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–Pichel et al. 1996), whereas in other cases the traditional classification drastically underestimates extant diversity (Ferris et al. 1996).

The assemblages of autotrophic micro-oranisms (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 (POULÍČKOVÁ et al. 2008a). Although epipelic eukaryotic algae were previously studied, e.g. diatoms (reproductive biology, cryptic speciation, geographic biodiversity and bioindication; POULÍČKOVÁ 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 *Komvophoron, Oscillatoria, Phormidium, Geitlerinema* and *Pseudanabaena* (ŠPAČKOVÁ et al. 2009; HAŠLER & POULÍČKOVÁ 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 (*Geilterinema, 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°3‘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 AxioImager 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’–CTCTGTGTGCCTAGGTATCC–3’) and P2 (5’–GGGGAATTTTCCGCAATGGG–3’) described previously in BOYER et al. (2002). These primers produce a ~1180 bp segment of the 16S rRNA 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 $ng.\mu l^{-1}$). 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

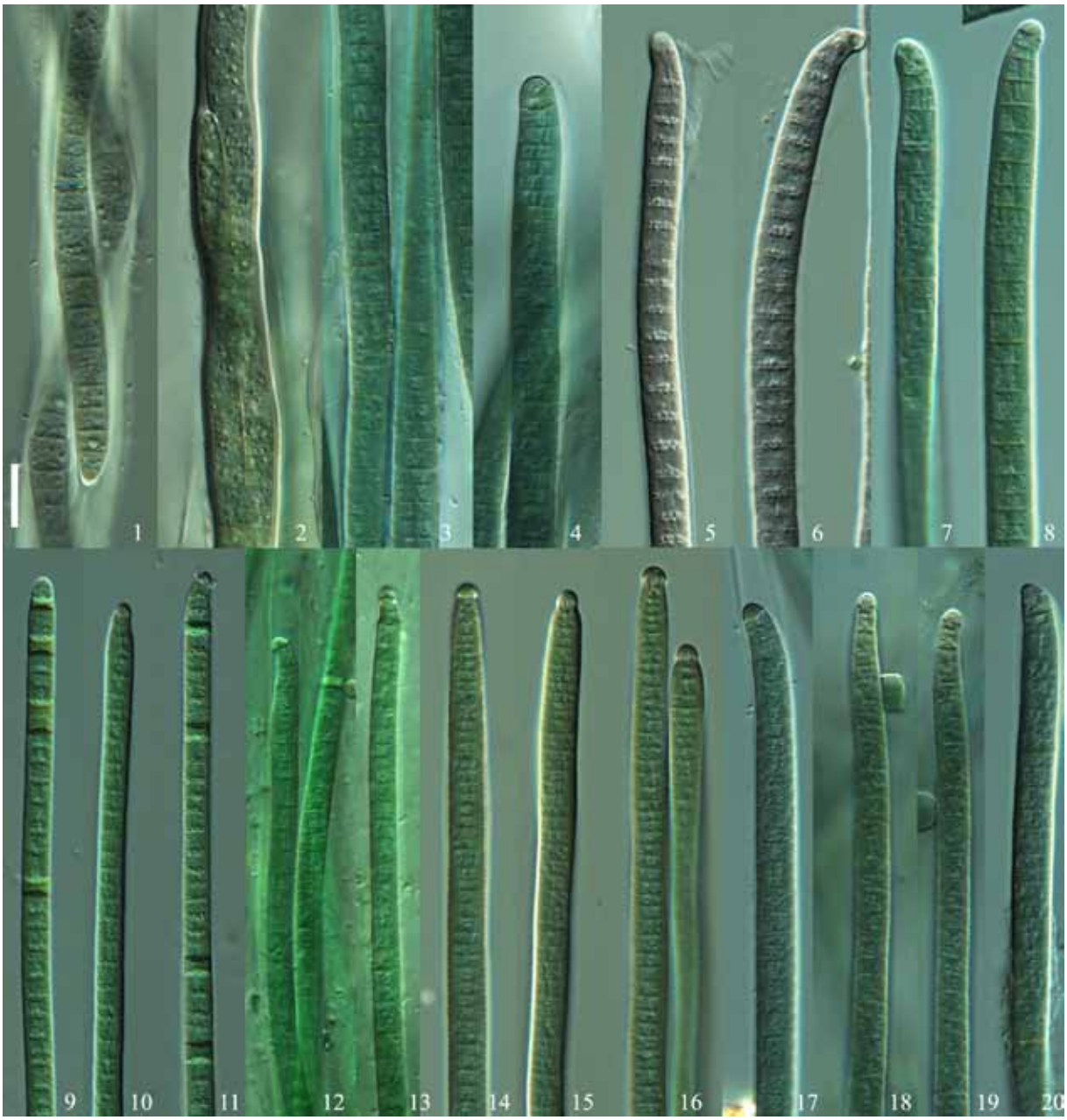
Morphological variability was studied in natural samples as well as in isolated strains. We did not observe extensive variability in filaments in studied morphospecies, especially in natural samples (Table 1). All strains produced single–trichome filaments, only seldom forming filaments of up to five trichomes (e.g. typical for *M. vaginatus*). Isolated strains usually formed fine mats (*Phormidium*, *Geitlerinema carotinosum*, *G. pseudoacutissimum*), 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 calyptra 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 calyptra. 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.

Phormidum autumnale sensu stricto (lacking the 11 bp insert) fell into two lineages sister to *M. vaginatus*, and included a GenBank



Figs 1–20. Variability of filamentous epipelic cyanobacteria: (1–2) *M. vaginatus*, strain P006; (3–4) *M. vaginatus*, strain P0R1; (5–6) *M. vaginatus*, strain P09; (7) *M. vaginatus*, strain P0B; (8) *M. vaginatus*, strain P0C; (9–11) *Ph. autumnale*, strain P00; (12–13) *M. vaginatus*, strain P007; (14–16) *Ph. autumnale*, strain P019; (17–20) *Ph. autumnale*, strain P012. Scale bar 10 mm.

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

Figs 21–39. Variability of filamentous epipelic cyanobacteria: (21) *Ph. formosum*, strain P0010; (22–23) *Ph. formosum*, strain P07; (24) *Ph. formosum*, strain P0A; (25) *Ph. formosum*, strain P001; (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)].

G. carotinosum P013 (Austria) from morphologically similar strains of *G. pseudacutissimum* originating from Italian Lakes Tovel and Monbino (Italy). The internal sequence similarity of the 16S rRNA gene in the *G. pseudoacutissimum* 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 *Geitlerinema* were more related to Phormidiaceae than to Pseudanabaenaceae. *Geitlerinema* sequenced by others were clearly

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).

Strain	Trichome end	Apical cell	Width mm	Cell L/W	C	S	GenBank access number	ITS length	Origin
Ph. autumnale									
P00	Attenuated	Rounded, conical, capitate	4–6	0.3–1	+	+	JQ712616 JQ347244	560	CZ Obectov
P012	Attenuated	Rounded, conical, capitate	5–7	0.5–1	+	+	JQ712612 JQ347240	556	CZ Chropyně
P007	Attenuated	Rounded, conical, capitate	4–5	0.5–1	+	+	JQ712604 JQ347232	547	CZ Vrah
P019	Attenuated	Rounded, conical, capitate	4–6	0.3–0.75	+	+	JQ712607 JQ347235	525	CZ Buková
Ph. formosum									
P0010	Shortly attenuated	Rounded, conical	4–5	0.5–1	–	–	JQ712600 JQ347228	645	CZ Naděje
P07	Shortly attenuated	Rounded, conical	4–6	0.5–1	–	–	JQ712606 JQ347234	635	CZ Velký Tisý
P0A	Shortly attenuated	Rounded, conical	4–5	0.5–1	–	–	JQ712603 JQ347231	642	CZ Tovačov
P001	Shortly attenuated	Rounded, conical	4–5	0.5–1	–	–	JQ712611 JQ347239	644	CZ Záhlinice 2
P010	Shortly attenuated	Rounded, conical	4–6	0.3–1	–	–	JQ712613 JQ347241	642	CZ Chropyně
M. vaginatus									
P006	Attenuated	Rounded, conical, capitate	6–7	0.5–1	+	+	JQ712615 JQ347243	566	CZ Obora
P0R1	Attenuated	Rounded, conical, capitate	6–7	0.3–1	+	+	JQ712610 JQ347238	557	CZ Buková
P09	Attenuated	Rounded, conical, capitate	5–7	0.5–1	+	+	JQ712605 JQ347233	553	CZ Rožmberk
P0B	Attenuated	Rounded, conical, capitate	6–7	0.5–1	+	+	JQ712609 JQ347237	577	CZ Horní Ves
P0C	Attenuated	Rounded, conical, capitate	6–7	0.5–1	+	+	JQ712601 JQ347229	581	CZ Bezedník
G. pseudacutissimum									
P004	Not attenuated	Rounded, pointed	1.5–2	1.5–3	–	–	JQ712617 JQ347245	447	I Lake Monbino

Table 1 Cont.

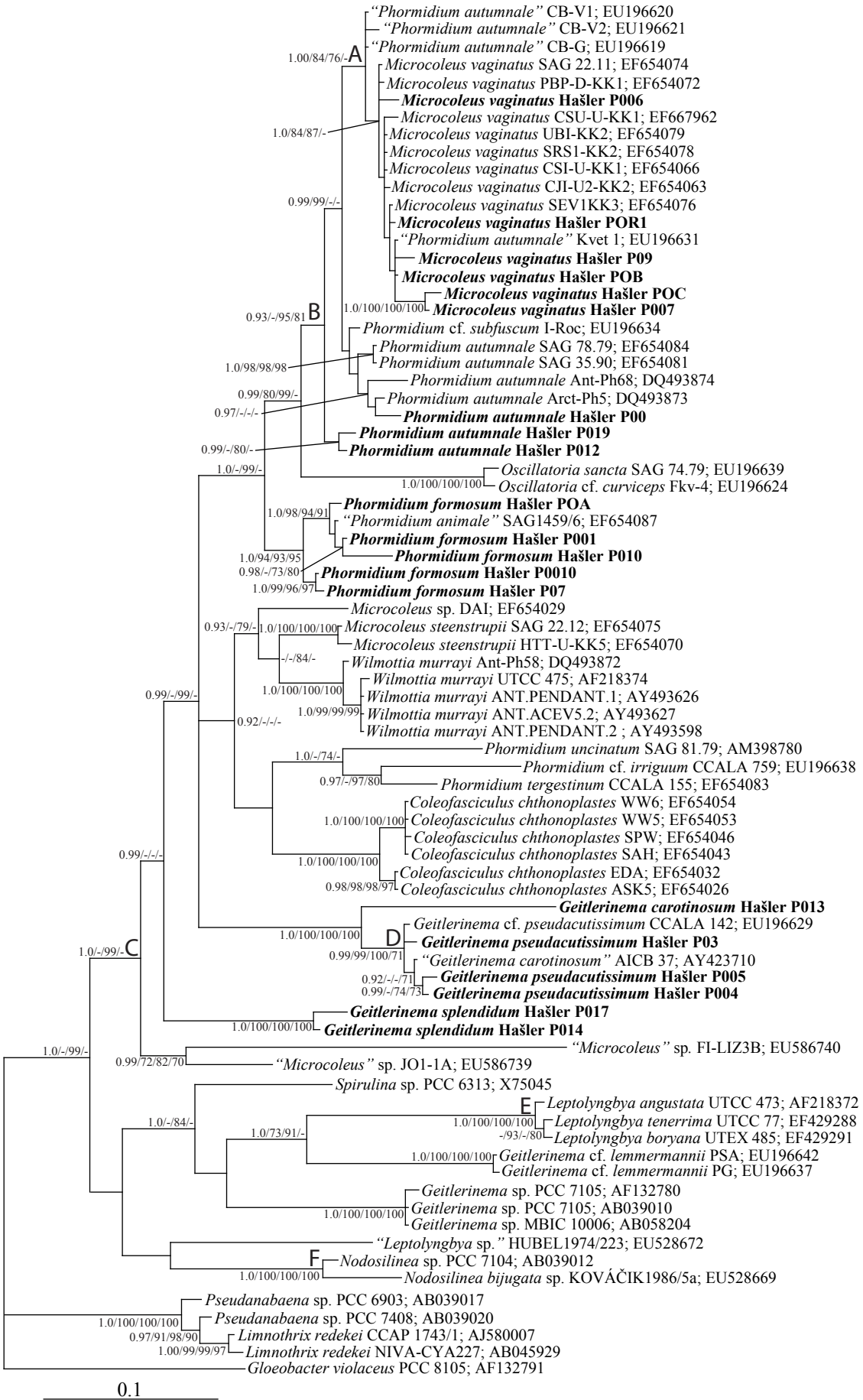
P005	Not attenuated	Rounded, pointed	1.5–2	1.5–3	–	–	JQ712608 JQ347236	451	I Lake Monbino
P03	Not attenuated	Rounded, pointed	1.5–2	1.5–3	–	–	JQ712614 JQ347242	461	I Lake Tovel
G. carotinosum									
P013	Not attenuated	Rounded, pointed	1.5–2	1.5–3	–	–	JQ712598 JQ347226	477	A Lake Untersee Lunz
P014	Attenuated, bent, screw-like	Rounded, capitate	2–3	1.5–3	–	–	JQ712602 JQ347230	493	I Lake Monbino
P017	Attenuated, bent, screw-like	Rounded, capitate	2–2.5	1.5–3	–	–	JQ712599 JQ347227	491	I Lake Tovel

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 heterogenity in D1–D1´and Box–B helices of *Ph. autumnale*, *Ph. formosum*, *M. vaginatus*, *G. carotinosum*, *G. pseduacutissimum* 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. autumnale*, *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. pseudoacutissimum*, 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 obsrved 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. pseudoacutissimum* 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).


"Phormidium autumnale" CB-V1; EU196620
"Phormidium autumnale" CB-V2; EU196621
"Phormidium autumnale" CB-G; EU196619
1.00/84/76/-A
Microcoleus vaginatus SAG 22.11; EF654074
Microcoleus vaginatus PBP-D-KK1; EF654072
***Microcoleus vaginatus* Hašler P006**
Microcoleus vaginatus CSU-U-KK1; EF667962
1.0/84/87/-
Microcoleus vaginatus UBI-KK2; EF654079
Microcoleus vaginatus SRS1-KK2; EF654078
Microcoleus vaginatus CSI-U-KK1; EF654066
Microcoleus vaginatus CJI-U2-KK2; EF654063
0.99/99/-/-
Microcoleus vaginatus SEV1KK3; EF654076
***Microcoleus vaginatus* Hašler POR1**
"Phormidium autumnale" Kvet 1; EU196631
***Microcoleus vaginatus* Hašler P09**
***Microcoleus vaginatus* Hašler POB**
***Microcoleus vaginatus* Hašler POC**
1.0/100/100/100
***Microcoleus vaginatus* Hašler P007**
0.93/-/95/81B
Phormidium cf. *subfuscum* I-Roc; EU196634
Phormidium autumnale SAG 78.79; EF654084
1.0/98/98/98
Phormidium autumnale SAG 35.90; EF654081
Phormidium autumnale Ant-Ph68; DQ493874
0.99/80/99/-
Phormidium autumnale Arct-Ph5; DQ493873
0.97/-/-/-
***Phormidium autumnale* Hašler P00**
***Phormidium autumnale* Hašler P019**
0.99/-/80/-
***Phormidium autumnale* Hašler P012**
1.0/-/99/-
Oscillatoria sancta SAG 74.79; EU196639
1.0/100/100/100
Oscillatoria cf. *curviceps* Fkv-4; EU196624
***Phormidium formosum* Hašler POA**
1.0/98/94/91
"Phormidium animale" SAG1459/6; EF654087
***Phormidium formosum* Hašler P001**
1.0/94/93/95
***Phormidium formosum* Hašler P010**
0.98/-/73/80
***Phormidium formosum* Hašler P0010**
1.0/99/96/97
***Phormidium formosum* Hašler P07**
Microcoleus sp. DAI; EF654029
0.93/-/79/-
1.0/100/100/100
Microcoleus steenstrupii SAG 22.12; EF654075
Microcoleus steenstrupii HTT-U-KK5; EF654070
-/-/84/-
Wilmottia murrayi Ant-Ph58; DQ493872
1.0/100/100/100
Wilmottia murrayi UTCC 475; AF218374
0.99/-/99/-
Wilmottia murrayi ANT.PENDANT.1; AY493626
1.0/99/99/99
Wilmottia murrayi ANT.ACEV5.2; AY493627
0.92/-/-/-
Wilmottia murrayi ANT.PENDANT.2 ; AY493598
Phormidium uncinatum SAG 81.79; AM398780
1.0/-/74/-
Phormidium cf. *irriguum* CCALA 759; EU196638
0.97/-/97/80
Phormidium tergestinum CCALA 155; EF654083
Coleofasciculus chthonoplastes WW6; EF654054
1.0/100/100/100
Coleofasciculus chthonoplastes WW5; EF654053
Coleofasciculus chthonoplastes SPW; EF654046
0.99/-/-/-
Coleofasciculus chthonoplastes SAH; EF654043
1.0/100/100/100
Coleofasciculus chthonoplastes EDA; EF654032
0.98/98/98/97
Coleofasciculus chthonoplastes ASK5; EF654026
***Geitlerinema carotinosum* Hašler P013**
Geitlerinema cf. *pseudacutissimum* CCALA 142; EU196629
1.0/100/100/100
D
***Geitlerinema pseudacutissimum* Hašler P03**
1.0/-/99/-C
0.99/99/100/71
"Geitlerinema carotinosum" AICB 37; AY423710
0.92/-/-/71
***Geitlerinema pseudacutissimum* Hašler P005**
0.99/-/74/73
***Geitlerinema pseudacutissimum* Hašler P004**
***Geitlerinema splendidum* Hašler P017**
1.0/100/100/100
***Geitlerinema splendidum* Hašler P014**
1.0/-/99/-
"Microcoleus" sp. FI-LIZ3B; EU586740
0.99/72/82/70
"Microcoleus" sp. JO1-1A; EU586739
Spirulina sp. PCC 6313; X75045
E
Leptolyngbya angustata UTCC 473; AF218372
1.0/-/84/-
1.0/100/100/100
Leptolyngbya tenerrima UTCC 77; EF429288
1.0/73/91/-
-/93/-/80
Leptolyngbya boryana UTEX 485; EF429291
1.0/100/100/100
Geitlerinema cf. *lemmermannii* PSA; EU196642
Geitlerinema cf. *lemmermannii* PG; EU196637
Geitlerinema sp. PCC 7105; AF132780
1.0/100/100/100
Geitlerinema sp. PCC 7105; AB039010
Geitlerinema sp. MBIC 10006; AB058204
"Leptolyngbya sp.*"* HUBEL1974/223; EU528672
F
Nodosilinea sp. PCC 7104; AB039012
1.0/100/100/100
Nodosilinea bijugata sp. KOVÁČIK1986/5a; EU528669
Pseudanabaena sp. PCC 6903; AB039017
1.0/100/100/100
Pseudanabaena sp. PCC 7408; AB039020
0.97/91/98/90
Limnothrix redekei CCAP 1743/1; AJ580007
1.00/99/99/97
Limnothrix redekei NIVA-CYA227; AB045929
Gloeobacter violaceus PCC 8105; AF132791
0.1

Discussion

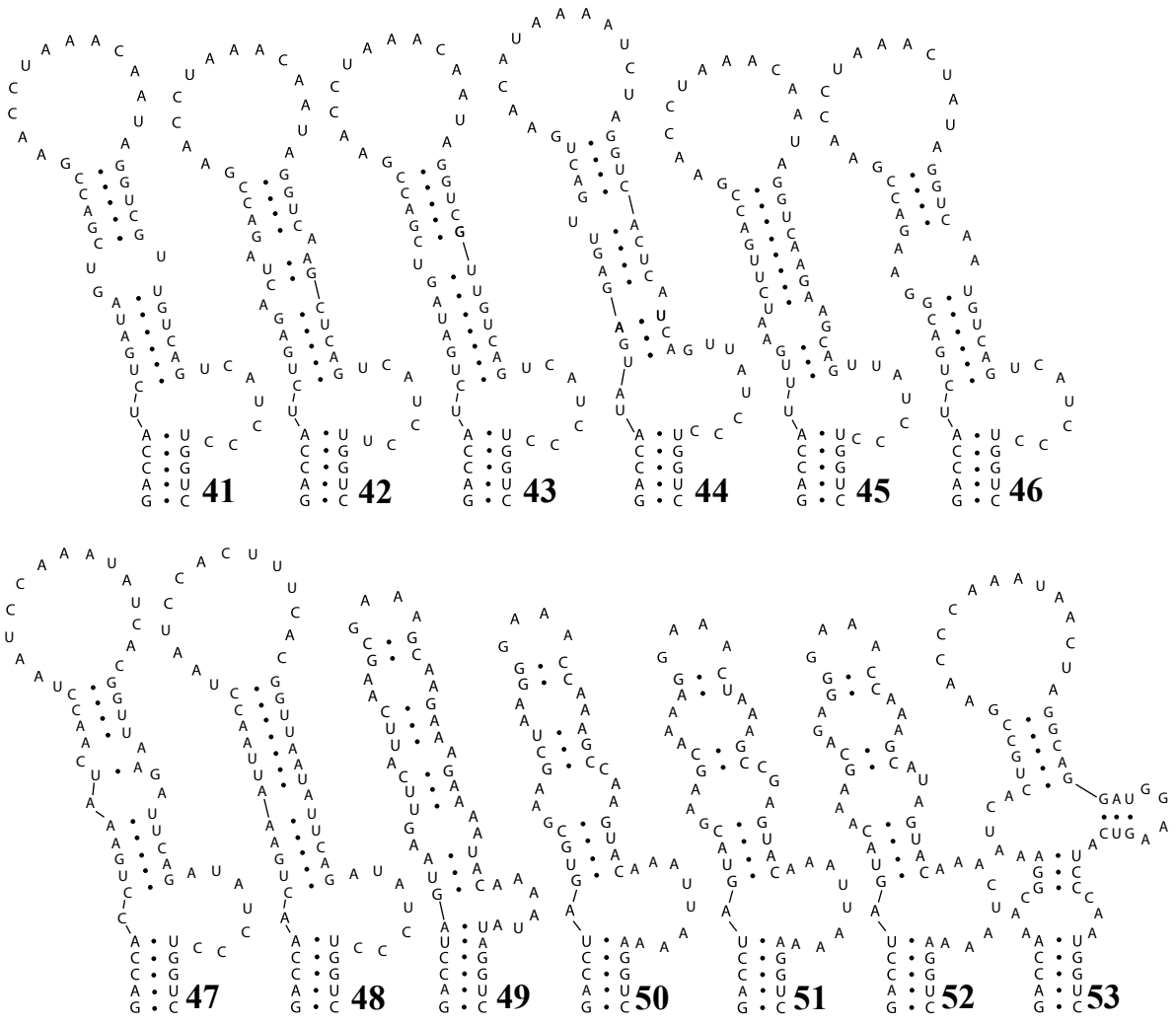
Morphological variability

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 (Hašler 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 (*Lyngbya 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; Siegesmund 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; Starmach 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. splendidium* 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

Fig. 40. Phylogram (Consensus Bayesian tree) based on 16S rRNA 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.



Figs 41–53. ITS secondary structures of D1–D1′ helices: (41–43) *M. vaginatus*, (41) strain P006, (42) strain P09, P007, (43) strain P0B, 0C, 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 P0A, P010, P001; (49) *G. carotinosum*, strain P013; (50–52) *G. pseudacutissimum*, (50) strain P03, (51) strain P005, (52) strain P004; (53) *G. splendidum*, strain P014 and P017.

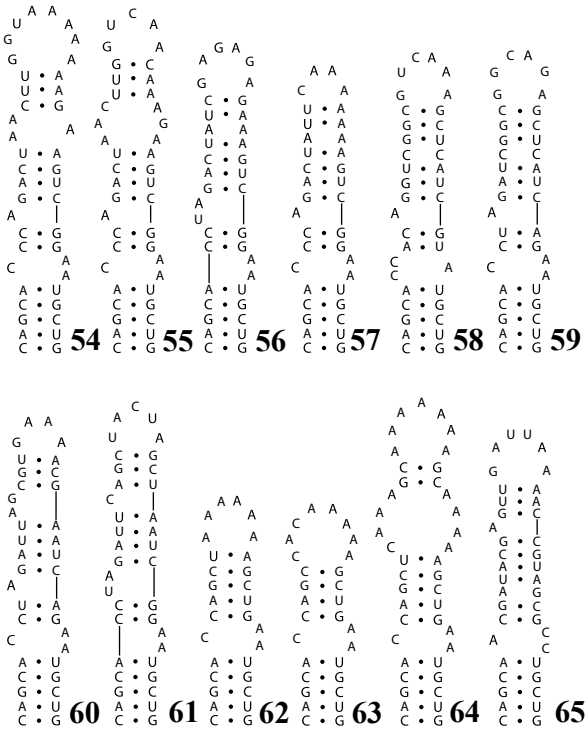
See, Lake Untersee, strain P013). The species was originally described as *Oscillatoria carotinosa* (GEITLER 1956), later combined as *Phormidium carotinosum*, subg. *Geitlerinema* (ANAGNOSTIDIS & KOMÁREK 1988). The diagnostic feature of *G. carotinosum*, carotenoid granules, was found in *G. pseudoacutissimum* 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



Figs 54–65. ITS secondary structures of Box B helices: (54–56) *M. vaginatus*, (54) strain P006, (55) strain P0B, P0C, (56) strain P09, P007; (57–59) *Ph. autumnale*, (57) strain P00, (58) strain P019, (59) strain P012; (60–61) *Ph. formosum*, (60) strain P0010, (61) strain P0A, P010, P001; (62–63) *G. pseudacutissimum*, (62) strain P03, (63) strain P004, P005; (64) *G. carotinosum*, strain P013; (65) *G. splendidum*, strain P014, P017.

the last decade (e.g. Boyer et al. 2002; Marquardt & Palinska 2007; Palinska & Marquardt 2008; Siegesmund et al. 2008). The majority of authors considered the *M. vaginatus–Ph. autumnale* complex as polyphyletic, although a high degree of morphological and genetic similarity between the two taxa was found. Important diacritical features overlap (trichome structure, cell dimensions, successive cell division, presence of calyptrae and sheaths, autecology). Analysis of the 16S rRNA gene presented here confirmed the relationship between *M. vaginatus* and *Ph. autumnale* recorded previously (Siegesmund et al. 2008). A number of workers have noted an 11 bp insert in the 16S rRNA gene (bp 423–433) of *M. vaginatus* (Garcia–Pichel et al. 2001; Boyer et al. 2002; Siegesmund et al. 2008), and this has been identified as an important synapomorphic feature defining the species. It was surprising to find this marker in our aquatic, epipelic strains, as *M. vaginatus* has been thought to be a soil species in arid soils in the past. Both phylogenetic analysis and the presence of the 11 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; Komárek & 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; Perkerson 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 clearly close 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. pseudoacutissimum,* 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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