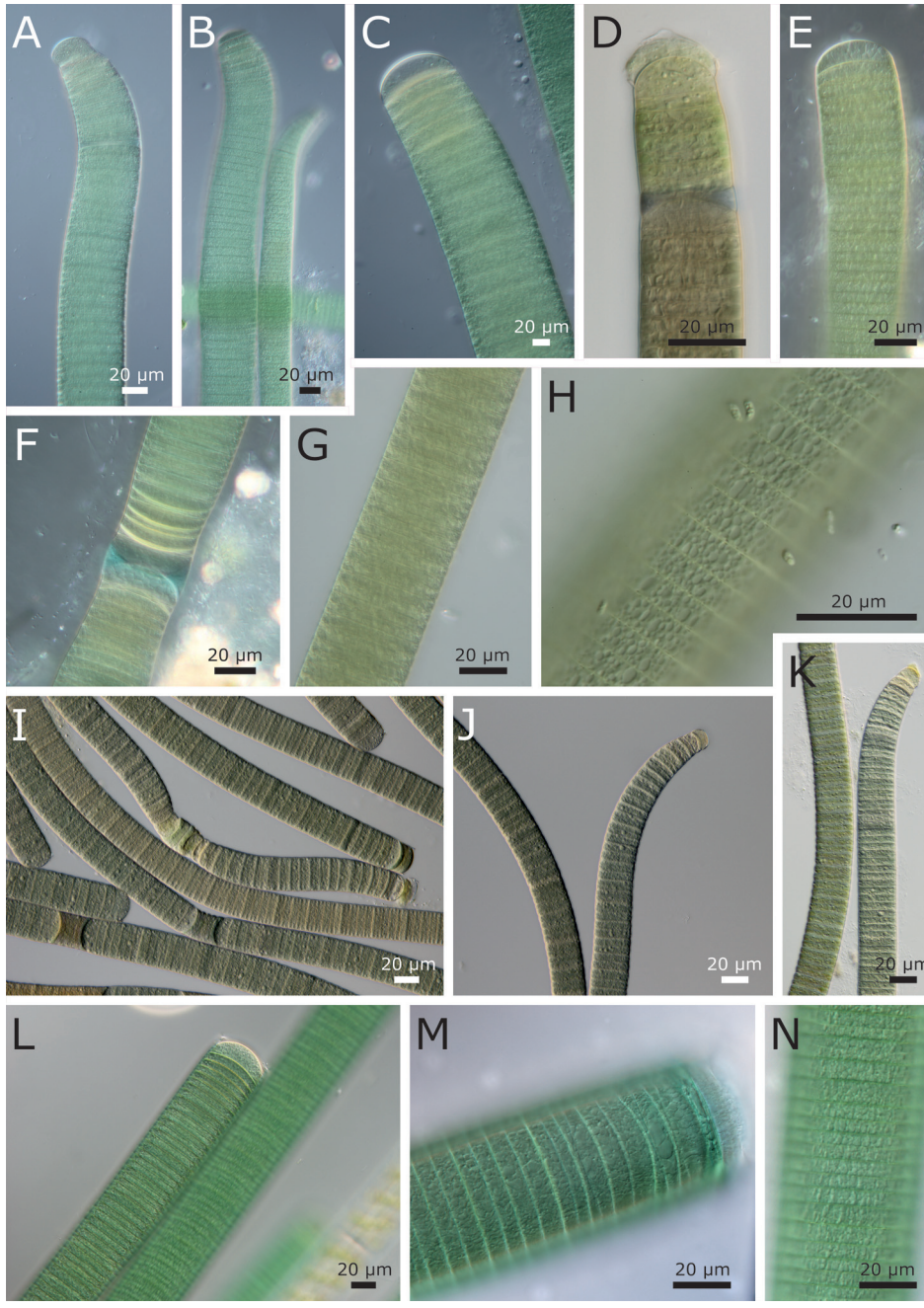

Fig. 2. – Morphology of different strains/fresh samples of *Oscillatoria* as viewed in light microscope. (A–C) *O. princeps* Rainbow Lake 2. (D) *O. princeps* NIVA-CYA 150. (E–H) *O. princeps* Parker Dam 2. (I–K) *O. princeps* NIVA-CYA 132. (L–N) *O. princeps* Strkovec.

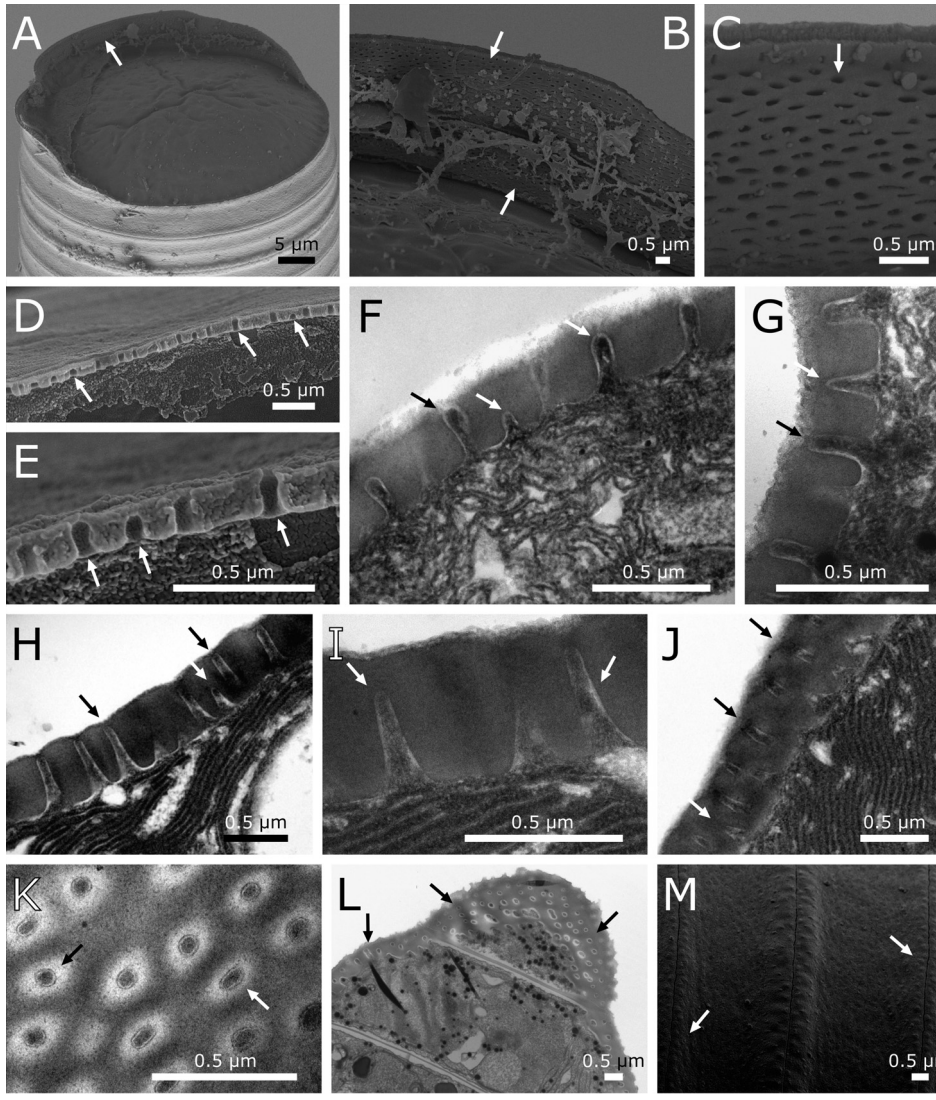

Fig. 3. – Large pores in thick cell wall of *Oscillatoria* visualized by the means of TEM and SEM. Arrows point out the position of the pores. (A–C) *O. princeps* Strkovec, SEM. (D–E) *O. princeps* CCALA 1115, SEM. (F–G) *O. princeps* CCALA 1115, TEM. (H–K) *O. princeps* Verde River 2, TEM. (L) *O. princeps*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

E c o l o g y: Blue-green mats first probably growing on the bottom of fresh-water reservoirs, later often released and floating at the water surface.

H o l o t y p e: 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).

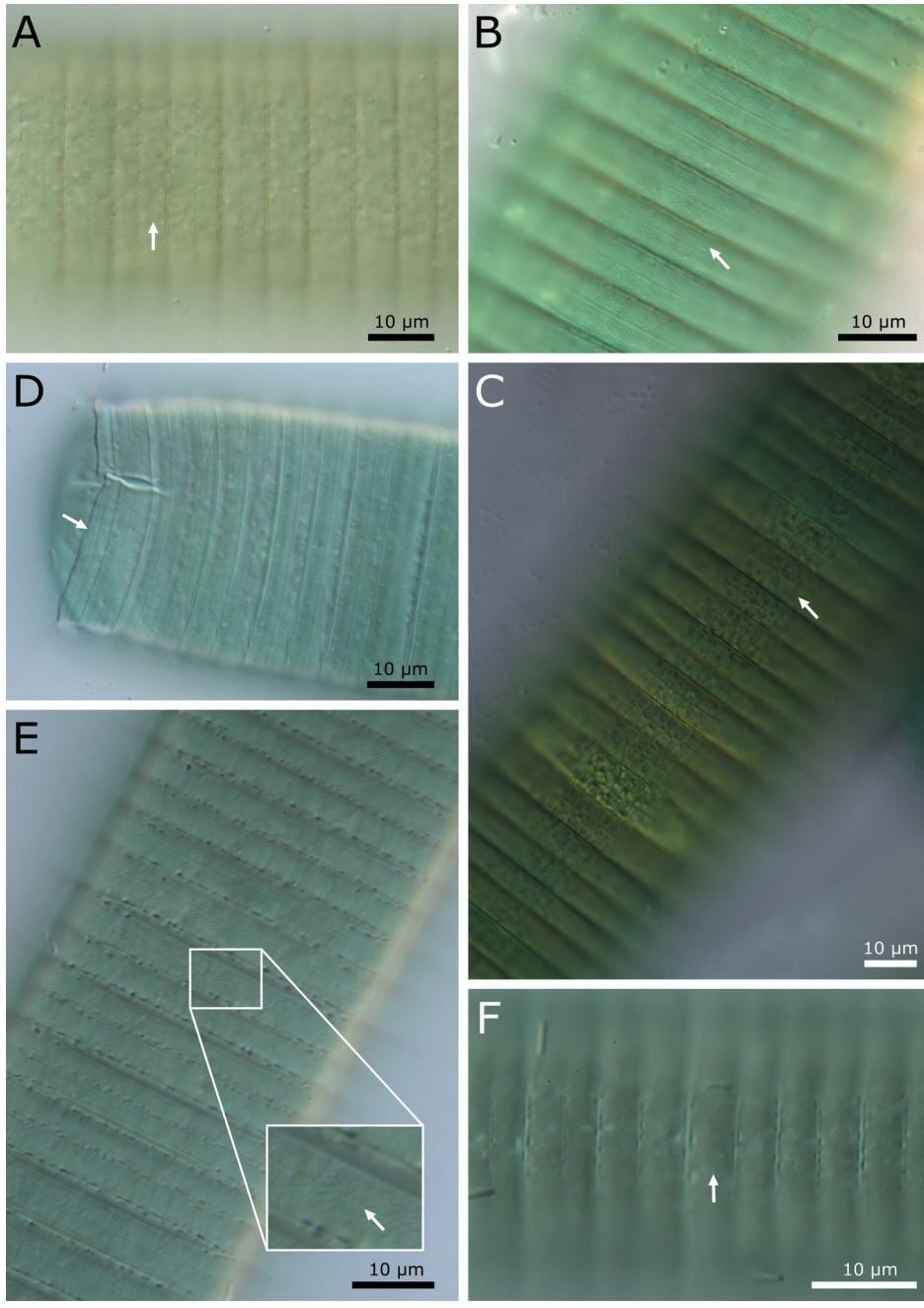

Fig. 4. – Detailed morphology of the surface of selected *Oscillatoria princeps* representatives visualized in light microscope. The presence of large pores in the thick cell wall can be noticed as roughly structured surface (arrows). (A) *O. princeps* Verde River 1. (B–C) *O. princeps* Strkovec. (D–F)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

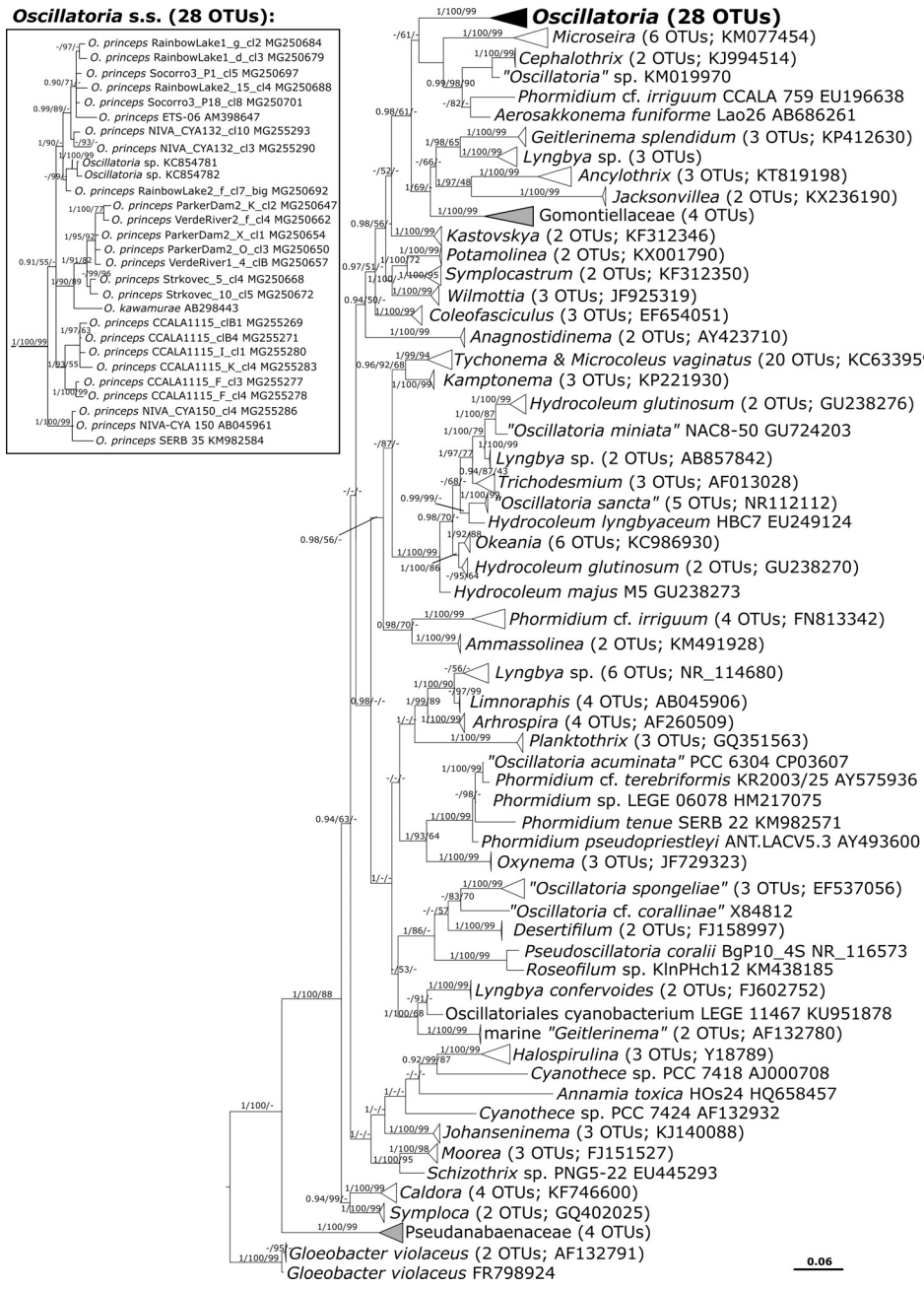

Fig. 5. – Phylogenetic analysis based on 184 sequences of the 1157 long fragment of the 16S rRNA gene. The position of the genus *Oscillatoria* is indicated by the black triangle. Branch support values are in following order: Bayesian posterior probability, Maximum Likelihood, and Maximum Parsimony bootstrap 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.

R e f e r e n c e s t r a i n: 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, Hoiczyk & 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 *Limnoraphis, 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–Limnoraphis*, *Microcoleus vaginatus* group–*Kamptonema*, 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 organizmu. V případě sinic mnoho typů existuje pouze ve formě obrázků, v lepším případě jako herbářové položky. S rychlým rozvojem mikroskopických metod a pokroku v molekulární biologii dochází k velkým změnám 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 nějž 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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