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Phylogenetic position of *Geitleribactron purpureum* (Synechococcales, Cyanobacteria / Cyanophyceae) and its implications for the taxonomy of Chamaesiphonaceae and Leptolyngbyaceae

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Abstract: Over the last decades, the taxonomy of cyanobacteria has been considerably improved and restructured due to the increase in data output from molecular phylogeny. Recently, a new protocol was developed that enables reliable sequencing of 16S rRNA genes in cultivation–resistant cyanobacteria using analysis of single cells, filaments, or colonies. In the current study, we examined a sample of a heteropolar unicellular cyanobacterium, *Geitleribactron purpureum*, from the holotype material (deep epilithon of Lake Tovel, Western Dolomites, Italy). We isolated and purified single colonies of *G. purpureum*, and subjected them to direct PCR and 16S rRNA gene sequencing. We obtained a congruent set of sequences that formed a unique, isolated cyanobacterial lineage, showing phylogenetic clustering among simple filamentous genera of the family Leptolyngbyaceae. We provide evidence for deep polyphyly in Chamaesiphonaceae, and suggest that *Geitleribactron* should be re–classified in the Leptolyngbyaceae.

Key words: Alps, carbonate lakes, *Geitleribactron*, heteropolar cyanobacteria, single–colony sequencing, unicellular cyanobacteria

Introduction

The taxonomy of cyanobacteria is passing through a difficult period of revision and restructuring with effects on all traditional taxa, as recently summarized by Komárek et al. (2014). One of the most challenging tasks of these revisions is the re–definition and splitting of polyphyletic morphogenera and families. The dominant practice in current cyanobacterial taxonomic work is based on the polyphasic approach and the monophyletic species concept (Johansen & Casamatta 2005; Osorio–Santos et al. 2014; Dvořák et al. 2015), which utilizes solely monophyletic taxa recognized on the basis of unique apomorphies (morphological and ultrastructural characters or biochemical and ecophysiological traits). Application of this taxonomic concept has already led to numerous descriptions of new genera and species (Komárek et al. 2014). Recently, Palinska & Surosz (2014) recommended molecular analysis of botanical type material from historical (and recent) herbaria to generate reference molecular data for existing cyanobacterial species and genera. Such a database might effectively reduce taxonomic confusion caused by phenotypic plasticity of cyanobacterial strains in culture versus natural habitats. Another group of researchers advocated caution and recommended keeping cyanobacterial strains designated with provisional generic names and strain numbers, without species epithets, until better understanding of cyanobacterial species diversity and evolutionary relationships is reached (Castenholz 1992; Castenholz & Norris 2005). This approach was used in the current *Bergey´s Manual of Systematic Bacteriology* (Castenholz 2001), which divided the phylum of cyanobacteria into five subsecti-

ons, each containing several form–genera. Temporary utilization of the standing botanical system was recommended in floristic and ecological studies lacking culture isolates (Castenholz 1992).

Among the groups whose modern revision has not yet been fully accomplished, one of the least understood are heteropolar unicellular cyanobacteria. According to Komárek et al. (2014), polarized cyanobacterial unicells are classified into three standalone families, each putatively placed in a different order: Chamaesiphonaceae (Synechococcales), part of the Dermocarpellaceae (Pleurocapsales), and Stichosiphonaceae (Chroococcales). The present study is concerned mainly with members of the Chamaesiphonaceae. Cyanobacteria from this family are supposed to be relatively simple, solitary, or colonial, exhibiting asymmetrical binary fission, producing one or few exocytes, and having parietally arranged thylakoids (Komárek & Anagnostidis 1998; Komárek et al. 2014). By contrast, Dermocarpellaceae and Stichosiphonaceae show irregular thylakoid patterns and extensive production of special reproductive cells, namely, baeocytes and exocytes, respectively. Nevertheless, the boundaries between these groups or genera within a single group are sometimes considered to be problematic. For example, Rosowski et al. (1995) observed a cyanobacterial strain resembling *Geitleribactron*, which formed long Y–shaped cells typical of *Cyanophanon* (both Chamaesiphonaceae). A recently described morphospecies *Chamaesiphon stratosus* Sant´Anna et al. from Brazil (Sant´Anna et al. 2011) showed a colony organization exhibiting features of both *Chamaesiphonopsis* (Chamaesiphonaceae) and *Godlewskia* (Stichosiphonaceae). Another study by Gold–Morgan et al. (2015) described a new coccoid heteropolar genus *Nisida* exhibiting a morphology that did not allow classification of the taxon in any of the existing families. The authors of all of the three above–mentioned studies were unable to sequence their material, and the authors of the latter two studies did not provide information on thylakoid arrangement. Without these essential pieces of evidence, no reliable taxonomic conclusions can be reached.

The Chamaesiphonaceae currently comprise five genera: *Chamaesiphon*, *Chamaesiphonopsis*, *Clastidium*, *Cyanophanon*, and *Geitleribactron* (Komárek et al. 2014). Although these cyanobacteria are not very often studied in detail, they are capable of establishing ecologically significant populations in certain biotopes. Species of *Chamaesiphon*, the most frequent of these genera, commonly colonize water vegetation and hard substrates in both running and stagnant, usually clear and cool, waters (Komárek & Anagnostidis 1998). Outside Europe, interesting reports of the genus come from, e.g., the Atlantic rainforest in Brazil (Sant´Anna et al. 2011), Himalaya (Komárek & Watanabe 1998), Mexico (Gold–Morgan et al. 1996), Australia (McGregor 2013), and even Antarctica (Komárek 2014). The abundance and relatively narrow ecological valence of *Chamaesiphon* species in stream biofilms have enabled their use in water quality assessment and bioindication (Barinova et al. 2008; Rott 2008; Niedermayr & Schagerl 2010; Loza et al. 2013a, b).

Another genus with some practical importance is *Geitleribactron*, although it is only rarely reported in the literature (Golubić 1967; Geitler 1970, 1975; Komárek 1975; Hällfors & Munsterhjelm 1982; Gold–Morgan et al. 1996; Karosienė & Kasperovičienė 2008; Biolo & Rodrigues 2011; Cantonati et al. 2014a). The four known *Geitleribactron* species are characterized by a very simple morphology: rod–shaped unicells lacking a sheath are attached to the substrate with an inconspicuous mucilaginous pad, cells divide somewhat asymmetrically, producing a single exocyte, and the thylakoids are arranged parietally (Komárek & Anagnostidis 1998; Cantonati et al. 2014a). The type species, *G. periphyticum* Komárek, is sometimes considered an expansive species in Europe, where it is potentially problematic for water–treatment facilities because its small size interferes with water filters (Kaštovský et al. 2010). Recently, a new species, *G. purpureum* Cantonati et Komárek, which is distinguishable from the generitype mostly by its bright purple color and habitat, was described from the epilithon of Lake Tovel, Italy (Cantonati et al. 2014a). This meromictic/oligomictic, carbonate mountain lake of the south–eastern Alps hosts a set of distinguishable phytobenthic communities along its depth/irradiance gradient (Cantonati et al. 2014b). Purple cyanobacterial species (*G. purpureum*, *Chlorogloea purpurea* Geitler) seem to have adapted to the low light availability at greater depths.

The major shortcoming in our knowledge of heteropolar unicellular cyanobacteria is the overall lack of molecular data. The only genus with a relatively good DNA sequence record is *Chamaesiphon* (Turner 1997; Loza et al. 2013b; Shih et al. 2013), although its type species (*C. confervicola* A. Braun in Rabenhorst) has not yet been analyzed using molecular methods. DNA sequence data on other genera are not available. Authors of recent studies agree on the resistance of these cyanobacteria to isolation into culture (Sant´Anna et al. 2011; Cantonati et al. 2014a; Gold–Morgan et al. 2015), probably because they have specific growth requirements. Until these problems are solved, cultivation–independent molecular approaches provide the only viable alternative to studying these species. One of these techniques, recently introduced by us for terrestrial cyanobacteria (Mareš et al. 2015), holds promise for the direct sequencing of the 16S rRNA gene in single cells or colonies present in morphologically distinct, uncultured cyanobacterial specimens.
In the current study, we examined a sample (colonies on cobble collected by SCUBA divers in Lake Tovel, Italy) of the type material of *Geitleribactron purpureum*, using the single–colony sequencing approach.

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Details concerning the ecology of the studied *G. purpureum* population and the associated microalgal assemblages were provided in previous studies (Cantonati et al. 2009; Cantonati et al. 2014a, b). The results of molecular analysis are discussed within the taxonomic framework of the Chamaesiphonaceae and other relevant cyanobacterial groups.

Material and Methods

Study site, sampling procedures, and morphological observations. Lake Tovel (south–eastern Alps, Adamello–Brenta Nature Park) is a carbonate (geological substratum: dolomite and limestone), meromictic (with a tendency to oligomixis), mountain lake (1178 m a.s.l.) affected by marked seasonal water–level fluctuations that never exceed 9 m (Cantonati et al. 2014c). Maximum depth is 39 m, average Secchi disk depth is about 10 m, and average lower limit of the euphotic zone is approximately 24 m (Cantonati et al. 2014b).

The cobble used in this study (Fig. 1a) was part of the holotype material of *Geitleribactron purpureum* (cobbles collected by SCUBA divers in June 2013 in the depth–distribution range of the species, 9–21 m). The holotype is stored in the collections of the Museo delle Scienze – MUSE of Trento, Italy (Code: cLIM009 PHYTOB 796) (Cantonati et al. 2014a).

An epilithic assemblage dominated by *G. purpureum* was scraped from the stone surface and observed using a Zeiss Axioskop 2 (Zeiss, Jena, Germany) light microscope at 1000× magnification equipped with a Zeiss Axiocam digital camera to document the morphology of the cells and colonies.

Molecular analysis. For molecular analysis, single small sub–colonies of *G. purpureum* containing approximately 5–20 cells were isolated from the same holotype stone used for the morphological analysis. The protocol followed exactly the procedures described by Mareš et al. (2015). Briefly, fresh material containing *G. purpureum* was scraped from the stone surface and homogenized with a needle in TE buffer. Using a microscope, six individual colonies were picked with a glass capillary, washed several times in TE buffer, and checked for contaminants. Clean colonies were placed individually into separate PCR tubes, and a partial 16S rRNA gene sequence was amplified in two steps using a semi–nested PCR protocol with cyanobacteria–specific primers (see Mareš et al. 2015 for details). Samples containing the PCR product of the predicted length (about 1 100 bp) were cloned into *E. coli* using the pGEM®–T Easy vector system (Promega, Madison, WI, USA), and the resulting plasmids were purified using NucleoSpin® Plasmid kit (Macherey–Nagel, Düren, Germany), and sequenced in SeqMe, s.r.o. (Dobříš,

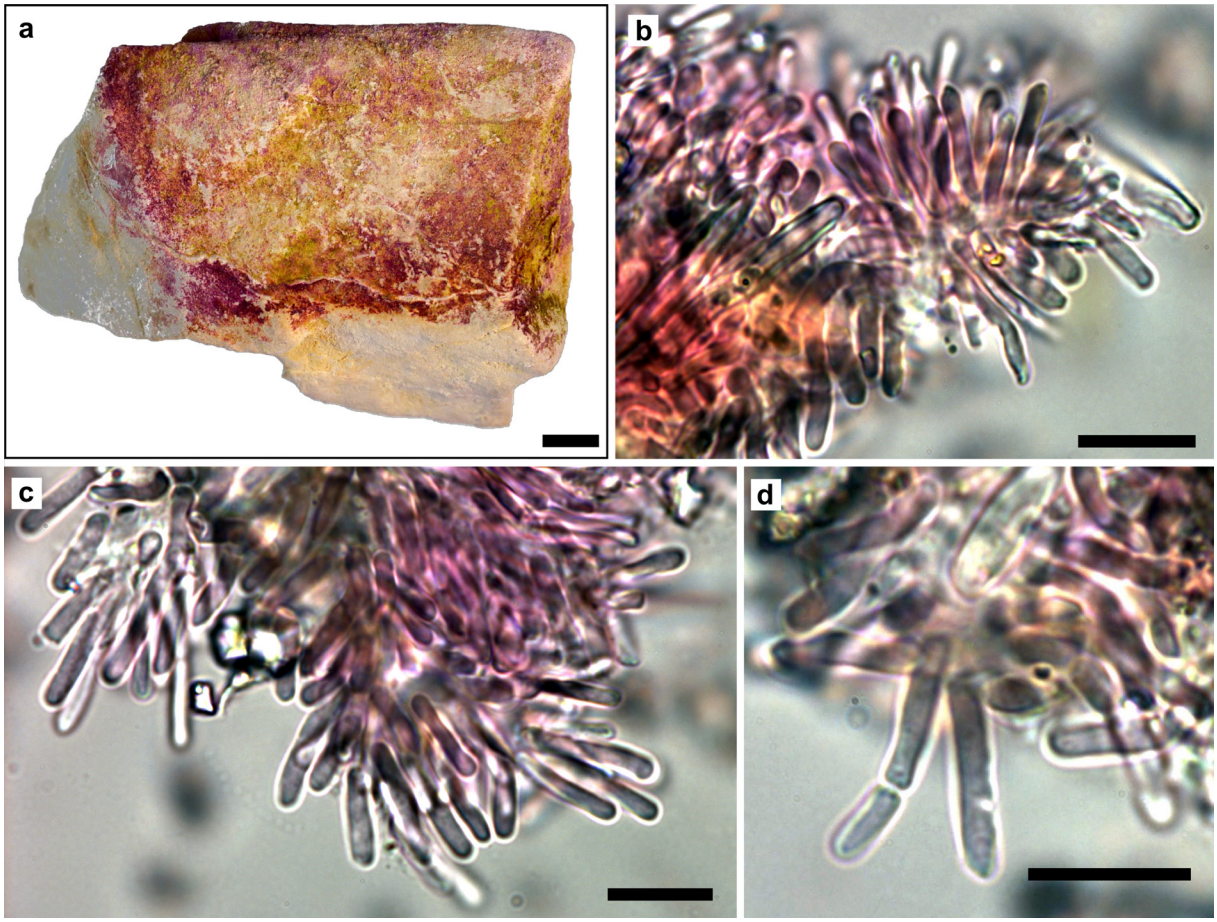

Fig 1. Morphology of *Geitleribactron purpureum* from the holotype material: (a) a cobble covered by a purple biofilm, dominated by *G. purpureum*; (b–c) typical stellate colonies of *G. purpureum*; (d) detail of asymmetrical binary fission. Scale bars: (a) 1 cm; (b–d) 10 µm.

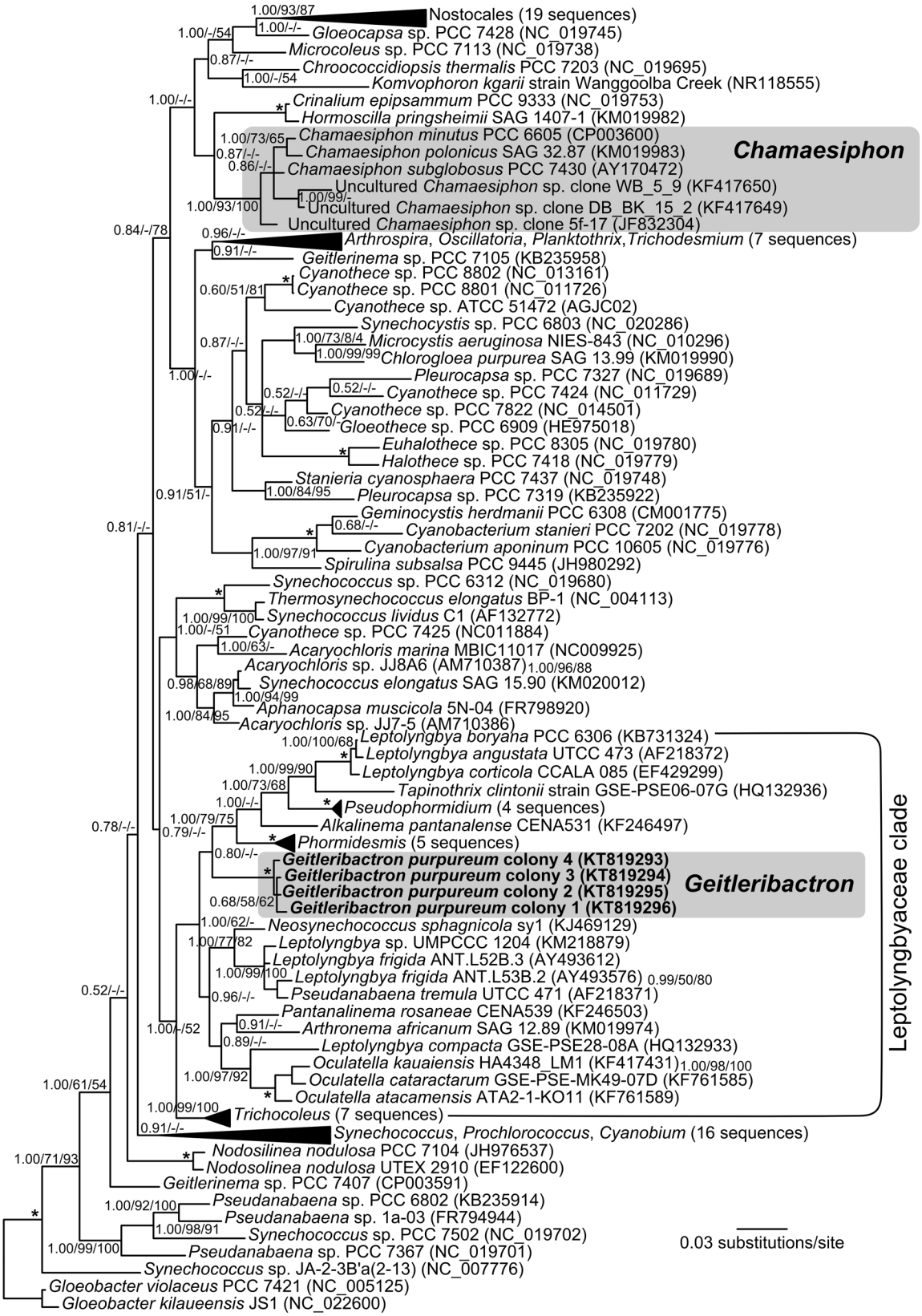

Fig 2. Phylogenetic tree (Bayesian Inference) of cyanobacteria inferred from a partial 16S rRNA gene alignment, showing the position of *Geitleribactron purpureum* in the Leptolyngbyaceae, and the distant position of *Chamaesiphon* species. Branch support values >50% are given near nodes in this shape: Bayesian Inference/Maximum Likelihood/Neighbor–Joining. Full support from all methods is marked with asterisks. Sequences obtained in this study are printed in bold.

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Czech Republic) using standard plasmid primers (T7promoter and SP6r).

The DNA sequences were aligned using the G–INS–i algorithm using the default parameters of MAFFT v. 7 (Katoh & Standley 2013) with a set of published *Chamaesiphon* sequences, and the sequences of members of other clusters of coccoid cyanobacteria, close BLAST hits, and a few representatives of all major cyanobacteria clades. The alignment produced by MAFFT was manually checked, and converted into a 1087 nucleotide long matrix of 128 sequences covering the major part of the 16S rRNA gene. Phylogeny was reconstructed using Bayesian Inference (BI), Maximum Likelihood (ML), and Neighbor–Joining (NJ) methods using the *Gloeobacter violaceus* strains as an outgroup for other members of the cyanobacteria. The BI calculation was executed in MrBayes 3.2.3 via MetaCentrum supercomputing grid (www.metacentrum.cz); two runs of eight Markov chains were performed for 1 275 000 generations, and sampled each 100 generations until the convergence criterion reached a value <0.01. The first 25% of the sampled data was discarded as burn–in. The ML analysis in RaxML v. 8 (Stamatakis 2014) was run on a CIPRES supercomputing facility (Miller et al. 2012) employing the general time–reversible + invariant + gamma (GTR+I+G) substitution model, with 1000 bootstrap pseudo–replications. The NJ analysis was run in SeaView v. 4 (Gouy et al. 2010) using the BioNJ algorithm (Gascuel 1997) and Jukes–Cantor substitution model, with 1000 bootstrap pseudo–replications. Nucleotide sequence identities were calculated as pairwise p–distances using the Sequence Identity Matrix tool in BioEdit v. 7 (Hall 1999).

Results

Morphology

The studied holotype material contained a microbial assemblage in the form of a purple–colored biofilm that was tightly attached to the parts of the stone exposed to light (Fig. 1a). The biofilm was dominated by *G. purpureum*, with minor contributions from other cyanobacteria and diatoms. Morphology and ultrastructure were thoroughly investigated and described in our previous study (Cantonati et al. 2014a). For the purpose of subsequent molecular analysis, we re–examined the morphology of *G. purpureum* in the sample used for the isolation and sequencing of single colonies. Several typical colonies of *G. purpureum* from the sample investigated in this study are shown in Fig. 1 b–d. These colonies were further homogenized, and small sub–colonies consisting of 5–20 cells free of contaminants were used for molecular analysis.

Molecular analysis

Six pure sub–colonies were successfully extracted from the biofilm and subjected to PCR amplification. Four of them provided a PCR product of the partial 16S rRNA gene, which was subsequently sequenced. The resulting sequences of *G. purpureum* were 1 095 bp long and highly uniform (sequence identity 99.0–99.6%). The sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers KT819293–296.

The phylogenetic tree resulting from BI analysis (with branch support values from other methods mapped on its nodes) is shown in Fig. 2. In this tree, *G. purpureum* formed a tight cluster (100% branch support) with strains of cyanobacterial genera such as *Leptolyngbya*, *Tapinothrix*, *Pseudophormidium*, *Phormidesmis*, *Oculatella*, *Pantanalinema*, *Alkalinema*, and *Arthronema*, and several sequences of unclear taxonomy, putatively placed in *Leptolyngbya* and *Pseudanabaena*. This clustering was supported using all methods (branch support BI/ML/NJ = 1.00/77/82). This group of strains, typified by *Leptolyngbya boryana* (Gomont) Anagnostidis et Komárek (strain PCC 6306), together with a sister branch of *Trichocoleu*s, represents the family Leptolyngbyaceae. It includes mostly simple and thin (≤3 μm wide) filamentous cyanobacteria, with the exception of *Neosynechococcus sphagnicola* Dvořák et al. (strain sy1), which is a rod–shaped coccoid cyanobacterium. Although *G. purpureum* 16S rRNA gene sequences were highly unique, and only 92.5–93.0% identical along the aligned region to the most similar sequences of leptolyngbyacean taxa (*Phormidesmis, Trichocoleus*), they were even less similar to the best–matching sequence of *Chamaesiphon* (91.0 %).

In our phylogeny, the rest of the currently accepted family Chamaesiphonaceae was represented by the only available sequenced genus, *Chamaesiphon*, which formed a well–supported cluster (branch supports >90%), recovered in a phylogenetic clade distant from Leptolyngbyaceae. Based on the BI topology, this cluster may be affiliated to Gomontiellaceae and other derived groups, and have a closer relationship with chroococcalean and nostocalean cyanobacteria than with synechococcalean types, including *G. purpureum*. Thus, our data provide strong evidence for a deep polyphyly in Chamaesiphonales.

Discussion

Heteropolar unicellular cyanobacteria are one of the least understood groups of blue–green algae (Gold–Morgan et al. 2015) despite recent phylum–wide revisionary efforts (Komárek et al. 2014). In the current study, we provide for the first time DNA sequence data for *Geitleribactron*, representing the second sequence record for Chamaesiphonaceae genera (in addition to several available *Chamaesiphon* sequences). Molecular data support the taxonomic position of *Geitleribactron* as a separate genus (Komárek 1975), because it formed an isolated phylogenetic lineage and showed only approximately 93% 16S rRNA sequence identity to those of its closest strains. Nevertheless, this conclusion is only preliminary and should be further verified by molecular analysis of the generitype, *G. periphy-*

ticum, in a future study. Ideally, such analysis should involve herbarium type material or material from the type locality of *G. periphyticum*, as recommended by Palinska & Surosz (2014). Because our study was based on a single population, additional molecular data from different localities are required to provide more insight into the variability within the genus. Given the evidence on morphological convergence and extreme polyphyly in other simple unicellular cyanobacteria (Dvořák et al. 2014a), monophyly of *Geitleribactron* needs to be verified by sequencing more of its species.

According to the phylogenetic reconstruction (Fig. 2), *G. purpureum* clearly clustered within a group of simple filamentous cyanobacterial genera, which are considered here as Leptolyngbyaceae. This clade, typified by *L. boryana* (type of the family), has been reported previously as a well–established lineage (Casamatta et al. 2005; Bohunická et al. 2011; Perkerson et al. 2011). The name Leptolyngbyaceae is in accordance with the standing cyanobacterial system (Komárek et al. 2014). Thus, it should be preferred to older names such as Pseudanabaenaceae (Perkerson et al. 2011; Vaz et al. 2015), which is now reserved for a group of several genera not including *Leptolyngbya* and related taxa. Recently, the group has been subject to intensive taxonomic research that has already yielded several new genera, such as *Phormidesmis* (Komárek et al. 2009), *Oculatella* (Zammit et al. 2012), *Alakalinema*, and *Pantanalinema* (Vaz et al. 2015). Clustering of a coccoid cyanobacterium inside a predominantly filamentous group is generally not surprising, since repeated emergence and loss of multicellularity in the course of cyanobacterial evolution has already been documented (Schirrmeister et al. 2011, 2013; Dvořák et al. 2014a). Nevertheless, only a single well–described example has been reported in Leptolyngbyaceae: the recently established genus *Neosynechococcus* (Dvořák et al. 2014b). Interestingly, this peculiar coccoid cyanobacterium is capable of forming extremely elongated cells (up to 20 µm *in situ* and up to 70 µm in culture), which is a feature that resembles the long cylindrical cells of *G. purpureum* (around 10–20 µm upon division (Fig. 1 b–d). Thus, the filiform shape observed in both genera may be a typical feature of unicellular Leptolyngbyaceae. However, *Neosynechococcus* and *Geitleribactron* did not form a monophyletic cluster in our phylogenetic tree, suggesting that the loss of ability to form long multicellular filaments may have occurred several times. Moreover, *G. purpureum* is characterized by its exclusively heteropolar growth. Simple heteropolar filamentous cyanobacteria are currently classified in a separate family, the Heteroleibleiniaceae, which comprises two genera: *Heteroleibleinia* and *Tapinothrix* (Komárek et al. 2014). Together with *Tapinothrix clintonii* Bohunická et Johansen (Bohunická et al. 2011), *G. purpureum* is the second example of a heteropolar cyanobacterium closely related to *Leptolyngbya sensu stricto*. Thus, the available evidence does not favor Heteroleibleiniaceae as a standalone family, and suggests it should be merged with Leptolyngbyaceae. This will again be possible only after careful analysis of a greater number of isolates, including the generitypes.

Our phylogeny, although it was based solely on partial 16S rRNA gene data, seems to unequivocally show a deep polyphyly in Chamaesiphonaceae. The sequenced members of *Chamaesiphon* clustered in a lineage distant from that of Leptolyngbyaceae. The 16S rRNA gene alone usually does not provide robust phylogenetic signal at higher taxonomic levels. Thus, multilocus or genome–wide comparisons are recommended for establishing reliable relationships among broader groups of cyanobacteria (Dvořák et al. 2015). Although the higher phylogenetic backbone was not statistically well supported in our current tree (Fig. 2), a similar branching of *Chamaesiphon* and *Leptolyngbya* was previously observed in both 16S rRNA–based (Loza et al. 2013b) and phylogenomic (Shih et al. 2013; Komárek et al. 2014) reconstructions. Therefore, we suggest the re–classification of the genus *Geitleribactron* to place it within the Leptolyngbyaceae. While our conclusion is likely correct, a future study employing molecular phylogenetic analysis of the type species of *Geitleribactron* and *Chamaesiphon* using multiple genomic loci will be required to obtain an ultimate validation.

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