Morphological and molecular study of epipelic filamentous genera
*Phormidium*, *Microcoleus* and *Geitlerinema* (Oscillatoriales, Cyanophyta/ Cyanobacteria)

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Abstract: Filamentous epipelic cyanobacteria were isolated from ponds and lakes in the Czech Republic, Austria and Italy. Morphological and genetic variation of 20 isolated strains within the genera *Geitlerinema*, *Microcoleus* and *Phormidium* were studied. Partial sequences of the 16S rRNA gene were used for phylogenetic analyses, and secondary structure of the 16S–23S ITS region was used to additionally define clades. Morphological and molecular were congruent, and we were able to identify the majority of strains correctly to species on the basis of morphological features. Overall diversity and morphological/genetic variation of epipelic species is not as high as described from other benthic habitats, possibly due to the relative microhabitat uniformity of lake/pond bottom sediments. The *M. vaginatus* clade is well defined by an 11 bp insert in 16S rRNA gene (bp 423–433) and populations from different ecological conditions differ in secondary structure in the 16S–23S ITS regions, particularly in Box–B helices. *Ph. autumnale* and the genus *Geitlerinema* appear to be polyphyletic as presently defined.

Key words: 16S rRNA, cyanobacteria, ecology, ITS, morphology, phylogeny, Oscillatoriales

Introduction

During most of the 19th and 20th centuries cyanobacterial taxonomy was based almost entirely on morphology (GEITLER 1932; ELENKIN 1938; DESIKACHARY 1959; STARMACH 1966; KONDRATEVA 1968). The taxonomic position of many morphologically–defined species is unclear and some genera urgently need revision (e.g. KOMÁREK & ANAGNOSTIDIS 1998; KOMÁREK & ANAGNOSTIDIS 2005). Moreover, the situation is complicated by a conflict between bacteriological and botanical nomenclatural rules and taxonomic practices (STANIER et al. 1978; RIPPKA et al. 1979; CASTENHOLZ 2001). The most progressive system utilizes a polyphasic approach (ANAGNOSTIDIS & KOMÁREK 1985; KOMÁREK & ANAGNOSTIDIS 1986; ANAGNOSTIDIS & KOMÁREK 1988; KOMÁREK & ANAGNOSTIDIS 1989; ANAGNOSTIDIS & KOMÁREK 1990; KOMÁREK 1994, 2003; KOMÁREK 2011), which includes a combination of morphological, ecological and molecular character sets. Recent molecular data support the validity of many genera, e.g. *Planktothrix*, *Pseudanabaena* (WILLAME et al. 2006), *Microcystis*, and *Spirulina* (KOMÁREK 2003, 2010) as defined by KOMÁREK & ANAGNOSTIDIS (1998, 2005), but at the species level we often have insufficient morphological, ecological and molecular data for reliable recognition of species–level diversity. In recent years, the analysis of the 16S rRNA gene sequences has demonstrated that morphological classification of cyanobacteria in some cases corresponds to phylogenetically coherent taxa (GARCIA–PICHL et al. 1996), whereas in other cases the traditional classification drastically underestimates extant diversity (FERRIS et al. 1996).

The assemblages of autotrophic microorganisms (cyanobacteria, algae) on bottom sediments of stagnant and running waters are called epipelon. These microorganisms perform a range of ecosystem functions including biostabilisation of sediments, regulation of...
benthic–pelagic nutrient cycling, and primary production (Pouličková et al. 2008a). Although epipelic eukaryotic algae were previously studied, e.g. diatoms (reproductive biology, cryptic speciation, geographic biodiversity and bioindication; Pouličková et al. 2008a, 2008b, 2009), epipelic cyanobacteria have been largely overlooked. The ecology of epipelic cyanobacteria is poorly understood. Species distribution is probably influenced by numerous environmental variables such as temperature, light irradiation, oxygen concentration, pH, sediment structure and chemical composition (e.g. Round 1953, 1957, 1961; Hašler et al. 2008). Autochthonous epipelic assemblages typically include 20 – 80% filamentous motile cyanobacteria during some seasons of the year, particularly Komvophoros, Oscillatoria, Phormidium, Geitlerinema and Pseudanabaena (Špačková et al. 2009; Hašler & Pouličková 2010).

We isolated 20 strains of filamentous epipelic cyanobacteria from ponds and lakes of different trophic status in three EU countries (Czech Republic, Austria and Italy). This project aims at taxonomic evaluation of the epipelic filamentous cyanobacteria (Geitlerinema, Microcoleus and Phormidium) based on morphological and molecular characters.

Materials and Methods

Strain isolation and morphological study. Altogether 48 sediment samples were taken during May 2007 using methods described by Hašler et al. (2008). The geographic position and environmental variables of the Czech sites were published by Hašler et al. (2008). Italian localities (Monbino, GPS: 46°7′28.191″N, 11°3′30.647″E; Lago di Tovel, GPS: 46°15′40.775″N, 10°56′57.851″E) were situated in Trento, near the border between Italy and Austria. The locality in Austria (Untersee) is situated at Lunz am See (GPS: 47°51′11.602″N, 15°2′3.256″E), southwest of Vienna. Strains of filamentous morphospecies were isolated following standard methods (Andersen et al. 2005). Cultures were maintained in 100 ml Erlenmeyer flasks under our standard laboratory conditions (temperature 22 ± 1 °C, illumination 20 mmol.m−2.s−1, light regime 12h light/12h dark, liquid Zehnder medium (Staub 1961). All strains were studied using a Zeiss Axiosmager light microscope (objectives EC Plan–Neofluar 40×/1.3 N.A., oil immersion, DIC; Plan–APOCHROMAT 100×/1.4 N.A., oil immersion, DIC); with images taken with a high resolution camera (AxioCam HRC 13MPx). During morphological evaluation we focused on these characters: trichome shape and width, presence of sheath, cell dimensions, cell wall constrictions, shape of apical cell, presence or absence of calyptra, and granulation of cells. At least 30 filaments of each strain were characterized.

DNA extraction. DNA extraction was performed using the protocol of Doyle & Doyle (1990). The integrity and quality of DNA was checked on 1.8% agarose gels. Concentrations of DNA samples were assessed using a NanoDrop ND–1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA).

DNA amplification and sequencing. PCR amplification of the partial 16S rRNA gene and full 16S–23S ITS region was performed using a combination of two primers P1 (5′–CTCTGGTGCTAGGTATCC–3′) and P2 (5′–GGGAATTTTCCGCAATGGG–3′) described previously in Boyer et al. (2002). These primers produce a ~1180 bp segment of the 16S RNA gene (bp 325–end) as well as the complete 16S–23S ITS region and 30 bases of the 23S rRNA gene. Total volume of the PCR reaction was 20 µl and it contained: 8.5 µl of sterile water, 0.5 µl of each primer (concentration 0.01 mM), 10 µl FastStart PCR master (Roche Diagnostics GmbH, Mannheim, Germany) and 0.5 µl of template DNA (50 ng µl−1). Conditions of the PCR reaction were: 1) initial denaturation for 4 min at 95 °C, 2) 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 57 °C, and extension for 1 min 50 s at 72 °C, and 3) a final extension for 7 min at 72 °C. PCR product was checked on 1.5% agarose gels stained with ethidium bromide. Finally, PCR product was purified using GenElute™ PCRClean–Up Kit (Sigma–Aldrich, Co., Saint Louis, Mo, USA) and sent away for commercial sequencing. Sequencing primers were same as primers for amplification.

Phylogenetic analyses. The sequences were assembled in BioEdit v 7.0.5 (Hall 2005) and gene sequence anomalies (e.g. chimeras) were detected using Mallard software (Ashelford et al. 2005). All sequences investigated in this study were deposited in GenBank (see accession numbers in Table 1). Additional sequences for further phylogenetic analysis were acquired from GenBank (http://www.ncbi.nlm.nih.gov/) using the following criteria: sequences had to be sufficiently long (at least 1013 bp) and freshwater species of Oscillatoriales sensu lato (Pseudanabaenales, Phormidiales, Oscillatoriales in newer taxonomy). Moreover, we tried to avoid poorly determined sequences (marked with sp.). Using these criteria, 78 sequences were chosen for analysis, a data set that was as large as possible given the time restraints of the phylogenetic analyses used. All sequences were initially aligned in Clustal X (Larkin et al. 2007) and manually corrected in BioEdit version 7.0.5 (Hall 2005). Gloeobacter violaceus PCC 8105 was selected
as the outgroup taxon. Phylogenetic analysis was carried out in Mr. Bayes 3.1 (Ronquist & Huelsenbeck 2003), PAUP* version 4.0b10 (Swofford 2001) and MEGA 5.02 (Tamura et al. 2007). Evolutionary models were selected on the basis of the BIC (Bayesian Information Criterion) model test implemented in MEGA 5.02. The evolutionary model used in Mr. Bayes was the GTR model with gamma–distributed rate variation across sites and a proportion of invariable sites. The analysis was run for 10 000 000 generations with sampling every 100th generation. Minimum evolution (ME) and maximum likelihood (ML) analyses were performed in MEGA 5.02 and maximum parsimony (MP) in PAUP*, gaps were treated as missing data. GTR+Γ model was used in ML analysis. Bootstrap resampling was performed using 1000 replications (ME, MP) or 500 replications (ML), respectively.

The secondary structures of different ITS regions (D1–D1´ helix and Box–B helix) were predicted with the Mfold web server version 3.2 (Zucker 2003) with temperature set to default conditions (37 °C) and draw mode at untangle with loop fix. Secondary structures were then drawn in Adobe Illustrator (CS–3).

Results

Morphology of investigated strains

Morphological variability was studied in natural samples as well as in isolated strains. We did not observe extensive variability in filaments in studied morphospecies, especially in natural samples (Table 1). All strains produced single–trichome filaments, only seldom forming filaments of up to five trichomes (e.g. typical for M. vaginatus). Isolated strains usually formed fine mats (Phormidium, Geitlerinema carotinosum, G. pseudacutissimum), macroscopic/microscopic fasciculated colonies (M. vaginatus, G. carotinosum, G. pseudacutissimum) or spherical colonies (G. splendidum). M. vaginatus often loses its fasciculated filaments in culture, and is then morphologically difficult to separate from Ph. autumnale given the similarities in cell dimensions, type of cell division, absence of constrictions at cross–walls, and presence of tapering and calyprata in mature trichomes. However, M. vaginatus (Figs 1–8) was distinguishable from Ph. autumnale (Figs 9–20) in the frequent presence of conspicuous granules at the cross–walls and generally wider trichomes. Trichomes of Ph. formosum (Figs 21–27) were intensely motile (gliding, rotating), constricted slightly at cross–walls, tapered towards apices which possessed rounded to rounded–conical apical cells lacking calyprata. Granulation was fine, if present. A strain of G. carotinosum (P013, Fig. 33) was isolated from Lunzer Untersee, from the same watershed as Geitler’s type material (Lunzer Untersee is hydrologically connected with the type locality Lunzer Obersee, Austria). Apical cells were rounded and conspicuous carotenoid granules were present at cross–walls in this strain. G. pseudacutissimum (Figs 28–32) contained fine carotenoid granules at cross–walls but to a lesser extent than in G. carotinosum. Apical cells were hooked or rounded–acuminate. Both strains of G. splendidum (Figs 34–39) did not differ from each other. They both possessed intensely motile attenuated trichomes, and were bent or screw–like at the ends with capitate or rounded apical cells.

Analysis of 16S rRNA and secondary structures of ITS

The PCR reactions yielded a partial 16S rRNA gene (size ~1100 bp) from every strain. Phylogenetic analysis included also comparable long sequences available in GenBank, particularly well defined freshwater strains of filamentous cyanobacteria (Fig. 40) from the families Pseudanabaenaceae, Phormidiaceae and Oscillatoriaceae. Positions of isolated species in the consensus Bayesian tree were in good agreement with their morphology. The Phormidiaceae formed a distinct clade, but members of the Pseudanabaenaceae formed a paraphyletic cline below the Phormidiaceae (Fig. 40). This made clear separation of Pseudanabaenaceae from the Phormidiaceae difficult.

M. vaginatus, as defined by both morphology and the 11 bp insert (bp 423–433), formed a distinct well–supported clade (Fig. 40, clade A). A single filamentous strain identified initially as Ph. autumnale P007 due to its slightly narrower trichome diameter had the 11 bp insert as well and was subsequently redesignated M. vaginatus. Three strains of Ph. autumnale in clade A (strains EU196619–21) had the same 11 bp insert and were isolated from puddles in the Czech Republic by other workers (Lokmer 2007). We conclude that they belong to M. vaginatus, and should be considered as such in future studies. All strains in this clade were 98% or more similar in their 16S rRNA gene sequence similarity.

Phormidium autumnale sensu stricto (lacking the 11 bp insert) fell into two lineages sister to M. vaginatus, and included a GenBank
sequence designated as Phormidium cf. subfuscum. The branch of Ph. autumnale including Ph. cf. subfuscum did not have good support. However, the clade with clearly calyptrate taxa (Fig. 40, clade B) had good bootstrap support. Oscillatoria sancta and Oscillatoria cf. curviceps do not have the capitate apices with calyptra, but both can have a thickened end cap which has been interpreted to be a calyptra (KOMÁREK & ANAGNOSTIDIS 2005). The clade that includes these two Oscillatoria and clade B, (Ph. autumnale and M. vaginatus) is also well supported.

The clade of Ph. formosum had high bootstrap support and 16S rRNA sequence data showed at least two lineages corresponding to their geographic origin. Both lineages had high bootstrap support.

The branch containing the calyptrate taxa and non–calyptrate Phormidium, along with a mixture of taxa including some Geitlerinema, Microcoleus, Coleofasciculus, Wilmottia and Phormidium species had good bootstrap support (Fig. 40, clade C).

Analysis of the 16S rRNA gene separated
G. carotinosum P013 (Austria) from morphologically similar strains of G. pseudacutissimum originating from Italian Lakes Tovel and Monbi- no (Italy). The internal sequence similarity of the 16S rRNA gene in the G. pseudacutissimum clade was 98.6–99.4% (Fig. 40, clade D). G. carotinosum had very low similarity to the taxa we place in G. pseudoacutissimum (including “G. carotinosum” AICB 37), with 16S rRNA similarity to each of those strains ranging 93.1–93.6% similar. G. splendidum formed a separate clade, which was strongly supported, although our two strains shared only 97.0% similarity. All our strains of Geitlerinina were more related to Phormidiaceae than to Pseudanabaenaceae. Geitlerinina sequenced by others were clearly

Figs 21–39. Variability of filamentous epipelic cyanobacteria: (21) Ph. formosum, strain P0010; (22–23) Ph. formosum, strain P007; (24) Ph. formosum, strain P0A; (25) Ph. formosum, strain P011; (26–27) Ph. formosum, strain P010; (28) G. pseudacutissimum, strain P03; (29–30) G. pseudacutissimum, strain P004; (31–32) G. pseudacutissimum, strain P005; (33) G. carotinosum, strain P013; (34–36) G. splendidum, strain P014; (37–39) G. splendidum, strain P017. Scale bars 10 mm [(21–32, 34–39), (33)].
Table 1. List of isolated strains of epipelic filamentous cyanobacteria [morphology: (L/W) length width ratio, (C) calyptra, (S) sheath, n=30; origin: (A) Austria, (CZ) Czech Republic, (I) Italy]. Molecular characteristics of isolated strains [length of 16S rRNA for all strains ~1031 bp; Gen Bank access number (16S rRNA+ITS), genes of tRNA^Ala and tRNA^Ile in all strains]. Measured environmental variables are shown in Hašler et al. (2008).

<table>
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<th>Strain</th>
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<th>Apical cell</th>
<th>Width mm</th>
<th>Cell L/W</th>
<th>C</th>
<th>S</th>
<th>GenBank access number</th>
<th>ITS length</th>
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<td>+</td>
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<td>CZ Obectov</td>
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<td>+</td>
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<td>+</td>
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<td>547</td>
<td>CZ Vrah</td>
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<td>+</td>
<td>JQ712607 JQ347235</td>
<td>525</td>
<td>CZ Buková</td>
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<td>Ph. formosum</td>
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<td>–</td>
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<td>–</td>
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<td>0.5–1</td>
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<td>JQ712617 JQ347245</td>
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in the Pseudanabaenaceae sister to *Leptolyngbya sensu stricto* (Fig. 40, clade E). *Nodosilinea* (Fig. 40, clade F) is part of a group of strains that were recently described as a new genus (*Perkerson et al. 2011*).

Analyses of secondary structures in 16S–23S ITS regions (size 447–645 bp) demonstrated both similarity and heterogeneity in D1–D1’ and Box–B helices of *Ph. autumnale*, *Ph. formosum*, *M. vaginatus*, *G. carotinosum*, *G. pseudacutissimum* and *G. splendidum*. Basal parts of all D1–D1’ helices in *Phormidium* and *Microcoleus* were formed by identical 5 bp basal helices (5’–GACCA–UGGUC–3’), followed by a unilateral bulge on the 3’ side (Figs 41–48). Generally, secondary structures of D1–D1’ helices of *Ph. formosum* and *M. vaginatus* were also similar in the formation of a large terminal loop (Figs 41–48). This is also consistent with our observations of this structure in isolates from desert soils. The region which was variable in the Phormidiaceae clade was the central helix, which contained various and differing small bilateral and unilateral bulges (Figs 41–48). D1–D1’ helices of *Ph. autumnale*, *Phormidium formosa* and *Microcoleus vaginatus* were strikingly similar in structure, demonstrating a close phylogenetic relationship between the three taxa (Figs 41–48). Secondary structure of the D1–D1’ helices in *Ph. formosum* demonstrated two lineages. One represented by strains P0010 and P07 (isolated from South Bohemia, Fig. 47) and the second represented by strains P00, P010, P001 (isolated from Central Moravia, Fig. 48), a result consistent with the 16S rRNA phylogeny (Fig. 40).

The genus *Geitlerinema* was quite variable in structure of D1–D1’. *G. carotinosum* (strain P013 isolated from Lunzer Untersee) differed in structure (Fig. 49) from *G. pseudacutissimum*, in which D1–D1’ helices demonstrated two lineages (Fig. 50–51). However, both species did have the typical 5’–GACCU–AGGUC–3’ basal helix characteristic of most cyanobacteria. Both strains of *G. splendidum* had an identical D1–D1’ helix, but these structures were very unique. They lacked the 3’–unilateral bulge found in almost all D1–D1’ helices in prokaryotes (Fig. 52). Furthermore, they had a small branch on the 3’ side of the central helix (Fig. 53).

Analysis of secondary structures in Box–B helices demonstrated a pattern similar to that observed for the D1–D1’ helices. All lineages had a conserved basal helix with sequence 5’–CAGCA–UGCUG–3’. *M. vaginatus* generally had longer helices than *Ph. autumnale* (Fig. 54–59). Dissimilarity was evident in the terminal loops, which varied in size and sequence. We found that structures of strains of *M. vaginatus* isolated from Central Moravia were different from those originating from Bohemia. We found some difference between strains of *P. formosum* originating from Bohemia (Fig. 60) and those isolated from Moravia (Fig. 61). Box–B helices differed widely among studied species of *Geitlerinema*. Two structures of Box–B helices were found in *G. pseudacutissimum* but they differed only by one base (Fig. 62–63). The structures of Box–B helices in all three species of *Geitlerinema* were different (Fig. 62–65).

<table>
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**Discussion**

**Morphological variability**

We studied morphological variability of filamentous cyanobacteria from the families Phormidiaceae (Phormidium, Microcoleus) and Pseudanabaenaceae (Geitlerinema), which were collected and isolated from the bottom sediments. The distribution of epipelic species has been found to be influenced primarily by sediment quality (HASLER et al. 2008). The proportion of fine mud tends to be higher at more eutrophic sites, sandy sediments are characteristic for oligo/dystrophic sites. Muddy or sandy–muddy sediments were inhabited by Ph. autumnale [Agardh] Trevisan ex Gomont, Ph. formosum (Bory ex Gomont) Anagnostidis et Komárek, M. vaginatus Gomont ex Gomont and G. splendidum (Greville ex Gomont) Anagnostidis. Sandy sediments were inhabited by G. carotinosum (Geitler) Anagnostidis and G. pseudacutissimum (Geitler) Anagnostidis.

Some of the species, Phormidium autumnale and Microcoleus vaginatus, seem to be widely distributed among sampling sites and exhibit overlapping morphological variation. A typical feature of M. vaginatus, fasciculate filaments, was consistently observed except in strain P007, which kept a single trichome per filament mode of life in culture. The similarity between Ph. autumnale and M. vaginatus was first discussed by Drouet (1962). He considered Ph. autumnale as a special single filament stage (ecophene) of M. vaginatus. The author studied eleven Phormidium–like species (Lynghya aerugineo-caerulea, Ph. autumnale, Ph. favosum, Ph. incrustanum, Ph. setchellianum, Ph. subsalsum, Ph. toficola, Ph. umbilicatum, Ph. uncinatum, Oscillatoria amoena, Os. beggiatoiformis) and postulated that all of them represented natural variability of M. vaginatus under different ecological conditions. Recent studies on Ph. autumnale and M. vaginatus have not supported Drouet’s opinion (e.g. Casamatta et al. 2005; Siegsmund et al. 2008). Our epipelic strains of M. vaginatus showed a narrow morphological variability under laboratory conditions in contrast to descriptions by Drouet (1962) or Komárek & Anagnostidis (2005). The strain P006 was the most representative of epipelic Microcoleus and we consider this strain as epitypic. Ph. autumnale did not exhibit high morphological variability in contrast to previous reports (e.g. Gomont 1888; Geitler 1932; Desikachary 1959; Star mach 1966; Kondrateva 1968; Komárek 1972; Anagnostidis & Komárek 1988, 2005). Cells were usually wider than long and granulation at cross–walls was fine. The single trichome per filament mode of life was typical. However, old cultures formed flat leathery mats. Ph. formosum represents Phormidium group No. III (following the classification published by Komárek & Anagnostidis 2005; p. 423, fig. 602). All strains were characterized by shortly narrowed and bent trichome ends with conically–attenuated or rounded apical cells without calyptra. Ph. formosum shows similarity to another species, e.g. Ph. animale, which belongs to group No II, having gradually narrowed trichome ends in contrast to Ph. formosum. Our strains of Ph. formosum and strain Ph. animale SAG 1459–6 (identical strains: CCAP 1459/6; UTEX 1309) were placed in the same cluster. Ph. animale was isolated before 1972 and morphology has been influenced by long–term cultivation. However, it seems to be similar to our epipelic strains of Ph. formosum. With respect to similar morphology and position in the same cluster, we conclude that the strain of Ph. animale should be referred to as Ph. formosum in future studies. In the case of Ph. formosum/Ph. animale morphological features may be insufficient to separate them as the key diagnostic feature (long vs. short trichome attenuation) appears variable.

Members of the genus Geitlerinema were originally described within the genus Phormidium. However, morphology, ultrastructure and physiology differ significantly (Anagnostidis & Komárek 1988; Anagnostidis 1989). We isolated two strains of G. splendidum with low morphological variability in contrast to variation described previously (e.g. Anagnostidis 1989). We had occasion to study populations of G. carotinosum quite close to the type locality (Austria, Lunz am

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Fig. 40. Phylogram (Consensus Bayesian tree) based on 16S RNA sequences (size ~1000 bp) originated from 20 strains of epipelic cyanobacteria (in bold). Bootstrap values are shown (from left to right) as follows: posterior probabilities ≥ 0.9 and for ≥ 70% minimum evolution, maximum parsimony, maximum likelihood. Sequences from GenBank which appear to us to be misidentified are in quotation marks.
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carotinosum, subg. Geitlerinema (originally described as Oscillatoria carotinosa. See, Lake Untersee, strain P013). The species was
strain P0010, strain P07, (48) strain P0A, P010, P001; (44–46) strain P0B, 0C, P0R1; (44–46) Figs 41–53. ITS secondary structures of D1–D1´ helices: (41–43) strain P006, (42) strain P09, P007, (43) strain P0B, OC, P0R1; (44–46) Ph. autumnale, (44) strain P00, (45) strain P019, (46) strain P012; (47–48) Ph. formosum, (47) strain P0010, strain P07, (48) strain P05, P010, P001; (49) G. carotinosum, strain P013; (50–52) G. pseudoacutissimum, (50) strain P03, (51) strain P005, (52) strain P004; (53) G. splendidum, strain P014 and P017.

See, Lake Untersee, strain P013). The species was originally described as Oscillatoria carotinosa (Geitler 1956), later combined as Phormidium carotinosum, subg. Geitlerinema (Anagnostidis & Komárek 1988). The diagnostic feature of G. carotinosum, carotenoid granules, was found in G. pseudoacutissimum as well. Morphology of both species is very similar. However, from our study it seems that trichome ends and type of thallus differ. While G. pseudoacutissimum from Italy formed fascicles and resembled Microcoleus–like thalli, G. carotinosum from Austria created single filaments. Trichome ends of G. carotinosum were usually rounded in contrast to G. pseudoacutissimum with conical apical cells. However, separation of these two taxa based on type/shape of apical cells can be a fairly subjective decision if used as a sole criterion.

In summary, it seems to be rather characteristic that within species, epipelic populations within this study are morphologically very similar, and consequently populations from different ponds and lakes can be reliably placed within the same species. We tried to verify this hypothesis using molecular methods (see below).

16S rRNA and secondary structures of 16S–23S ITS

Molecular data for the epipelic species under study were congruent with morphology. However the autecology and distribution of individual species shows the patterns discussed below.

Numerous papers focusing on phylogeny of Phormidium–like taxa have been published during
bp insert distinguished all strains of *M. vaginatus* from *Ph. autumnale*. *M. vaginatus* P007 was morphologically similar to *P. autumnale* and was identified by us as that taxon at first due to its narrower trichome width. In previous studies, *M. vaginatus* was recorded as cosmopolitan, occurring mainly in subaerophytic habitats, soils, moist walls, stones, etc. (e.g. GARCIA–PICHEL et al. 2001; KOMAREK & ANAGNOSTIDIS 2005). Our epipelic strains from the Czech Republic clustered together with desert soil strains from the USA (BOYER et al. 2002; SIEGESMUND et al. 2008). It seems possible that cryptic diversity is present in the clade we currently call *M. vaginatus*, and this diversity is not resolved in the 16S rRNA phylogeny. Morphological differences between strains are evident in our work (Figs 1–8). More detailed study of these aquatic strains (ITS, rbcL, physiology) may allow taxonomic recognition of these strains in the future. Secondary structures of 16S–23S ITS regions were different in epipelic and desert soil strains, the highest variation being found in Box–B helices (cf. SIEGESMUND et al. 2008; fig. 4). We conclude that differences in 16S–23S ITS regions show at least two lineages, one adapted for short periods of desiccation in contrast to a second lineage adapted for long hot periods. In general, genetic variation in the ITS region seems to be a useful feature for distinguishing populations of cyanobacteria with respect to geographical and habitat preferences.

On the other hand our results support the purported cosmopolitanism of *Ph. autumnale*. Comte et al. (2007) did not find any genetic or morphological differences between Arctic and Antarctic *Phormidium*–like strains, and their sequences belong to the same clade as our epipelic strain P00. We postulate that one worldwide–distributed genotype might exist, which co–occurs with genotypes adapted for particular geographical and environmental conditions, as in the case of genetically different strains Hašler P012 and P019. Secondary structures in the ITS region are considered as informative (BOYER et al. 2001, 2002; ŘEHÁKOVÁ et al. 2007; PERRKSON et al. 2011), and can serve as an additional taxonomic character. As with previously mentioned authors, we did not find high variability in D1–D1’ helices, but Box–B helices showed divergent patterns, which corresponded to the topology of our tree. Differences found between clones from Moravia (P00, P010, P001) and Bohemia (P0010 and P07) cannot be explained by ecology, as all
localities are eutrophic fishponds with large bird colonies causing organic pollution. However the geographical distance between both regions is approximately 400 km and the ponds belong to different watersheds and geological units.

Ph. formosum has not been sufficiently studied by molecular methods. Only three sequences of 16S rRNA have been submitted to GenBank. Our strains formed a well supported clade with Ph. animale SAG 1459/6 which may have been misidentified. Secondary structures of Box–B helices in Ph. formosum had a specific pattern, different from Ph. autumnale and previously described similar filamentous cyanobacteria (cf. Siegesmund et al. 2008).

In the first molecular studies on Geitlerinema (e.g. Meyers et al. 2007; Bittencourt–Oliveira et al. 2009), the authors did not discuss the position of the genus within the order Oscillatoriales. In a more recent study (and the most thorough on this genus), the authors indicated that Geitlerinema was polyphyletic, with Geitlerinema sensu stricto (including the freshwater G. splendidum) in the Pseudanabaenaceae (Perkerson et al. 2010). However, their phylogeny included no Microcoleus or Phormidium taxa, and consequently the familial placement of Geitlerinema remains uncertain. Our strains of G. carotinosum, G. pseudacutissimum and G. splendidum were in an uncertain position between the Phormidiaceae and Pseudanabaenaceae. While some Geitlerinema strains were closely clear to Leptolyngbya in the Pseudanabaenaceae, others were sister to the Phormidiaceae (clade containing Microcoleus, Phormidium, Wilmottia and Coleofasciculus). Two problematic strains originally assigned to Microcoleus (FI–LIZ3B and JO1–1A) by Boyer et al. (2002) are certainly not in that species, and this further confuses the placement of our Geitlerinema strains. The most interesting result of our phylogenetic analysis is that our Geitlerinema splendidum strains (Hašler P014, Hašler P017) are sister to the clade that includes the remainder of our Geitlerinema strains (under 2 µm in diameter), as well as all of the Oscillatorineae (Phormidiales and Oscillatoriales). Geitlerinema is currently very problematic as it occupies three clades, two between Pseudanabaenaceae (Synechococcineae) and Phormidiaceae (Oscillatorineae) and one clade within the Pseudanabaenaceae. Studies conducted thus far suggest that Geitlerinema has a thylakoid structure belonging to the Pseudanabaenaceae (Komárek & Anagnostidis 2005). More study on the taxa transitional between the two families (indeed between two subclasses – Synechococcineae and Oscillatorineae! – see Hoffmann et al. 2005) is certainly needed.

We suggest the revision of the genus Geitlerinema based on material collected from more localities and ecological conditions. Our data show that the genus is not a monophyletic group. This would certainly be consistent with the conclusions of Perkerson et al. (2010) who looked at more putative Geitlerinema than us. Sequences of 16S rRNA from G. carotinosum and G. pseudacutissimum confirmed the validity of recognizing these as separate species. Description of both species based on morphology is almost identical (Komárek & Anagnostidis 2005). However, both species are clearly separated with strong bootstrap support. This finding is supported by analysis of secondary structures in D1–D1’ and Box–B helices. It seems that G. carotinosum has been observed only in the type locality and connected lakes in Lunz am See. By contrast, G. pseudacutissimum is known from the Czech Republic (Lužnice River, strain CCALA 142) and from Italy (Lakes Tovel and Monbino). Despite some limitation (number of strains under study) we do not agree with Willame et al. (2006) that G. splendidum and G. carotinosum are closely related. Our results are supported by differences in secondary structures in ITS and have a high bootstrap support.

This study showed that for a number of species good agreement between morphology and phylogeny existed at the species level. M. vaginatus, P. autumnale, P. formosum, G. pseudacutissimum, and G. carotinosum all formed monophyletic groups consistent with their morphology. What was surprising was that aquatic members of the M. vaginatus clade were found, and these were fairly indistinguishable morphologically from P. autumnale. These two taxa differ primarily in sheath and filament characteristics, and these are very variable depending on environmental cues. The sheaths tend to disappear in culture, and actually are not very evident in aquatic populations. The fasciculation clear in soil populations of M. vaginatus was only weakly expressed in the epipelon. The strong difference in biotopes (desert soil, Czech lakes) suggests separate lineages, but these lineages were not separable by phylogenetic analysis of the 16S rRNA gene sequence. More study of these populations is certainly of interest,
as it is at the center of the physiological variability possible in multiple populations of a single species, or the alternative, cryptic species within a genus.

Finally, this study shows that taxonomic revision is almost certainly inevitable in the group of taxa currently encompassed in Phormidium and Microcoleus. These two taxa share the same starting point (Gomont 1892). Microcoleus vaginatus has cell division similar to the Oscillatoriaceae, and is very different from the majority of species in the genus which have cell division similar to Phormidiaceae. The type species of Phormidium is P. lucidum, which also has cell division closer to Oscillatoriaceae than Phormidiaceae. Thus, the types for both Microcoleus and Phormidium are in the Oscillatoriaceae as presently defined in Komárek & Anagnostidis (2005), leaving the vast majority of species in both genera needing revision. Phormidium and Microcoleus are also confused, and a recommendation has even been made to retypify Phormidium with P. autumnale (Komárek & Anagnostidis 2005), which would place both types in a highly supported monophyletic clade. Clearly, this problematic group of species, genera, and even families is in need of further study and revision!

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