

## Phylogenetic Relationships of Some Filamentous Cyanoprokaryotic Species

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**ABSTRACT:** The polyphasic approach is the most progressive system that has been suggested for distinguishing and phylogenetically classifying Cyanoprokaryota (Cyanobacteria/Cyanophyta). Several oscillatoriacean genera (*Lyngbya*, *Phormidium*, *Plectonema*, and *Leptolyngbya*) have problematic phylogenetic position and taxonomic state because of their heterogeneity and polyphyletic nature. To accurately resolve the phylogenetic relationship of some filamentous species (*Nodosilinea bijugata*, *Phormidium molle*, *Phormidium papyraceum*), we have performed phylogenetic analyses based on 16S rRNA gene and the phycocyanin operon (PC-IGS) by using maximum-likelihood (ML) tree inference methods. These analyses were combined with morphological re-evaluation. Our phylogenetic analyses support the taxonomic separation of genus *Nodosilinea* from the polyphyletic genus *Leptolyngbya*. Investigated *Nodosilinea* strains always formed a coherent genetic cluster supported with a high bootstrap value. The molecular phylogeny confirmed also the monophyly of the *Wilmottia* group. In addition, data reveal that although *P. papyraceum* is morphologically similar to *Wilmottia murrayi*, this species is genetically distinct. Strains from the newly formed genus *Phormidesmis* and some *Phormidium priestleyi* strains were clustered in a separate clade different from the typical *Phormidium* species, but without strong bootstrap support.

**KEYWORDS:** Cyanoprokaryota, 16S rRNA gene, phycocyanin operon, phylogeny, systematics, taxonomy

**CITATION:** Stoyanov et al. Phylogenetic Relationships of Some Filamentous Cyanoprokaryotic Species. *Evolutionary Bioinformatics* 2014;10:39–49 doi: 10.4137/EBO.S13748.

**RECEIVED:** December 1, 2013. **RESUBMITTED:** January 12, 2014. **ACCEPTED FOR PUBLICATION:** January 19, 2014.

**ACADEMIC EDITOR:** Jike Cui, Associate Editor

**TYPE:** Original Research

**FUNDING:** Author(s) disclose no funding sources.

**COMPETING INTERESTS:** Author(s) disclose no potential conflicts of interest.

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### Introduction

Cyanoprokaryota (Cyanobacteria/Cyanophyta) are the most ancient and widespread group of photosynthetic prokaryotes, which had a major role in the evolution of the plant kingdom and Earth's atmosphere. They inhabit almost all biotopes including lakes, rivers, streams and reservoirs,<sup>1</sup> oceans and estuaries,<sup>2</sup> as well as extreme habitats such as hot springs,<sup>3–9</sup> alkaline lakes,<sup>10,11</sup> desert soils,<sup>12,13</sup> and even glaciers,<sup>14</sup> where their role as primary producers is of great importance.

Cyanoprokaryotic organisms (blue-green algae) are similar to both bacteria and algae, and have characteristics linking them to both groups. Like most bacteria, they are characterized by the absence of a differentiated nucleus and have a rigid cell wall containing murein. On the other hand, like plants and algae, they perform photosynthesis, releasing free oxygen. Traditionally, they were classified according to the International Code of Botanical Nomenclature using a combination

of phenotypic and ecological characters, but later on, these organisms were accepted as bacteria and classified according to the International Code of Nomenclature of Prokaryotes.<sup>15,16</sup>

Despite the long taxonomic history, the present state of the cyanoprokaryotic systematics is still complicated by the two separate systems, the traditional botanical system and the bacteriological system, which produce many misinterpretations and confusions.<sup>16,17</sup> Thus, within the last decade, the efforts of the nomenclatural specialists were directed to establish a unified nomenclatural system that would be acceptable for both bacteriologists and botanists. The polyphasic taxonomy, which includes a combination of morphological, ultrastructural, ecophysiological, biochemical, and molecular characters, was the most progressive system that has been suggested.<sup>15,17</sup>

The necessity of new data and re-evaluating criteria for continual revision of the present classification defines the combination of molecular and morphological characters as an



essential tool for correct identification of the cyanoprokaryotic organisms. The analysis of 16S rRNA gene sequences is the most commonly used approach for distinguishing and phylogenetically classifying Cyanoprokaryota and particularly the genus level.<sup>18</sup> In most cases, the clusters based on 16S rRNA gene sequences correspond to traditional, morphologically characterized genera.<sup>17</sup>

Cyanoprokaryota are generally classified into five orders, Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, and Stigonematales. This classification is based on the formation of filaments, heterocytes, akinetes, and true branches.

Order Oscillatoriales are filamentous prokaryotes without heterocytes and akinetes. The taxonomic state of this group is most problematic because of their heterogeneous and polyphyletic nature.<sup>19</sup> Many of the morphological characters that are used to distinguish the genera within the order are not stable and can be influenced by environmental conditions.<sup>20</sup> This defines the need to re-evaluate the taxonomic status of some genera (especially from the LPP-B group: *Lyngbya*/*Phormidium*/*Plectonema* and *Leptolyngbya*) applying the polyphasic approach.<sup>17,19,21,22</sup>

*Leptolyngbya* was created as a new genus<sup>23</sup> comprising several species of the traditional genera *Lyngbya*, *Phormidium*, and *Plectonema* with very thin trichomes (0.5–3.5 µm wide) and peripherally arranged thylakoids. The intrageneric classification of *Leptolyngbya* species is still difficult, because morphologically they are very simple and often the molecular analyses of 16S rRNA gene sequences show controversial phylogenetic position among the other cyanoprokaryotic species from the LPP group. Several authors<sup>18,20,22,24–29</sup> have already discussed the heterogeneity of this genus and suggest re-evaluation and revision of the *Leptolyngbya* species and those from the LPP group using the polyphasic approach, and taxonomic corrections for sequences published in the GenBank.

*Leptolyngbya bijugata* (Kong.) Anagnostidis et Komárek was described for the first time by Kongisser<sup>30</sup> as *Phormidium bijugatum* Kong. In 1988, after revision of the order Oscillatoriales, Anagnostidis and Komárek transferred this species to the newly created genus *Leptolyngbya*.<sup>23</sup> Later, based on morphological and ecological features, Perkerson et al proposed a new combination (*Nodosilinea bijugata* (Kong.) comb. nov.) although their analyses showed that its internal transcribed spacer (ITS) sequence and structure is very distinctive from the other *Nodosilinea* species.<sup>31</sup> The new genus *Nodosilinea* comprises four species separated from genus *Leptolyngbya*. This reopened the question about the taxonomic status of *L. bijugata*, which is not clearly supported and delimited by molecular analyses.

In 2009, another new genus (*Phormidesmis*) was separated from the genus *Phormidium* based on morphological, ultrastructural, and molecular analyses.<sup>32,33</sup> Currently, genus *Phormidesmis* comprises the tropical cyanobacterium *Phormidesmis* (formerly *Phormidium*) *molle* as a type species and Antarctic strains of the species *Phormidesmis* (formerly *Phormidium*)

*priestleyi*. The 16S rRNA gene sequences of these species that have been used for the phylogenetic analyses probably are not deposited in the GenBank because such information cannot be found, and therefore these data are not available for other researchers. Moreover, in fact, the taxonomical status of *Phormidium molle* is still unclear.

After taxonomic revision of 23 cyanobacterial strains isolated from Antarctica, which resemble to *Phormidium murrayi*, Strunecky et al.<sup>34</sup> transferred *P. murrayi* to a new genus named *Wilmottia*. Up to now, this genus includes only one species, *Wilmottia murrayi*. It has been noted that many other *Leptolyngbya* and *Phormidium* species (including *Phormidium papyraceum*) are morphologically similar to *W. murrayi* and could be generically identical with *Wilmottia*.<sup>34</sup> Therefore, the aim of this study was to re-evaluate the phylogenetic relationships and taxonomic status of *N. bijugata* (= *L. bijugata*, = *P. bijugatum*), *Phormidesmis molle* (= *Phormidium molle*) and *P. papyraceum* (similar to *W. murrayi*) by using DNA sequence analyses (16S rRNA gene, phycocyanin operon) and morphological features.

## Materials and Methods

### Cyanoprokaryotic strains and culturing conditions.

*P. bijugatum* PACC 8602 (= *N. bijugata*, = *L. bijugata*), *Phormidium molle* PACC 8140 (= *Phormidesmis molle*), *P. papyraceum* PACC 8600, *Phormidium autumnale* PACC 5522, and *Phormidium uncinatum* PACC 8693 were obtained from the Plovdiv Algal Culture Collection (PACC) at the Plovdiv University, Bulgaria. The strains were grown on Z-medium<sup>35</sup> under identical conditions at 22 °C, light intensity of 10–20 µmol photon s<sup>-1</sup> m<sup>-2</sup> provided by 40 W cool-white fluorescent tubes and a light/dark cycle of 12/12 hours for six weeks. To check the nodule formation capability, cultures were maintained under low-light conditions as described by Perkerson et al.<sup>31</sup>

### DNA isolation, PCR amplification, and sequencing.

Genomic DNA was extracted from 40 mg of fresh cyanoprokaryotic mass either by Genomic DNA Purification Kit (Fermentas Life Sciences) following the manufacturer's instructions or by using the xanthogenate-SDS (XS) extraction protocol.<sup>36</sup> DNA concentration and purity was measured on a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The integrity of the extracted DNA was visualized on an agarose gel by ethidium bromide staining and UV transillumination (MiniBIS Pro gel documentation system, DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

The 16S rRNA gene was amplified using primers pA (5'-AGAGTTTGATCCTGGCTCAG-3')<sup>37</sup> and B23S (5'-CTTCGCCTCTGTGTGCCTAGGT-3').<sup>38</sup> The intergenic spacer (IGS) and flanking coding regions of the phycocyanin locus were amplified using the primers PCβF (5'-GGCTGCT-TGTTTACGCGACA-3') and PCαR (5'-CCAGTACCAC-CAGCAACTAA-3').<sup>39</sup> PCR reactions were conducted using PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare,

Buckinghamshire, UK) where the final mixture contained 1.5 U of *Taq* DNA polymerase, 10 mM of Tris-HCl pH 9, 50 mM of KCl, 1.5 mM of MgCl<sub>2</sub>, 200 μM of each dNTP, 5 pmol of each of the two primers, 100 ng of genomic DNA, and water to a final volume of 25 μL (for IGS) or 50 μL (for 16S rRNA gene). Amplification was done in a TC-412 thermal cycler (Technique, Cambridge Ltd., UK) using the following programs: (1) for the 16S rRNA gene, initial denaturation for 5 minutes at 95 °C, followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 55 °C, and 1.5 minutes at 72 °C, and final elongation step of 5 minutes at 72 °C and (2) for the *cpcBA*-IGS region, the thermal cycling was performed with an initial denaturation at 94 °C for 5 minutes followed by 40 cycles of 94 °C for 20 seconds, 58 °C for 30 seconds, 72 °C for 1 minute with a final elongation step of 72 °C for 5 minutes. All PCR products were analyzed by electrophoresis in a 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer with GeneRuler™ 100 bp DNA Ladder Plus as the size marker (Fermentas Life Sciences), stained with ethidium bromide, and visualized under UV trans-illumination. After visualizing the bands under UV light, amplified products were cut out of the gel and purified using a DNA extraction kit (Fermentas Life Sciences). Purified products of 16S rDNA and *cpcBA*-IGS were sent for sequencing (Eurofins MWG Operon, Ebersberg, Germany). Sequencing was done employing the same primers used for PCR amplification. The sequences were deposited in the GenBank (National Center for Biotechnology Information (NCBI)) under the following accession numbers: KF770966 *N. bijugata* PACC 8602 (= *P. bijugatum*, = *L. bijugata*), KF770967 *Phormidium molle* PACC 8140 (= *Phormidesmis molle*), KF770968 *P. papyraceum* PACC 8600, KF770969 *P. autumnale* PACC 5522, and KF770970 *P. uncinatum* PACC 8693.

**Phylogenetic analyses.** Nucleotide sequences of 16S rRNA gene and the *cpcB*-IGS-*cpcA* locus of the phycocyanin operon (PC-IGS) obtained from DNA sequencing were compared with other cyanobacterial sequences from the NCBI database designated as *Phormidium*, *Wilmottia*, *Nodosilinea*, *Phormidesmis*, *Leptolyngbya*, or *Lyngbya* by using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). For the purpose of phylogenetic analyses, we have selected mainly NCBI sequences of identified and described members of these genera at the species level trying to avoid sequences of those members determined only at the generic level.

Multiple sequence alignment was performed using the ClustalW tool within alignment function of MEGA 5 phylogenetic package. Phylogenetic trees were computed by MEGA 5.2.2 using the minimum evolution (ME), maximum parsimony (MP), maximum-likelihood (ML), and neighbor-joining (NJ) algorithms.<sup>40</sup> All algorithms were performed with 1,000 bootstrap replicates. Nucleotide positions containing gaps and missing data were eliminated from the data set (complete deletion option). The evolutionary distances were computed using the maximum composite

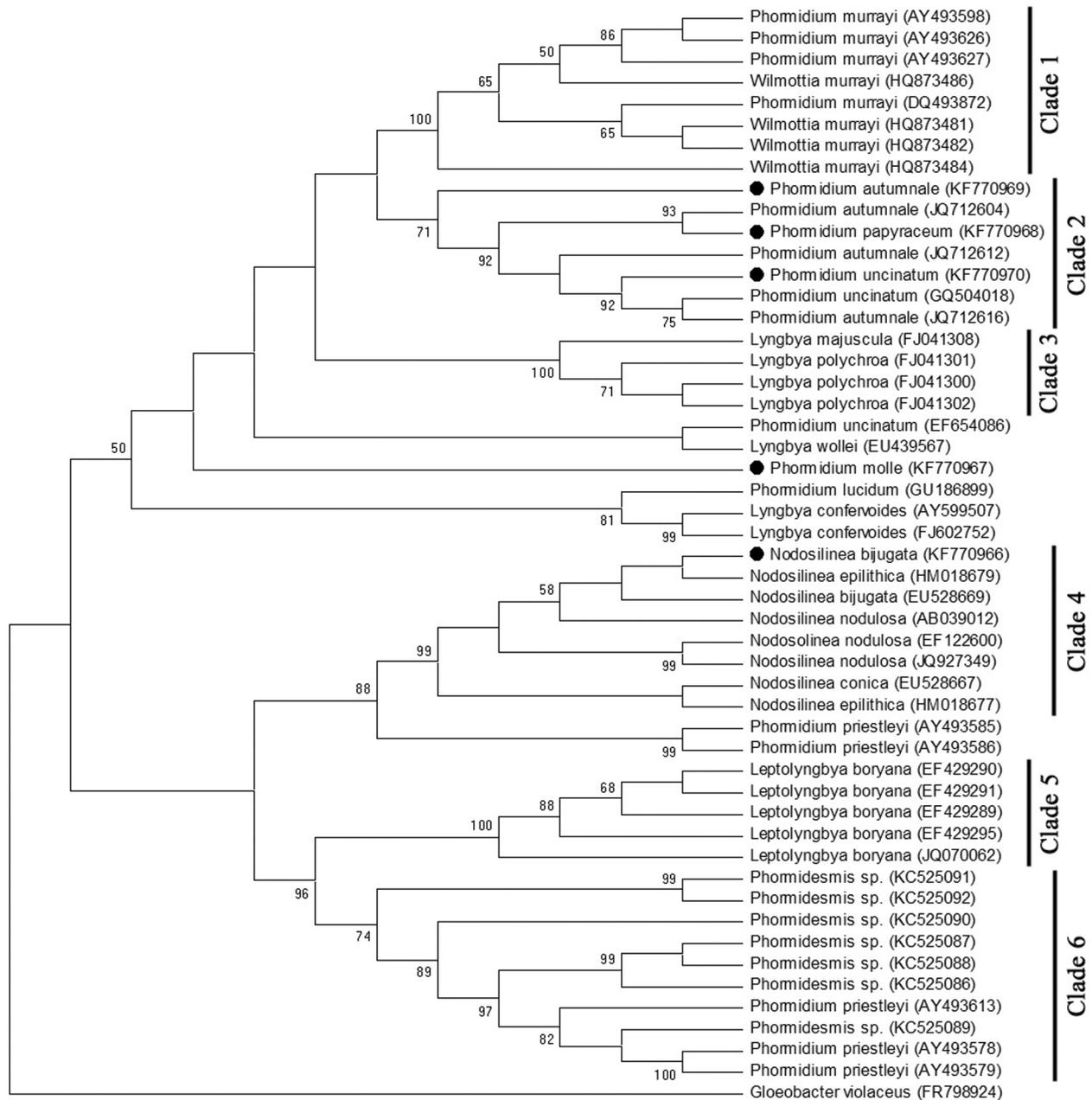
likelihood method. The ME trees were searched using the close-neighbor-interchange (CNI) algorithm. MP trees were generated applying the tree-bisection-reconnection (TBR) method at a search level of 1 with random tree addition. For ML, the general time reversible (GTR) model with corrected invariable sites (I), gamma distribution shape parameters (G), and nearest-neighbor-interchange algorithm were selected. *Gloeobacter violaceus* (FR798924) was selected as the outgroup taxon for the construction of trees with the 16S rRNA gene sequences. In the PC-IGS tree, *Cyanobacterium* sp. (AJ401183) was used as an outgroup.

**Morphological characterization.** Morphological investigations were performed using a Magnum-T microscope equipped with 3 Mpx high definition digital camera Si-3000 and software (Medline Scientific, UK). Cyanoprokaryotic species were scored for a range of diacritical morphological traits including cell shape and size of intercalary and terminal cells, width and length of intercalary cells, presence or absence of sheaths, constrictions at the cross-wall and necridic cells, color of the sheath, number of trichomes per filament, presence or absence of false branching, and heterocytes. Cell measurements were made based on the optical images. At least 30 filaments of each species were characterized. The taxonomic works of Anagnostidis and Komárek<sup>23</sup> and Komárek and Anagnostidis<sup>20</sup> as well as the description of Perkinson et al.<sup>31</sup> were used.

## Results

To accurately resolve the phylogenetic relationship of investigated filamentous cyanoprokaryotic species, separate phylogenetic analyses, based on 16S rRNA gene and tPC-IGS, were performed. The phylogenetic trees were constructed by using four different methods: ME, MP, ML, and NJ. Because these methods gave similar clustering, we represented only the ML trees.

The partial 16S rRNA gene sequences (from 1252 to 1371 bp) determined in this study for five *Phormidium*/*Leptolyngbya* species (GenBank accession numbers KF770966–KF770970) were compared with 16S rRNA gene sequences of well-defined representatives of the genera *Phormidium*, *Lyngbya*, *Leptolyngbya*, *Nodosilinea*, *Phormidesmis*, and *Wilmottia* available in the GenBank. Six distinct large clades could be distinguished in the phylogenetic reconstruction, here named Clade 1, Clade 2, Clade 3, Clade 4, Clade 5 and Clade 6 (Fig. 1). The bootstrap support values for the major lineages of the phylogenetic tree calculated by using different methods (ME, NJ, MP, and ML) are given in Table 1. The first clade (Clade 1) comprises *Phormidium* strains (100% bootstrap support) that were recently separated in a new genus, *Wilmottia*, with the single species *W. murrayi*.<sup>34</sup> The second clade (Clade 2) includes typical *Phormidium* species, and it was supported by a bootstrap value of 71%. Our strain of *P. papyraceum* was also clustered in this group (not in Clade 1), although it was reported that this species is morphologically similar



**Figure 1.** Phylogenetic tree based on 16S rRNA gene sequences of representative oscillatorial species from the genera *Phormidium*, *Wilmottia*, *Phormidesmis*, *Leptolyngbya*, *Nodosilinea*, and *Lyngbya*, and reconstructed using the ML analysis by applying the GTR+I+G evolutionary model. The numbers above branches indicate bootstrap support (>50%) from 1,000 replicates. The sequences determined in the present study are indicated with filled circles. The sequence of *G. violaceus* was used as out-group. GenBank accession numbers are indicated in parentheses.

to *W. murrayi* and could be placed in the *Wilmottia* group.<sup>34</sup> Clade 3, which had a maximal statistical support of 100%, consists of typical *Lyngbya* strains. There were other *Lyngbya* species that were not clustered in this clade, which confirms the polyphyly of the genus. Clade 4 is formed by a group of cyanoprokaryotic strains that were recently transferred to the new genus, named *Nodosilinea*.<sup>31</sup> This clade was supported by a bootstrap value of 99% (Fig. 1). Our strain of *N. bijugata* (= *L. bijugata*, = *P. bijugatum*) was also placed in Clade 4. The fifth clade (Clade 5), supported by a 100% bootstrap value, includes strains identified as *Leptolyngbya boryana*. This clade

showed a sister relationship with Clade 6 (96% bootstrap support), which encompasses Antarctic strains of *Phormidium priestleyi* and soil cyanoprokaryotic strains from Atacama Desert (Chile) identified as *Phormidesmis* sp. Our strain *Phormidesmis molle* (= *Phormidium molle*) is in a separate branch (Fig. 1) and has no specific phylogenetic relationship with the strains from this clade (*Phormidesmis* group). A close relationship of two Antarctic *P. priestleyi* strains to the *Nodosilinea* group (Clade 4) could be also observed. Therefore, the analyses showed that some strains identified as *P. priestleyi* had no clear phylogenetic support for relationship with genus *Phormidesmis* (Clade 6).

**Table 1.** Bootstrap support (%) for the major lineages of the phylogenetic tree presented in Figure 1 by using ME/NJ/MP/ML methods.

LINEAGE	ME	NJ	MP	ML
Clade 1	100	100	100	100
Clade 2	69	68	54	71
Clade 3	100	100	100	100
Clade 4	98	98	91	99
Clade 5	100	100	100	100
Clade 6	70	67	68	74
Clades 1 and 2	33	31	43	41
Clades 5 and 6	97	96	94	96
Clades 4, 5, and 6	49	48	56	49

**Abbreviations:** ME, minimum evolution; NJ, neighbor joining; MP, maximum parsimony; ML, maximum likelihood.

To confirm the phylogenetic position and separation of the genus *Nodosilinea*, 16S rRNA gene sequences of 15 *Nodosilinea* strains were aligned together with the available GenBank 16S rRNA gene sequences of well-defined *Leptolyngbya* species, and the inferred phylogeny was determined (Fig. 2). *Nodosilinea* strains formed a distinct clade supported by a bootstrap value of 95%, which indicates that their separation from genus *Leptolyngbya* and assignment to a new genus (*Nodosilinea*)<sup>31</sup> was appropriate. This clade also includes several *Leptolyngbya* species (*Leptolyngbya antarctica*, *Leptolyngbya saxicola*, *Leptolyngbya mycoidea*, *Leptolyngbya margaretheana*, and *Leptolyngbya halophila*) isolated from Antarctica, Portugal, and Italy.

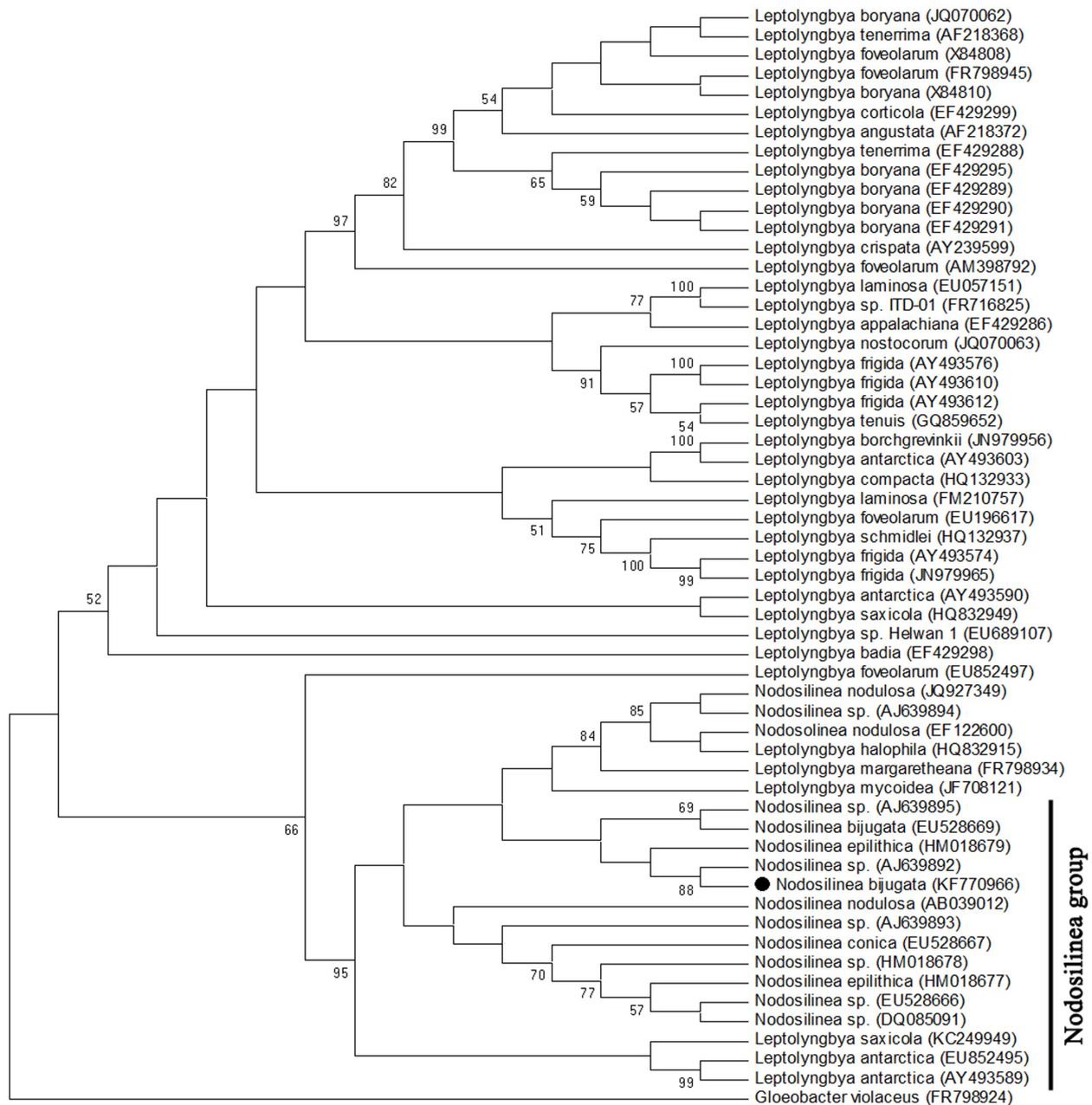
Next, we analyzed the phylogenetic relationship between genus *Phormidium* and the newly formed genera *Phormidesmis* and *Wilmottia*, which were separated from genus *Phormidium* (Fig. 3). The 16S rRNA gene sequences from all *Phormidesmis* strains, *Wilmottia* strains, and well-defined *Phormidium* species available in the GenBank as well as the 16S rRNA gene sequences determined in this study were compared. From the phylogram (Fig. 3) it is clear that *W. murrayi* strains form a separate branch (Clade 1) supported in 100% of the bootstrap trees. Two *W./P. murrayi* strains (with GenBank accession numbers GQ504024 and EU852498) were clustered with other *Phormidium* species in different clades. This is probably because of incorrect identification of the strains. Strains ascribed to the genus *Phormidesmis* were clustered together with several *P. priestleyi* strains and strains named as “Antarctic bacterium” (Clade 2), but without strong bootstrap support. The whole clade was supported by a bootstrap value of 82%. Other *P. priestleyi* strains were grouped in Clade 3 with two *N. bijugata* strains and *Phormidium persicinum* through a node supported by a bootstrap value of 99%. Our strain *Phormidesmis molle* (= *Phormidium molle*) again was clustered in a separate branch as in Figure 1 without relatives from the *Phormidesmis* strains. Nevertheless, the phylogenetic

analyses based on 16S rRNA gene sequences clearly show that the strains identified as *Phormidesmis* as well as the *P. priestleyi* strains that were suggested to be transferred to genus *Phormidesmis*<sup>33</sup> had no strong phylogenetic support. Similar to the phylogenetic analysis presented in Figure 1, our strain *P. papyraceum* was grouped again with typical *Phormidium* species (Fig. 3), but not with the *Wilmottia* strains.

The alignment of the limited number of *cpcB*-IGS-*cpcA* sequences (from 605 to 664 bp) of strains from the investigated genera available in the public databases showed three distinct large clades (Fig. 4). The first clade (Clade 1) consisted of two subclades, in which are included mainly typical *Phormidium* strains and two *Lyngbya* strains. These subclades are supported by bootstrap values of 100 and 93%, respectively. The second clade (Clade 2) also includes two subclades (*Leptolyngbya* and *Phormidium*) supported by the bootstrap values of 100 and 99%, respectively. Most of our previously published *cpcB*-IGS-*cpcA* sequences<sup>41</sup> of the strains, included also in this study, are grouped in Clade 3 with a high bootstrap support of 100% (Fig. 4). Although this clade consists members of the genus *Phormidium*, our *N. bijugata* strain (= *L. bijugata*, = *P. bijugatum*) was clustered here, but with low bootstrap support. Unfortunately, there were not available PC-IGS sequences from *Phormidesmis* and *W./P. murrayi* strains in the GenBank, but our strain *Phormidesmis molle* (= *Phormidium molle*) (AY466128) was clustered separately outside from the main clades.

In addition to the phylogenetic analyses, investigated cyanoprokaryotic strains were morphologically re-evaluated (Table 2) using the main diacritical features such as shape and size of the cells, sheaths around the trichomes and its color, morphology of the apical cells, constrictions at the cross-wall, presence or absence of false branching, etc. The characteristics of all strains matched the species description (followed after Komárek and Anagnostidis).<sup>20</sup> Trichomes of *N. bijugata* and *Phormidium molle* were distinctly constricted, whereas in the other species (*P. papyraceum*, *P. autumnale*, and *P. uncinatum*) they were slightly constricted or not constricted (Table 2). All strains formed fine mats. Filaments in all studied strains can be straight or curved (in varying degrees), and the sheath is always thin and colorless. Most of the apical cells were rounded or conical. Calyptra was observed on the apical cell in *P. autumnale* and *P. uncinatum*. The species *N. bijugata*, *Phormidium molle*, and *P. papyraceum* lacked calyptra. False branching was not observed. Species that were re-evaluated in the present study are shown in Figure 5.

The performed analysis confirmed that these morphological characters cannot be used separately for taxonomic purposes. The only morphological feature that distinguishes representatives of the genera *Phormidium* and *Leptolyngbya* is the size of the cells, which can vary under different environmental or culture conditions. It was accepted that the modern system of the cyanoprokaryotic organisms must be based on the molecular definition of genotypes in correlation



**Figure 2.** Phylogenetic tree based on 16S rRNA gene sequences of *Leptolyngbya* and *Nodosilinea* species. The tree was reconstructed using the ML analysis by applying the GTR+I+G evolutionary model. The numbers above branches indicate bootstrap support (>50%) from 1,000 replicates. The sequences determined in this study are indicated with filled circles. The sequence of *G. violaceus* was used as out-group. GenBank accession numbers are indicated in parentheses.

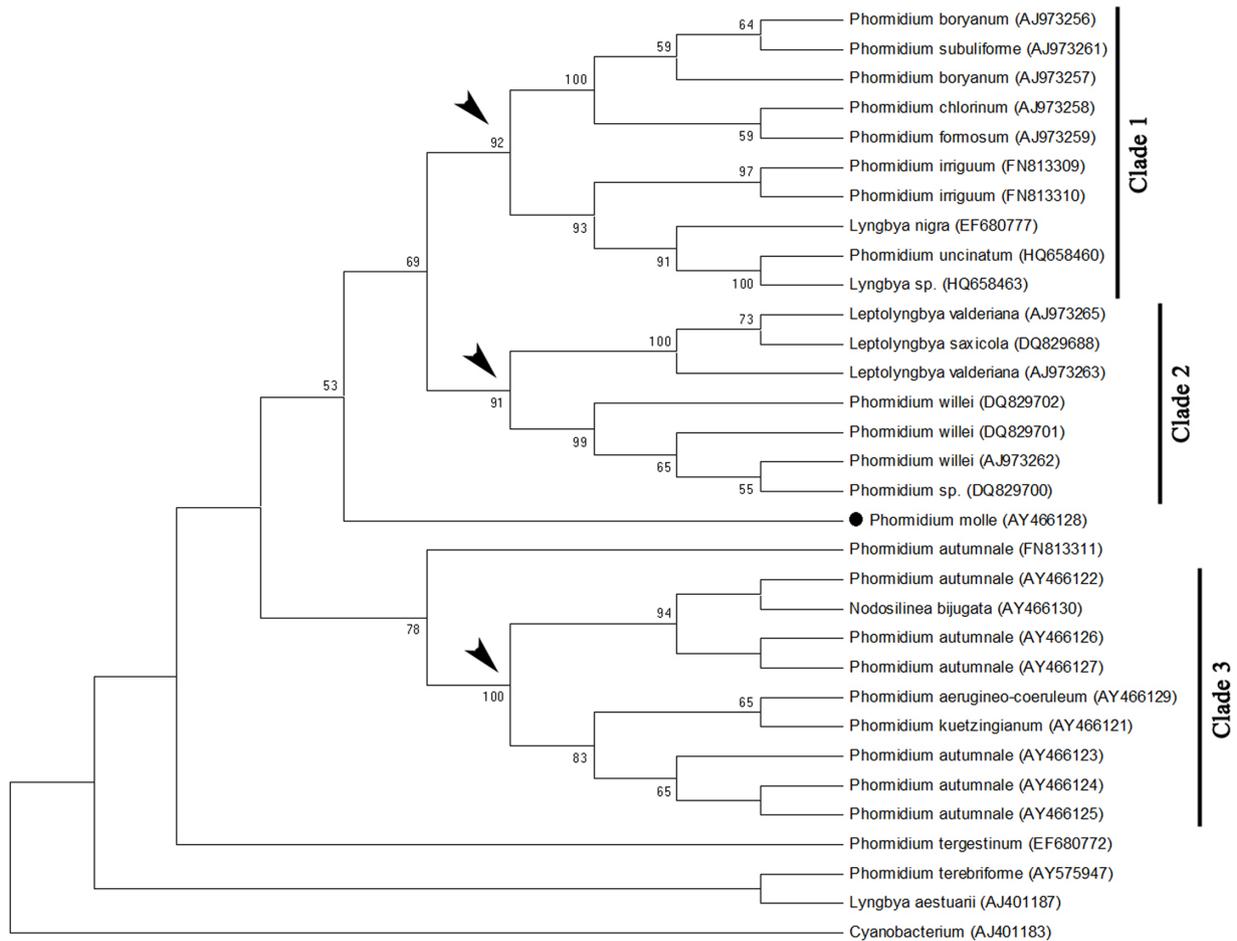
with phenotypic, biochemical, ultrastructural, and ecological characters.<sup>21</sup>

## Discussion

In the present study, we have performed phylogenetic analyses based on the 16S rRNA gene and *cpcB*-IGS-*cpcA* locus of the phycocyanin operon combined with morphological re-evaluation of some filamentous cyanobacteria with problematic taxonomy. The correct identification of a single species requires genetic, morphological, and ecological uniformity. Our phylogenetic analyses support the taxonomic separation of

genus *Nodosilinea* from the polyphyletic genus *Leptolyngbya*.<sup>31</sup> Investigated *Nodosilinea* strains always formed a coherent genetic cluster supported with a high bootstrap value. *N. bijugata* (= *L. bijugata*, = *P. bijugatum*) was transferred in this genus based on the morphological and ultrastructural features, although the studied strain was distinct from the other *Nodosilinea* strains in terms of 16S–23S gene sequences and its secondary structure.<sup>31</sup> Now, based on the 16S rRNA gene sequence, we confirm that this species belongs to genus *Nodosilinea*. The main diacritic morphological character of this genus is that the filaments form nodules under low-light





**Figure 4.** Phylogenetic tree based on the available PC-IGS sequences of *Phormidium*, *Leptolyngbya*, *Nodosilinea*, and *Lyngbya* species. The tree was reconstructed using the ML analysis by applying the GTR+I+G evolutionary model. The numbers above branches indicate bootstrap support (>50%) from 1,000 replicates. *Cyanobacterium* sequence was used as out-group. GenBank accession numbers are indicated in parentheses.

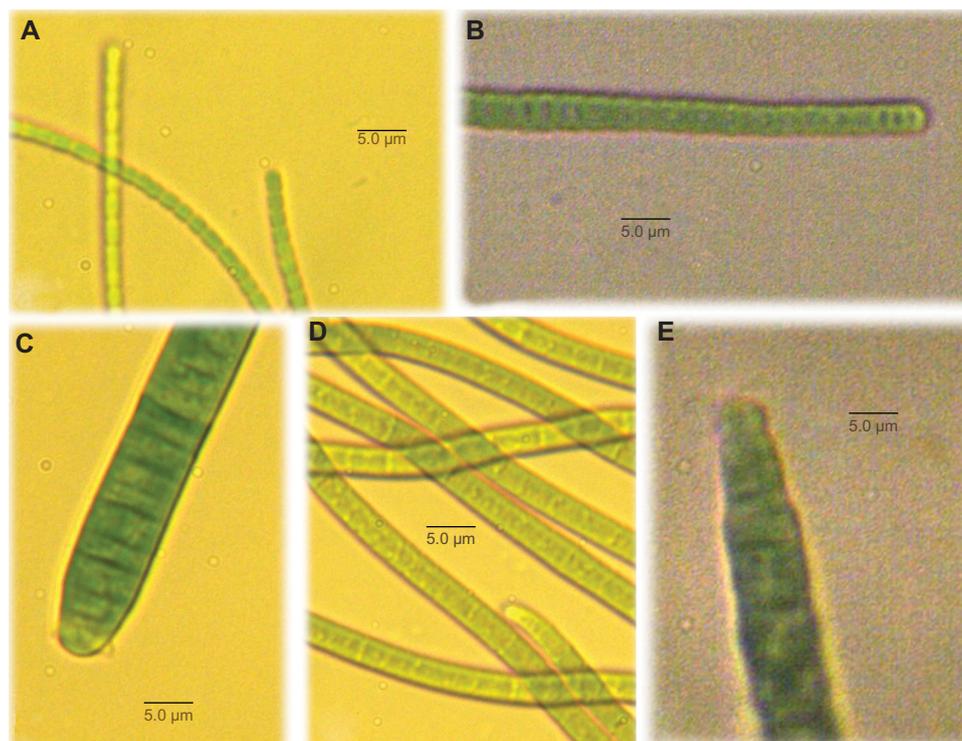
conclusion from this phylogeny is that *P. papyraceum* does not belong to genus *Wilmottia*.

Genus *Phormidesmis* was separated from genus *Phormidium* on the basis of combined molecular (16S rRNA gene) and

phenotype analyses of the tropical type-species *Phormidium molle*.<sup>32,33</sup> Unfortunately, the used 16S rRNA gene sequence of the type species for the phylogenetic analyses performed by these authors is not available in the public databases. In our

**Table 2.** Morphological characters of investigated cyanoprokaryotic strains.

SPECIES	STRAIN	GENBANK ACCESSION NUMBERS	CELL SHAPE	CELL WIDTH (µM)	CELL LENGTH (µM)	SHEATH	APICAL CELL TYPE	CONSTRICTION
<i>N. bijugata</i> (= <i>P. bijugatum</i> , = <i>L. bijugata</i> )	PACC 8602	KF770966	Cylindrical, isodiametric	1.0–1.9	1.4–2.4	Thin, colorless	Rounded, without calyptra	Distinct
<i>Phormidium molle</i> (= <i>Phormidesmis</i> <i>molle</i> )	PACC 8140	KF770967	Cylindrical, isodiametric	1.5–2.0	2.7–3.3	Thin, colorless	Rounded, without calyptra	Distinct
<i>P. papyraceum</i>	PACC 8600	KF770968	Wider than long	6.4–7.7	2.3–4.8	Firm, colorless	Conical, without calyptra	Not constricted
<i>P. autumnale</i>	PACC 5522	KF770969	Isodiametric	3.0–5.1	1.3–4.2	Thin, colorless	Rounded or conical with calyptra	Slightly constricted
<i>P. uncinatum</i>	PACC 8693	KF770970	Isodiametric	5.5–7.1	2.3–5.2	Thin, colorless	Rounded or conical with calyptra	Not constricted



**Figure 5.** Light microphotographs of investigated cyanoprokaryotic species. (A) *N. bijugata* PACC 8602 (= *L. bijugata*, = *P. bijugatum*), (B) *Phormidium molle* PACC 8140 (= *Phormidesmis molle*), (C) *P. papyraceum* PACC 8600, (D) *P. autumnale* PACC 5522, and (E) *P. uncinatum* PACC 8693. Scale bar, 5 µm.

phylogenetic analyses, we used all available 16S rRNA gene sequences of strains assigned as *Phormidesmis*, and in addition, the 16S rRNA gene sequence of our own strain identified as *Phormidium molle*. Results showed that *Phormidesmis* strains and some *P. priestleyi* strains formed a separate clade different from the typical *Phormidium* species, but without strong bootstrap support (Figs. 1 and 3). On the other hand, these *Phormidesmis* strains were grouped in a sister clade to the *Leptolyngbya* (Fig. 1) supported by a bootstrap value of 96%. Komárek et al. showed that the arrangement of thylakoids in *Phormidesmis molle* (= *Phormidium molle*) is parietal (typical for the pseudanabaenacean types including *Leptolyngbya*), which is different from the radial arrangement of thylakoids in Phormidiaceae.<sup>33</sup> At the same time, it is mentioned that the arrangement and density of thylakoids are dependent on the environmental factors (salt concentration, light intensity, age of cells). The authors suggested also that the Antarctic *P. priestleyi* strains have to be eliminated from the genus *Phormidium* and transferred to genus *Phormidesmis*. Although several *P. priestleyi* strains are grouped together with *Phormidesmis*, our phylogenetic analyses reveal that other *P. priestleyi* strains are intermixed with *Phormidium* and *Nodosilinea*. In addition to the *P. priestleyi* strains, several other *Phormidium* species were listed as morphologically corresponding to the genus *Phormidesmis* including *Phormidium macedonicum* Čado 1959.<sup>33</sup> This species was also given as morphologically similar to *W. murrayi*.<sup>34</sup>

In all performed phylogenetic analyses, our strain *Phormidesmis molle* (= *Phormidium molle*) was clustered separately without relatives from other *Phormidium* or *Phormidesmis* strains. On the other hand, the morphological analysis showed that this strain has all morphological characters (according to the description of Komárek and Anagnostidis)<sup>20</sup> to be classified as *Phormidium molle*. These data imply that we have to designate *Phormidium molle* as a cryptic species.<sup>17,21,42</sup> Surprisingly, when we blasted its 16S rRNA gene sequence with all available sequences in the GenBank, the analysis showed 98% similarity with *Tolypothrix* sp. (Nostocales). The *cpcB*-IGS-*cpcA* sequence was 85% similar to the phycocyanin locus of *Tolypothrix* sp. and 100% similar to *P. autumnale* strains. Because false branching and presence of heterocytes (typical for Nostocales) were not observed, a possible explanation of this phenomenon is horizontal exchange of genetic material.<sup>43</sup>

Because there is a limited number of 16S rRNA gene sequences currently available for *Phormidesmis* strains and their genetic relationships are still incomplete, it is premature to reclassify them in a separate genus (*Phormidesmis*) based mainly on morphological and ultrastructural features that could vary depending on the environmental factors.

## Conclusions

Using the principles of the polyphasic taxonomy, our analyses reveal that the cyanoprokaryotic species *N. bijugata*



(= *L. bijugata*, = *P. bijugatum*) was correctly transferred to the newly formed genus *Nodosilinea*. The phylogenetic reconstructions based on 16S rRNA gene sequences confirm the separation of the *W./P. murrayi* strains from genus *Phormidium* as a new generic level (genus *Wilmottia*). Although *P. papyraceum* is morphologically similar to *W. murrayi*, it is a genetically distinct species related more closely to the typical *Phormidium* strains, and therefore, this species should be kept within genus *Phormidium*. Because the available 16S rRNA gene sequences of strains identified as *Phormidesmis* and those of *P. priestleyi* strains were clustered in distinct branches without significant bootstrap support, as well as in the absence of 16S rRNA gene sequence data of the type-species *Phormidium molle*, at this stage we suggest all *Phormidesmis* and *P. priestleyi* strains to be classified as *Phormidium* species. In addition, the phylogenetic analyses based on 16S rRNA gene and PC-IGS sequences of our *Phormidesmis molle* (= *Phormidium molle*) strain placed it in a separate branch without specific phylogenetic relationship with the other *Phormidesmis* or *P. priestleyi* strains.

### Author Contributions

RM, BD, and IT conceived and designed the experiments. PS, DM, RM, BD, and IT analyzed the data. PS, BD, and IT wrote the first draft of the manuscript. RM and DM contributed to the writing of the manuscript. PS, DM, RM, BD, and IT agreed with manuscript results and conclusions. RM, BD, and IT jointly developed the structure and arguments for the paper. BD and IT made critical revisions and approved the final version. All authors reviewed and approved the final manuscript.

### DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

### REFERENCES

- Mur LR, Skulberg OM, Utkilen H. Cyanobacteria in the Environment. In: Chorus I, Bartram J, eds. *Toxic Cyanobacteria in Water. A guide to their Public Health Consequences and Management*. London: E&FN Spon; 1999.
- Hoffman L. Marine cyanobacteria in tropical regions: diversity and ecology. *Eur J Phycol.* 1999;34(4):371–9.
- Lacap DC, Smith GJ, Warren-Rhodes K, Pointing SB. Community structure of free-floating filamentous cyanobacterial mats from the Wonder Lake geothermal springs in the Philippines. *Can J Microbiol.* 2005;51(7):583–9.
- Lau E, Nash CZ, Vogler DR, Cullings KW. Molecular diversity of cyanobacteria inhabiting coniform structures and surrounding mat in a Yellowstone hot spring. *Astrobiology.* 2005;5(1):83–92.
- Sompong U, Hawkins PR, Besley C, Peerapornpisal Y. The distribution of cyanobacteria across physical and chemical gradients in hot springs in northern Thailand. *FEMS Microbiol Ecol.* 2005;52(3):365–76.
- Finsinger K, Scholz I, Serrano A, et al. Characterization of true-branching cyanobacteria from geothermal sites and hot springs of Costa Rica. *Environ Microbiol.* 2008;10(2):460–73.
- McGregor GB, Rasmussen JP. Cyanobacterial composition of microbial mats from an Australian thermal spring: a polyphasic evaluation. *FEMS Microbiol Ecol.* 2008;63(1):23–35.
- Mohamed ZA. Toxic cyanobacteria and cyanotoxins in public hot springs in Saudi Arabia. *Toxicon.* 2008;51(1):17–27.
- Ward DM, Ferris MJ, Nold SC, Bateson MM. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiol Mol Biol Rev.* 1998;62(4):1353–70.
- Jungblut AD, Hawes I, Mountfort D, et al. Diversity within cyanobacterial mat communities in variable salinity meltwater ponds of McMurdo Ice Shelf, Antarctica. *Environ Microbiol.* 2005;7(4):519–29.
- Lopez-Archilla AI, Moreira D, Lopez-Garcia P, Guerrero C. Phytoplankton diversity and cyanobacterial dominance in a hypereutrophic shallow lake with biologically produced alkaline pH. *Extremophiles.* 2004;8(2):109–15.
- Garcia-Pichel F, Lopez-Cortes A, Nubel U. Phylogenetic and morphological diversity of cyanobacteria in soil desert crusts from the Colorado plateau. *Appl Environ Microbiol.* 2001;67(4):1902–10.
- Bowker MA, Reed SC, Belnap J, Phillips SL. Temporal variation in community composition, pigmentation, and F(v)/F(m) of desert cyanobacterial soil crusts. *Microb Ecol.* 2002;43(1):13–25.
- Stibal M, Sabacka M, Kastovska K. Microbial communities on glacier surfaces in Svalbard: impact of physical and chemical properties on abundance and structure of cyanobacteria and algae. *Microb Ecol.* 2006;52(4):644–54.
- Anagnostidis K, Komárek J. Modern approach to the classification system of Cyanophytes 1—Introduction. *Arch Hydrobiol.* 1985;38–39:291–302.
- Oren A. A proposal for further integration of the cyanobacteria under the Bacteriological Code. *Int J Syst Evol Microbiol.* 2004;54(Pt 5):1895–902.
- Komárek J. Cyanobacterial taxonomy: current problems and prospects for the integration of traditional and molecular approaches. *Algae.* 2006;21(4):349–75.
- Wilmotte A, Herdman M. Phylogenetic relationships among the cyanobacteria based on 16S rRNA sequences. In: Garrity GM, Boone DR, Castenholz RW, eds. *Bergey's Manual of Systematic Bacteriology*. New York: Springer-Verlag; 2001:487–599.
- Sciuto K, Andreoli C, Rascio N, La Rocca N, Moro I. Polyphasic approach and typification of selected Phormidium strains (Cyanobacteria). *Cladistics.* 2012;28:357–74.
- Komárek J, Anagnostidis J. *Cyanoprokaryota –2. Teil/2nd Part: Oscillatoriales*. Vol 19/2. Heidelberg: Elsevier/Spektrum; 2005.
- Komárek J. Recent changes (2008) in cyanobacterial taxonomy based on a combination of molecular background with phenotype and ecological consequences (genus and species concept). *Hydrobiologia.* 2010;639:245–59.
- Palinska K, Deventer B, Hariri K, Lotocka M. A taxonomic study on Phormidium-group (cyanobacteria) based on morphology, pigments, RAPD molecular markers and RFLP analysis of the 16S rRNA gene fragment. *Fottea.* 2011;11(1):41–55.
- Anagnostidis J, Komárek J. Modern approach to classification system of cyanophytes. 3. Oscillatoriales. *Arch Hydrobiol.* 1988;53:327–472.
- Casamatta DA, Johansen JR, Vis ML, Broadwater ST. Molecular and morphological characterization of ten polar and near polar strains within the Oscillatoriales (Cyanobacteria). *J Phycol.* 2005;42:421–38.
- Komárek J. Phenotype diversity of the cyanobacterial genus *Leptolyngbya* in the maritime Antarctic. *Pol Polar Res.* 2007;28(3):211–31.
- Sciuto K, Rascio N, Andreoli C, Moro I. Polyphasic characterization of ITD–01, a cyanobacterium isolated from the Ischia Thermal District (Naples, Italy). *Fottea.* 2011;11(1):31–9.
- El Semary N. Microscopic, molecular, and biochemical investigations to characterize a benthic cyanoprokaryote *Leptolyngbya* strain from Egypt. *Microsc Res Tech.* 2013;76:249–57.
- Albertano P, Kovacic L. Is the genus *Leptolyngbya* an homogeneous taxon? *Arch Hydrobiol.* 1994;75:37–51.
- Taton A, Grubisic S, Ertz D, et al. Polyphasic study of Antarctic cyanobacterial strains. *J Phycol.* 2006;42:1257–70.
- Kongisser RA. *On the Methods of Studying Phytoplankton [In Russian]*. Vol 54. Trav. Soc. Nat. Leningrad; 1925.
- Perkerson RB, Johansen JR, Kováčik L, Brand J, Kastovsky J, Casamatta DA. A unique pseudanabaenalean (cyanobacteria) genus *Nodosilinea* gen. nov. based on morphological and molecular data. *J Phycol.* 2011;47(6):1397–412.
- Turicchia S, Ventura S, Komarkova J, Komárek J. Taxonomic evaluation of cyanobacterial microflora from alkaline marshes of northern Belize. 2. Diversity of oscillatorialean genera. *Nova Hedwigia.* 2009;89(1–2):165–200.
- Komárek J, Kastovski J, Ventura S, Turicchia S, Smarda J. The cyanobacterial genus *Phormidesmis*. *Algal Stud.* 2009;129:41–59.
- Strunecky O, Elster J, Komarek J. Taxonomic revision of the freshwater cyanobacterium "*Phormidium*" *murrayi* = *Wilmottia murrayi*. *Fottea.* 2011;11(1):57–71.
- Staub R. Ernährungsphysiologisch-antokologische Untersuchungen an der planktonischen Blualge *Oscillatoria rubescens* DC. *Schweiz Z Hydrol.* 1961;23:82–198.



36. Tillett D, Neilan BA. Xantogenate nucleic acid isolation from cultured and environmental Cyanobacteria. *J Phycol.* 2000;36:251–8.
37. Edwards U, Rogall T, Blocker H, Emde M, Bottger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* 1989;17(19):7843–53.
38. Lepère C, Wilmotte A, Meyer B. Molecular diversity of Microcystis strains (Cyanophyceae, Chroococcales) based on 16S rDNA sequences. *Syst Geogr Plants.* 2000;70:275–83.
39. Neilan BA, Jacobs D, Goodman AE. Genetic diversity and phylogeny of toxic cyanobacteria determined by DNA polymorphisms within the phycocyanin locus. *Appl Environ Microbiol.* 1995;61(11):3875–83.
40. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* 2011;28:2731–9.
41. Teneva I, Dzhambazov B, Mladenov R, Schirmer K. Molecular and phylogenetic characterization of Phormidium species (Cyanoprokaryota) using the cpcB-IGS-cpcA locus. *J Phycol.* 2005;41:188–94.
42. Johansen JR, Casamatta DA. Recognizing cyanobacterial diversity through adoption of a new species paradigm. *Algol Stud.* 2005;117:71–93.
43. Rudi K, Skulberg OM, Jakobsen KS. Evolution of cyanobacteria by exchange of genetic material among phyletically related strains. *J Bacteriol.* 1998;180(13):3453–61.