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ORIGINAL PAPER

Genotypic and phenotypic diversity of cyanobacteria assigned to the genus *Phormidium* (Oscillatoriales) from different habitats and geographical sites

Jürgen Marquardt · Katarzyna A. Palinska

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Abstract In this study, 30 strains of filamentous, non-heterocystous cyanobacteria from different habitats and different geographical regions assigned to diverse oscillatorian genera but here collectively referred to as members of the *Phormidium* group have been characterized using a polyphasic approach by comparing phenotypic and molecular characteristics. The phenotypic analysis dealt with cell and filament morphology, ultrastructure, phycoerythrin content, and complementary chromatic adaptation. The molecular phylogenetic analyses were based on sequences of the 16S rRNA gene and the adjacent intergenic transcribed spacer (ITS). The sequences were located on multiple branches of the inferred cyanobacterial 16S rRNA tree. For some, but not all, strains with identical 16S rDNA sequences, a higher level of discrimination was achieved by analyses of the less conserved ITS sequences. As shown for other cyanobacteria, no correlation was found between position of the strains in the phylogenetic tree and their geographic origin. Genetically similar strains originated from distant sites while other strains isolated from the same sampling site were in different phylogenetic clusters. Also the presence of phycoerythrin was not correlated with the strains' position in the phylogenetic trees. In contrast, there was some correlation among inferred phylogenetic relationship, original environmental habitat, and morphology. Closely related strains came from similar ecosystems and shared the same morphological and ultrastructural features. Nevertheless, structural properties are insufficient in themselves for identification at the genus or species level since some phylogenetically distant members also showed similar morphological traits. Our results reconfirm that the *Phormidium* group is not phylogenetically coherent and requires revision.

Keywords Cyanobacteria · Oscillatoriales · *Phormidium* · Phylogeny · Taxonomy

J. Marquardt · K. A. Palinska (✉)
Geomicrobiology, ICBM,
Carl von Ossietzky University of Oldenburg,
Carl-von-Ossietzky-Str. 9–11, 26111 Oldenburg, Germany
e-mail: k.palinska@uni-oldenburg.de

Introduction

The genus *Phormidium* Kützing 1843 has been used to encompass widely diverse morphotypes. For the Oscillatoriacean cyanobacteria, including those discussed in this communication, Maurice Gomont's Monographie des Oscillatoriées (Gomont 1892) is usually considered the earliest comprehensive source for their taxonomic classification. Gomont's scheme was followed with minor modifications by Geitler (1932), Frémy (1934), Elenkin (1949), Desikachary (1959), Starmach (1966) and Umezaki (1961).

The criteria traditionally prescribed for classification of genera in Oscillatoriaceae relied predominantly on the quality of external sheaths and colony formation rather than on cellular features. This left considerable freedom for interpretation errors, and opened the possibility that some species may have been described separately and placed in more than one genus. The genus *Phormidium* Kützing is characterized by thin, hyaline, mucous, partly diffluent or completely dissolved sheaths that cause filaments to stick together in mat-like layers.

It includes a large number of species from freshwater and marine environments, with different cell sizes and proportions, and different end cell morphology as well as different degrees of constrictions at cross-walls. Forms with thin, hyaline, firm but externally sticky sheaths, and with filaments, occasionally falsely branched, arranged in upright bundles were placed in the genus *Symploca* Kützing. The genus *Lyngbya* Agardhi included forms with well defined, thick sheaths and separated filaments. Difficulties in making generic distinction in view of possible environmental influences on sheath quality prompted Bourrelly (1970) to abolish the genera *Phormidium* and *Symploca*, and to transfer all species described within these genera to *Lyngbya* on the grounds of priority. Drouet (1968) radically revised the systematics of the Oscillatoriaceae, under the a priori assumption that differences among cyanobacterial phenotypes are mostly environmental modifications of a limited number of genotypes. The result was a drastic reduction of the number genera and species considered unacceptable by many authors (see Anagnostidis and Komarek 1985) and some of the taxonomic oversimplifications were experimentally proven to be unjustified (Stam and Venema 1977; see also Castenholz and Waterbury 1989).

Rippka et al. (1979) introduced a classification system of generic assignments for cyanobacteria by accepting or modifying generic definitions derived from the study of field populations (Geitler 1932). The resulting systematic scheme was based on axenic cultures, with limited applicability to morphological and ecological diversity of cyanobacteria observed in nature. Generic distinctions within Oscillatoriaceae were not completely resolved, but left as complexes LPP-groups A and B (for *Lyngbya–Phormidium–Plectonema*). This system was adopted with minor modifications and the addition of a few, well characterized generic descriptions derived from studies of natural populations in the first and second editions of Bergey's Manual of Systematic Bacteriology (Castenholz and Waterbury 1989; Castenholz et al. 2001). A major difference between these two editions, however, was the recognition (Castenholz et al. 2001) of the genera *Leptolyngbya* Anagnostidis and Komarek 1988, *Geitlerinema* Anagnostidis 1989 and *Microcoleus* Desmazières 1823 for some members previously assigned to the LPP-group B.

A major revision of cyanobacteria (cyanoprokaryotes) was recently introduced by Anagnostidis and Komárek (1985, 1988) and Komarek and Anagnostidis (2005). The system of the traditional genera complex that included *Phormidium, Lyngbya, Plectonema* ("LPP-group" of Rippka et al. 1979) and *Oscillatoria* was revised by introduction of new criteria such as cell proportions and division patterns, occurrence of gas vesicle clusters (aerotopes), motility, and manner of trichome disintegration. The earlier emphasis on sheaths was deprecated. As a consequence, a large number of species (about 440) were transferred from the above four "Geitlerian" genera to 18 newly defined generic entities, most of them acquiring corresponding name changes. Notably, the genus *Phormidium* was defined by Komárek and Anagnostidis (2005) as having, among other characteristics, radially oriented thylakoids in transversal sections of the cells. Unfortunately, this feature has not yet been proven for the majority of the numerous species described from both nature and culture studies. *Leptolyngbya* and *Geitlerinema* were created as a new genus and subgenus of *Phormidium*, respectively (Anagnostidis and Komarek 1988), to include a large number of oscillatorialean species with trichomes up to 3 μm wide. Narrow-celled species exhibiting little or no motility of the Geitlerian genera: *Phormidium, Plectonema* and *Lyngbya* were placed into the newly created genus *Leptolyngbya* Anagnostidis and Komarek 1988. Species with similar cell dimensions that show rapid gliding motility by rotation and contain peripheral thylakoids were placed into *Geitlerinema*, elevated to generic status in 1989 (Anagnostidis 1989).

Another problematic genus is *Microcoleus* Desmazières 1823, used for the taxonomic assignment of filamentous non-heterocystous cyanobacteria that form bundles of filaments united by a common gelatinous sheath (Geitler 1932; Castenholz et al. 2001; Anagnostidis and Komarek 1988; Komarek and Anagnostidis 2005). However, their trichome morphology may correspond to many different genera of the Oscillatoriales. Furthermore, under laboratory culture conditions sheath formation may be altered or lost, as shown for *Microcoleus chthonoplastes* (Garcia-Pichel et al. 1996) and generic assignment will be impossible, if based on this trait.

Cyanobacterial systematics are currently in a state of confusion. It is recognized among workers in this field that the identification of any particular species based solely on morphology is highly dubious and impractical. Apart from the nomenclatural difficulties mentioned above, cyanobacteria maintained in culture may also loose or no longer express certain characteristic features observed in nature (Palinska et al. 1996; Garcia-Pichel et al. 1996). In order to overcome some of these problems and to permit identification of cyanobacteria at the genetic level, different molecular tools have been applied for their distinction at different

taxonomic levels (e.g. Iteman et al. 2002; Neilan et al. 1995; Rajaniemi et al. 2005; Schönhuber et al. 1999; Stam 1980; Turner 1997; Zehr et al. 2003). With the goal of establishing in the future taxonomy of cyanobacteria supported by phylogenetic relationships, a polyphasic approach, combining phenotypic and genotypic characterizations has been recommended (Wilmotte and Herdman 2001; Stackebrandt 2001; Turner 1997; Palinska et al. 1996).

In this study, 30 strains of filamentous, non-heterocystous, cyanobacteria of the *Phormidium* group coming from different habitats (freshwater and marine, terrestrial crusts, mats, and turfs) and different geographical regions (Europe, Australia, South and North America, Asia, and Antarctica) have been characterized using a polyphasic approach comparing phenotypic, and molecular characteristics. The combination of modern phylogenetic research with classical taxonomic procedures was expected to improve the basis for classification of this group of microorganisms.

Materials and methods

Cyanobacterial material and culture conditions

The cyanobacterial strains listed in Table 1 were cultured in 50 ml Erlenmeyer flasks at ambient light and temperature, except strain *Oscillatoria* sp. CCMEE 416 which was kept at 12°C. The culture media were either BG11 or ASNIII (Rippka et al. 1979) as specified in Table 1. The strains were not bacteria-free and are available from the appropriate culture collection upon request. To test for the ability of the organisms to perform complementary chromatic adaptation, samples were kept for 6 weeks in red or green light using filters described by Tandeau de Marsac (1977).

Light microscopy, scanning electron microscopy and ultrastructural studies

Light microscopy studies were performed with a Zeiss Axioskop 50 microscope equipped with transmitted light, phase contrast, and Nomarski interference contrast illumination. Photomicrographs were taken using a Nikon Digital Camera DXM 1200. For scanning electron microscopy (SEM), samples were fixed in 4% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2), and dehydrated through a series of ethanol–water solutions. The samples were then critical-point dried in a Balzers Union (CPD 010) apparatus before gold sputtering (SCD 030, Balzers Union). The samples were examined with a ZEISS DSM 940 or a Hitachi S-450 scanning electron microscope operated at 10 or 20 kV and with working distances of 7–9 mm. The light microscopy and SEM studies were used to characterize cell sizes, cell forms, and the degree of constriction at cell junctions.

For ultrastructural studies by transmission electron microscopy (TEM), samples were prepared and embedded according to the procedures described previously (Surosz and Palinska 2004) and examined with a Zeiss EM 109 or EM 902A transmission electron microscope.

Isolation of genomic DNA

Twenty-five milliliter of the cyanobacterial cultures were spun down in a tabletop centrifuge and resuspended in 1 ml TESC (10 mM Tris, 1 mM EDTA, 20 mM NaCl, 2% cetyltrimethylammonium bromide, pH 8.0). After addition of lysozyme (1% final concentration), the samples were incubated at 37°C for 1 h. After 10 cycles of freeze (in liquid nitrogen) and thaw (at 65°C), 5 µl Proteinase K (100 µg/ml) and 90 µl 10% sodium dodecyl sulphate was added and the samples were incubated at 52°C for 150 min. The samples were centrifuged in a microcentrifuge at 12,000*g* for 5 min and the supernatants were extracted twice with phenol, phenol/chloroform, and chloroform. The DNA was precipitated from the aqueous phase with 0.6 volumes of 2-propanol, washed with 70% ethanol, vacuum dried, and stored in 100µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

PCR amplification and sequencing

Primers PLG1.1 and PLG2.1 described by Nadeau et al. (2001) were used for amplification of partial 16S rRNA genes. The Intergenic Transcibed Spacer (ITS) between the 16S and 23S rRNA genes was amplified with primers 322 and 340 (Iteman et al. 2000). The reaction volume was 100 µl and contained: 1 × RED-Taq PCR Buffer, 200 µM of each deoxynucleotide, 200 µg BSA, 500 ng of each oligonucleotide primer, 5 U of RED Taq DNA polymerase (Sigma-Aldrich), and 1 µl of DNA extract. After an initial denaturation step (4 min at 94°C), 31 incubation cycles followed, each consisting of 1 min at 94°C, 1 min at 52°C, and 1.5 min (8 min at the last cycle) at 72°C. The presence of PCR products was detected by standard agarose gel electrophoresis and ethidium bromide staining. Amplification products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). In cases of ITS amplification the entire PCR product was put on agarose gel, electrophorized, appropriate bands

Table 1 Compilation of the cyanobacterial strains examined, their isolation site and the medium used for their cultivation

No.	Taxon	Strain[a]	Geographical origin	Habitat	Medium
Cluster 1					
1	*Phormidium fragile*	OL 03	Germany, Mellum Island (North Sea)	Microbial mat	ASNIII
Cluster 2					
2	*Phormidium* sp.	OL M10	Germany, Mellum Island (North Sea)	Microbial mat	ASNIII
3	*Phormidium* sp.	OL S3	Germany, Schillig (North Sea)	Microbial mat	ASNIII
4	*Phormidium* sp.	OL 05	Germany, Mellum Island (North Sea)	Microbial mat	0.5 × ASNIII
5	*Phormidium* sp.	OL S5	Germany, Schillig (North Sea)	Microbial mat	0.5 × ASNIII
6	*Phormidium* sp.	OL 32	Germany, Mellum Island (North Sea)	Microbial mat	0.5 × ASNIII
7	*Phormidium persicinum*	SAG 80.79	USA, Woods Hole, MA	Sea water	ASNIII
Cluster 3					
8	*Phormidium* sp.	AA	Israel, Nizzara	Desert crust, on sand dunes	BG11
9	*Oscillatoria* sp.	CCMEE 416	Antarctica, Marble Point	Freshwater organism	BG11
Cluster 4					
10	*Phormidium* sp.	SAG 37.90	Switzerland Pass Bernina	On rock	BG11
Cluster 5					
11	*Phormidium* sp.	CCMEE 327	Italy, Valle del Ciavin (Tyrol)	Rock with chasmoendolithic growth	BG11
12	*Leptolyngbya foveolarum*	CCALA 081	Switzerland, Pass Bernina	On stone	BG11
13	Cyanobacterium (Oscillatoriaceae)	CCMEE 315	Australia	Quartz with chasmoendolithic growth	BG11
14	*Phormidium foveolarum*	SAG 1462–1	India, Faridpur	Soil	BG11
15	*Leptolyngbya* sp.	PCC 73110	(No information)	(Freshwater organism)	BG11
Cluster 6					
16	*Leptolyngbya* sp. without cluster assignment	CCALA 094	Nepal, Pokhara	Stone (granite)	BG11
17	*Leptolyngbya* sp.	PCC 8936	France, Mediterranean coast	Salt marsh, Salicornia habitat	ASNIII
Cluster 7					
18	*Phormidium* sp.	OL S4	Germany, Schillig (North Sea)	Microbial mat	ASNIII
19	*Phormidium* sp.	OL 81	Germany, Mellum Island (North Sea)	Microbial mat	ASNIII
Cluster 8					
20	*Phormidium* sp.	OL S12	Germany, Schillig (North Sea)	Microbial mat	ASNIII
21	*Phormidium* sp.	OL S6	Germany, Schillig (North Sea)	Microbial mat	ASNIII
22	*Phormidium* sp.	OL 75	Germany, Mellum Island (North Sea)	Microbial mat	0.5 × ASNIII
23	*Phormidium* sp.	OL "sphere"; Brehm et al. (2003)	North Sea	Microbial mat	ASNIII
Cluster 9					
24	*Phormidium animale*	CCALA 140	Italy, crater of the volcano Vesuvius	Soil	BG11
Cluster 10					
25	*Phormidium autumnale*	CCAP 1462/10	Antarctica, South Orkney Islands	Soil	BG11
26	*Oscillatoria* sp.	PCC 6407	USA, California	Freshwater	BG11
27	*Phormidium inundatum*	SAG 79.79	France	Thermal water	BG11
Cluster 11					
28	*Phormidium tergestinum*	CCALA 155	Spain, Mallorca	Concrete	BG11
29	*Phormidium* cf. *nigrum*	CCALA 147	Czech Republic, Sumperk	Tropical aquarium, periphyton on *Myriophyllum*	BG11
30	*Phormidium unicatum*	SAG 81.79	France	Freshwater	BG11

For better comparison, the strains are in the same order as in Fig. 1

[a] The strains come from the following culture collections: *AA* culture collection of Prof. Aharon Abeliovich, Ben Gurion University of the Negev, Beer-Sheeva, Israel; *CCALA* culture collection of autotrophic organisms, Trebon, Czech Republic; *CCAP* culture collection of algae and protozoa, Dunstaffnage Marine Laboratory, Oban, UK; *CCMEE* culture collection of microorganisms from extreme environments, University of Oregon, Eugene, OR, USA; *OL* Culture collection of Geomicrobiology group, University of Oldenburg, Germany; *PCC* Pasteur Culture Collection of Cyanobacteria, Institut Pasteur, Paris, France; *SAG*Sammlung von Algenkulturen Göttingen, Germany

(the large and small ITS bands) excised and DNA extracted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

DNA samples were directly sequenced in both directions by a commercial sequencing laboratory. The primers were the same as for amplification combined with F primer Phor-580 and R primer Phor-710 designed in this study for 16S rRNA as well as primers 323 (Iteman et al. 2000) and ITS30o and tRNA-F designed here for ITS fragments. The sequences of the primers are given in Table 2. The GenBank accession numbers of sequences determined in this study are given in Table 3. Sequence similarities were calculated online with ClustalW (Higgins et al. 1994) at http://www.ebi.ac.uk/clustalw/index.html.

Phylogenetic analysis

Phylogenetic analysis of the 16S rRNA gene was performed with the help of the software ARB (Ludwig et al. 2004, available online at http://www.arb-home.de) using the "ssu_jan04_corr_opt.arb" database. A fragment of approximately 925 nt (corresponding to *Escherichia coli* K12 16S rRNA residues 269–1215 and to residues 1003153–1004077 of the genome of *Anabaena variabilis* ATCC 29413, GenBank acc. no. NC007413) was aligned to the 16S rRNA alignment of the ARB database with the integrated aligner of the software using other cyanobacteria as reference organisms. The alignment was checked by eye and corrected manually. The aligned sequences were included into the cyanobacterial sub-tree (containing 1315 sequences) of the Parsimony tree "tree_1000_jan05" of the ARB database using the cyanobacterial filter with the "Parsimony (Quick Add Marked)" function of the software. Additionally, our sequences and selected GenBank entries were aligned online with clustalW at default configuration and the 925 nt fragment given above was used for the calculation of a Neighbor-Joining tree (Jukes and Cantor distance estimation, all sequence position considered, but insertions and deletions not taken into account, 2000 bootstrap replicates) with the software TREECON for Windows 1.3b (Van de Peer and De Wachter 1994). *E. coli* was used as outgroup and positioned with the "Single sequence (forced)" method.

Pigment extraction and spectroscopy

Cyanobacterial cultures were centrifuged for 5 min at 12,000*g* in a microcentrifuge and resuspended in 50 mM Tris/HCl (pH 8.0) with 250 mM NaCl and 10 mM EDTA. Cells were disrupted by sonication for 5–10 min on ice with a Branson Sonifier Cell Disruptor B15. Triton X-100 was added to a final concentration of 0.5% and the samples were incubated at 28°C for 30 min. After centrifugation at 12,000*g* for 5 min, absorbance spectra of the supernatant were recorded with a Hitachi U-3000 spectrophotometer from 400 to 750 nm.

Results

Molecular phylogeny

We analysed a 925 bp part of the 16S rRNA gene and the entire ITS of 30 filamentous, non-heterocysteous cyanobacterial strains. PCR amplification of the internal transcribed spacer (ITS) between the 16S rRNA and 23S rRNA genes yielded one or three products upon electrophoresis in agarose gels. In the latter case, two PCR products represented true ITS regions of different sizes, while the third one was probably a heteroduplex as described by Iteman et al. (2000). When rerun on an agarose gel this band yielded also the two other bands. However, no efforts were made to examine this in more detail. There were five strains with

Table 2 Primers used for amplification and sequencing of 16S rDNA and ITS

Primer	Sequence (5′–3′)	Target site[a]	DNA to be sequenced	References
PLG 1.1	ACGGGTGAGTAACRCCTRA	1003007–1003025	16S rDNA	Nadeau et al. (2001)
Phor-580	GCGAAAGGGATTAGATACCC	1003639–1003658	16S rDNA	This study
Phor-710	CCGTCAATTCCTTTGAGTTTC	1003788–1003768	16S rDNA	This study
PLG 2.1	CTTATGCAGGCGAGTTGCAGC	1004195–1004174	16S rDNA	Nadeau et al. (2001)
322	TGTACACACCGCCCGTC	1004252–1004268	ITS	Iteman et al. (2000)
323	ATTAGCTCAGKTGGTTAG	1004529–1004546	ITS	Iteman et al. (2000)
tRNA-F	GTWTAGCTCAGTTGGTAGAG	1004679–1004698	ITS	This study
ITS30o	GAACCTTGACAACTGCATA	1004823–1004841	ITS	This study
340	CTCTGTGTGCCTAGGTATCC	1004953–1004934	ITS	Iteman et al. (2000)

[a] Reference sequence is the genome of *Anabaena variabilis* ATCC 29413 (GenBank acc. no. NC007413)

three products: *Phormidium foveolarum* SAG 1462-1 (no. 14, cluster 5), *Leptolyngbya* sp. PCC 73110 (no. 15, cluster 5), *Phormidium animale* CCALA 140 (no. 24, cluster 9), *Phormidium* cf. *nigrum* CCALA 147 (no. 29, cluster 11), and *Phormidium unicatum* SAG 81.79 (no. 30, cluster 11). The lengths of the spacers identified and sequenced are given in Table 3. The ITS of all strains giving a single PCR band upon electrophoresis harbored $tRNA^{Ile}$ and $tRNA^{Ala}$ genes, except the one of *Phormidium tergestinum* CCALA 155 (no. 28, cluster

Table 3 Molecular characteristics of the examined cyanobacterial strains

No.	Name	Strain	GenBank accession numbers		Length of ITS (bp)	Presence of		% identity after pairwise alignment within clusters[a]	
			16S rDNA	ITS		$tRNA^{Ile}$	$tRNA^{Ala}$	16S rDNA	ITS
Cluster 1								94.5–97.5	–
1	*Phormidium fragile*	OL 03	AM398794	AM398972	695	+	+		
Cluster 2								93.6–100	69.2–100
2	*Phormidium* sp.	OL M10	AM398791	AM398969	447	+	+		
3	*Phormidium* sp.	OL S3	AM398785	AM398962	447	+	+		
4	*Phormidium* sp.	OL 05	AM398798	AM398977	447	+	+		
5	*Phormidium* sp.	OL S5	AM398787	AM398965	447	+	+		
6	*Phormidium* sp.	OL 32	AM398793	AM398971	447	+	+		
7	*Phormidium persicinum*	SAG 80.79	AM398783	AM398960	442	+	+		
Cluster 3								97.5–98.5	85.3
8	*Phormidium* sp.	AA	AM398777	AM398947	508	+	+		
9	*Oscillatoria* sp.	CCMEE 416	AM398781	AM398957	526	+	+		
Cluster 4								98.8	–
10	*Phormidium* sp.	SAG 37.90	AM398795	AM398973	579	+	+		
Cluster 5								95.3–100	69.7–100[b]
11	*Phormidium* sp.	CCMEE 327	AM398775	AM398945	503	+	+		
12	*Leptolyngbya foveolarum*	CCALA 081	AM398792	AM398970	552	+	+		
13	Cyanobacterium (Oscillatoriaceae)	CCMEE 315	AM398797	AM398975	504	+	+		
14	*Phormidium foveolarum*	SAG 1462–1	AM398778	AM398949	477	+	+		
				AM398948	285	–	–		
15	*Leptolyngbya* sp.	PCC 73110	AM398786	AM398964	447	+	+		
				AM398963	285	–	–		
Cluster 6								94.1	–
16	*Leptolyngbya* sp.	CCALA 094	AM398803	AM398976	446	+	+		
without cluster assignment									
17	*Leptolyngbya* sp.	PCC 8936	AM398800	AM398979	496	+	+		
Cluster 7								96.0–100	100
18	*Phormidium* sp.	OL S4	AM398788	AM398966	527	+	+		
19	*Phormidium* sp.	OL 81	AM398790	AM398968	527	+	+		
Cluster 8								95.3–100	98.5–100
20	*Phormidium* sp.	OL S12	AM398789	AM398967	527	+	+		
21	*Phormidium* sp.	OL S6	AM398802	AM398978	528	+	+		
22	*Phormidium* sp.	OL 75	AM398784	AM398961	527	+	+		
23	*Phormidium* sp.	OL "sphere"[a]	AM398796	AM398974	527	+	+		
Cluster 9								99.0	–
24	*Phormidium animale*	CCALA 140	AM398799	AM398956	540	+	+		
				AM398955	421	+	–		
Cluster 10								94.9–99.8	64.6–91.4
25	*Phormidiumautumnale*	CCAP 1462/10	AM398804	AM398959	561	+	+		
26	*Oscillatoria* sp.	PCC 6407	AM398782	AM398958	630	+	+		
27	*Phormidium inundatum*	SAG 79.79	AM398801	AM398954	632	+	+		
Cluster 11								93.4–98.4	51.0–57.6[b]
28	*Phormidium tergestinum*	CCALA 155	AM398776	AM398946	453	+	–		
29	*Phormidium* cf. *nigrum*	CCALA 147	AM398779	AM398951	560	+	+		
				AM398950	303	–	–		
30	*Phormidium unicatum*	SAG 81.79	AM398780	AM398953	622	+	+		
				AM398952	316	–	–		

[a] For 16S rDNA all strains shown in Figs. 1 and 2 were considered, for ITS only the strains in the Table

[b] Short ITS sequences not considered

11), whose ITS contained only the $tRNA^{Ile}$ gene. In the case of multiple PCR products, the longer spacers also contained the two tRNA genes but both were absent from the shorter spacers, except in strain *Phormidium animale* CCALA 140 (no. 24, cluster 9) where the $tRNA^{Ile}$ gene was still present (Table 3).

Using the ARB software, we added our 16S rDNA sequences to a cyanobacterial Parsimony tree containing 1,315 entries. The strains we investigated did not form a separate cluster but were scattered over multiple branches of the resulting tree. Some strains as *Phormidium fragile* (no. 1 in Tables 1, 3 and 4, cluster 1 in Figs. 1 and 2) and *Phormidium* spp. OL S4 and OL 81 (nos. 18 and 19, cluster 7) grouped with unicellular cyanobacteria of the genus *Synechococcus*. Due to its large size the entire phylogenetic tree cannot be shown here. A partial tree with selected strains is given in Fig. 1. The 16S rDNA sequence data for these strains were also used to calculate a Neighbor-Joining tree with the TREECON software (Fig. 2). The topology of the inferred tree differed from that determined by maximum parsimony analysis, especially in the partitions defined by the deeper branches where the bootstrap values in the Neighbor-Joining tree are small. However, the two trees do share identical clusters that are strongly supported by high bootstrap values. The clusters containing strains that were examined in this study are indicated with brackets and numbers in Figs. 1 and 2. Although *Oscillatoria sancta* PCC 7515 clusters with nos. 25–27 (cluster 10) in the Neighbor-Joining tree with fairly high bootstrap support (87%), it was not included in the demarcation of cluster 10 in Figs. 1 and 2 since it was closer to cluster 8 in the Parsimony tree (Fig. 1).

Some strains showed identical 16S rDNA sequences, as in the case of *Phormidium* sp. strains from the North

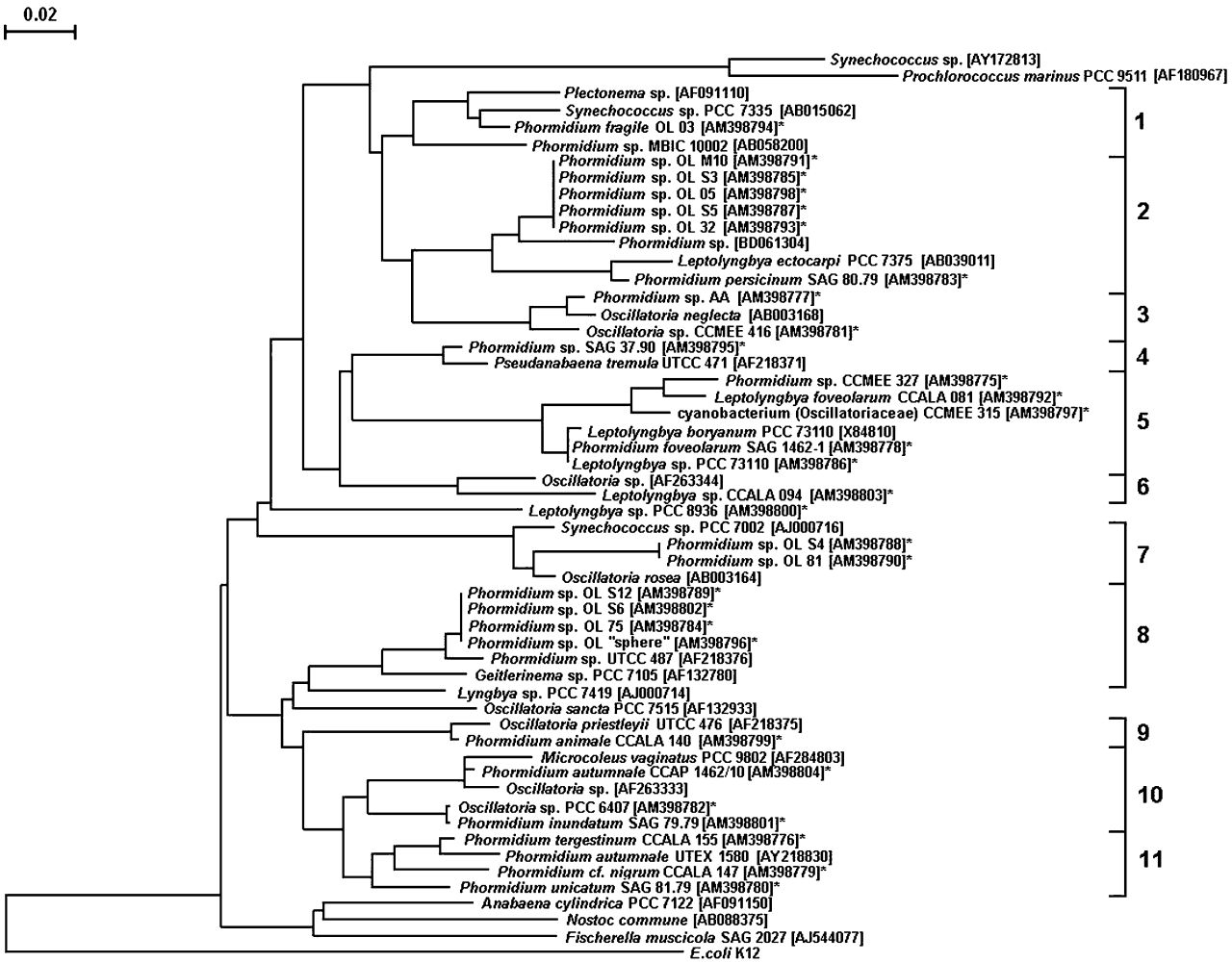

Fig. 1 Maximum parsimony tree based on partial 16S rRNA gene sequences (925 bp) as inferred with the ARB software, see Material and methods for details (Ludwig et al. 2004). GenBank accession numbers are in square brackets. The strains examined in this study are marked with asterisks. *Bar* substitutions per site

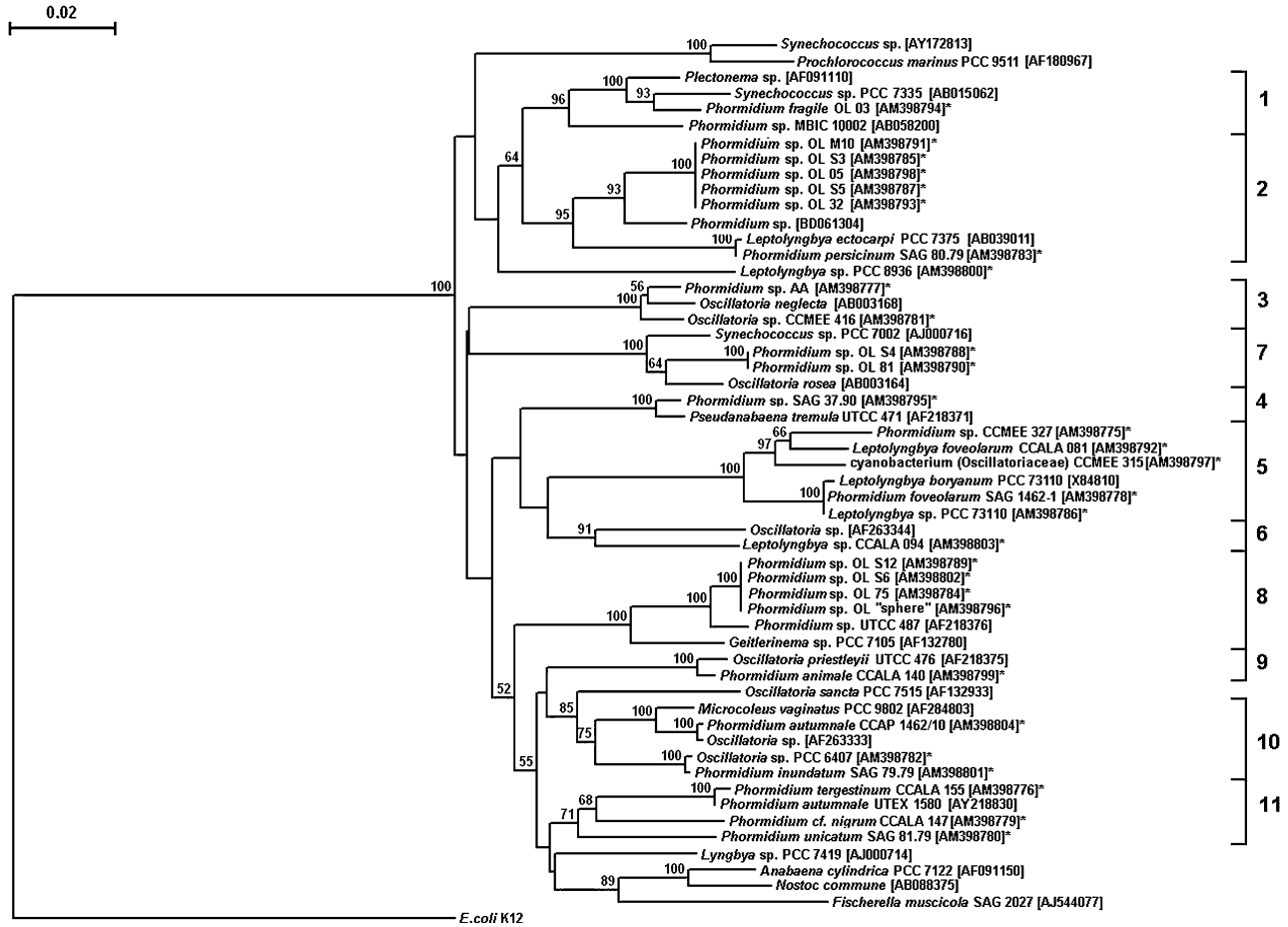

Fig. 2 Neighbor-Joining tree based on partial 16S rRNA gene sequence (925 bp) as inferred with the TREECON software (Van de Peer and De Wachter 1994). GenBank accession numbers are in *square brackets*. The strains examined in this study are marked with *asterisks*. The *numbers at the nodes* show bootstrap support as percentages based on 2,000 resampled data matrices. Only bootstrap values greater than 50% are given. *Bar* substitutions per site

Sea OL M10, OL S3, OL 05, OL S5, and OL 32 (nos. 2–6, cluster 2), *Phormidium foveolarum* SAG 1462-1 and *Leptolyngbya* sp. PCC 73110 (nos. 14 and 15, cluster 5), *Phormidium* sp. OL S4 and OL 81 (nos. 18 and 19 cluster 7), *Phormidium* sp. strains OL S12, OL S6, OL 75, and OL "sphere" (nos. 20–23, cluster 8). In most of these cases also the ITS sequences were identical. In the case of the strains of cluster 8 the ITS sequences of *Phormidium* sp. OL 75 (no. 22) and *Phormidium* sp. OL "sphere" (no. 23) were identical, while those of *Phormidium* sp. OL S12 (no. 20) and *Phormidium* sp. OL S6 (no. 21) differed in only one nucleotide. However, there were seven, respectively eight, nucleotide differences between both groups.

At least 99% identity were found between 16S rDNA sequences of *Phormidium ectocarpi* PCC 7375 and *Phormidium persicinum* SAG 80.79 (no. 7, cluster 2), *Oscillatoria priestleyi* UTCC 4762 and *Phormidium animale* CCALA 140 (no. 24, cluster 9), *Oscilatoria* sp. (acc. no. AF263333) and *Phormidium autumnale* CCAP 1462/10 (no. 25, cluster 10), and *Oscilatoria* sp. PCC 6407 and *Phormidium inundatum* SAG 79.79 (nos. 26 and 27, cluster 10). In the latter case the strains showed identities of 99.8% for the 16S rRNA gene and 91.4% identity for the less conserved ITS sequence.

Generally, the degrees of pairwise identity within single clusters varied between 93.4 and 100% for the rDNA and between 32.9 and 100% for the ITS sequences (Table 3). The similarity between rDNA sequences of strains from different clusters was 85.6–94.2% identity. For the ITS sequences the corresponding values were between 28.7 and 70.0%. Shorter and longer spacers from the same strain differed not only in the presence or absence of tRNA genes (Table 3) but also in their nucleotide sequences. They showed a degree of identity of 47.0% for strains *Phormidium foveolarum* SAG 1462-1 and *Leptolyngbya* sp. PCC 73110 (nos. 14 and 15, cluster 5), 72.8% for *Phormidium animale* CCALA 140 (cluster 9), 49.6% for *Phormidium* cf. *nigrum* CCALA 147, and 43.6% for

Phormidium unicatum SAG 81.79 (nos. 29 and 30, cluster 11). An example is shown in Fig. 3.

Morphology

The morphology of the strains was analyzed by light microscopy and scanning electron microscopy (SEM). Some examples of typical morphologies are shown in Fig. 4, and the results of these investigations are compiled in Table 4. The cell sizes were quite variable. Filament diameters ranged from less than 1 μm to more than 6 μm. Thin filaments were typical for organisms of clusters 1–5 and 7 (cf. Fig. 4a). All had cell diameters of less than 2 μm. Strains of clusters 6 and 8–11 had filament diameters of more than 2 μm. However, for clusters 1, 4, 6 and 9 only one strain was examined. Therefore, it remains unclear if this size range is typical for all strains in these clusters. The broadest filaments (4.5–6.3 μm) were found in cluster 10 (cf. Fig. 4c).

As summarized in Table 4, the cells of the filaments were either cylindrical (Fig. 4a, a cell length/diameter ratio >1), slightly longer than wide to isodiametric (length/diameter ≥ 1) isodiametric (Fig. 4b, length/diameter ≈1), or slightly shorter than wide to disc-shaped (Fig. 4C, length/diameter <1). The cell shape usually was correlated with the filament diameter. Narrow filaments (diameter <2 μm) contained isodiametric to cylindrical cells, while broad filaments (diameter >3 μm) contained isodiametric to disc-shaped cells. In filaments with a diameter between 2 and 3 μm either of the two cell types occurred. While cells of strains in cluster 8 were isodiametric to cylindrical, those of *Leptolyngbya* sp. CCALA 094 (no. 16, cluster 6), *Phormidium animale* CCALA 140 (no. 24, cluster 9) and

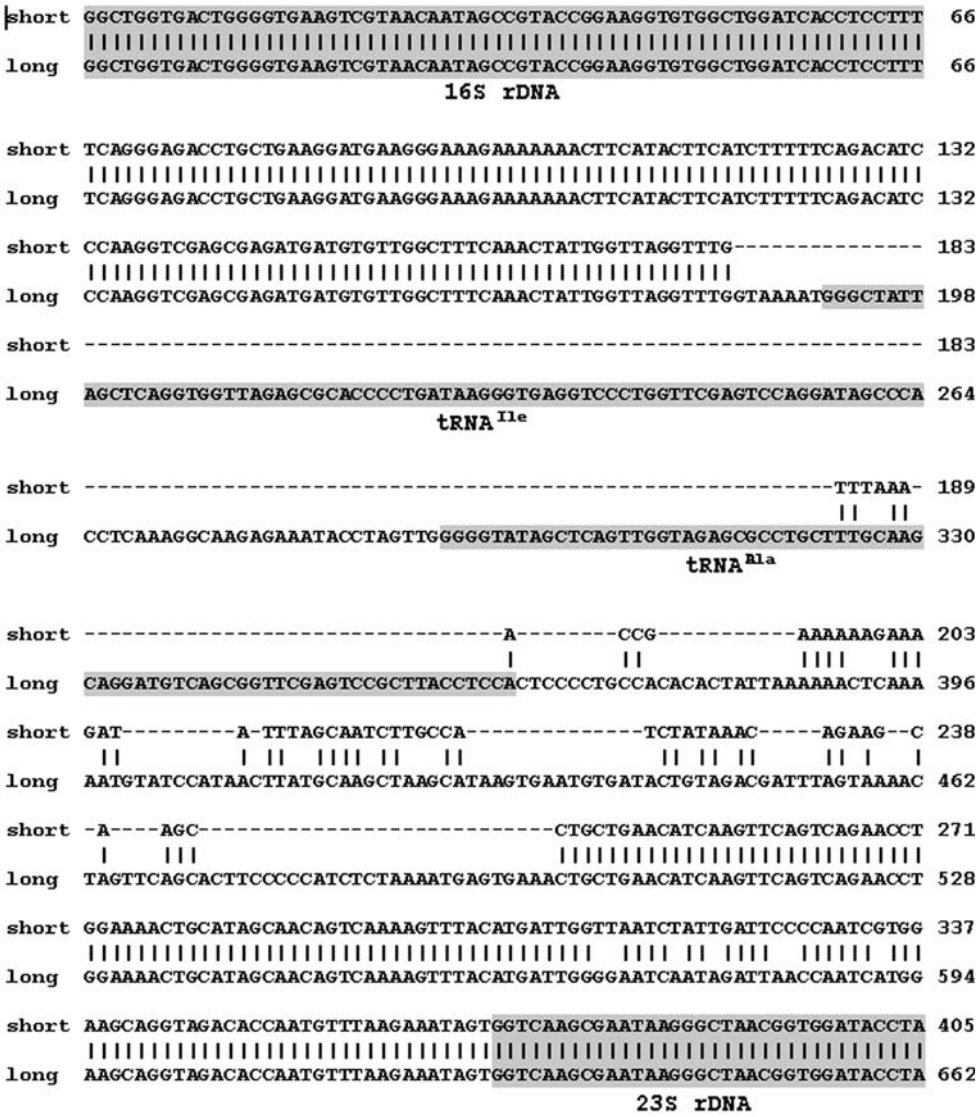

Fig. 3 Pairwise alignment with ClustalW of longer and shorter ITS and adjacent rDNA regions of *Phormidium* cf. *nigrum* CCALA 147. rDNA and tRNA genes are in *shaded areas*. The ITS regions show 49.6% identity

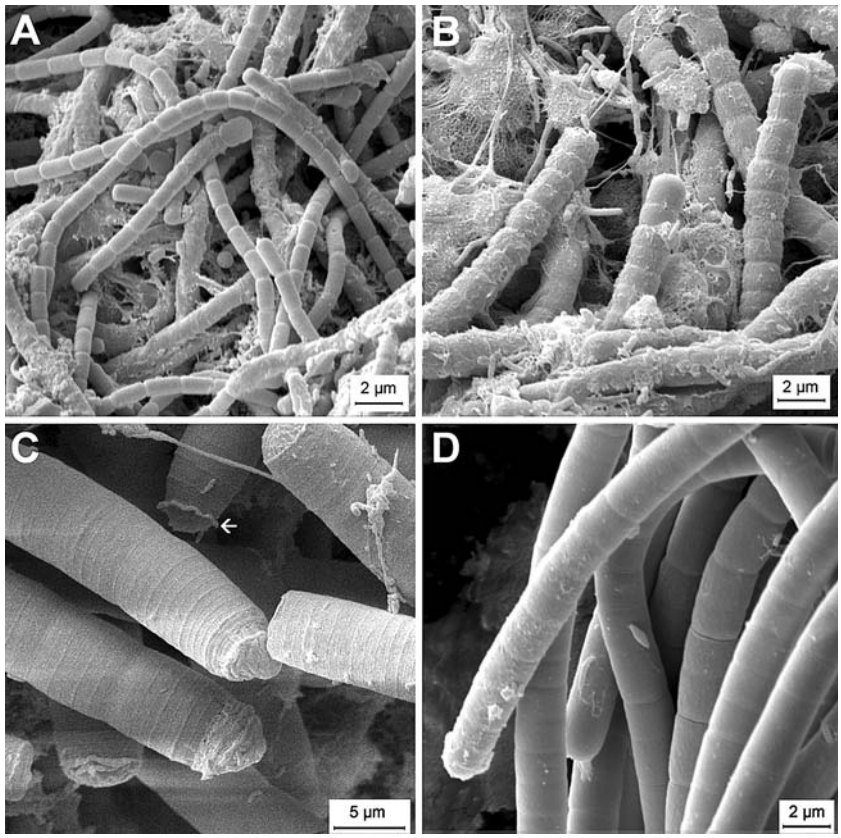

Fig. 4 Scanning electron micrographs of four *Phormidium* strains representing different trichome diameters, cell shapes, and degrees of cross-wall constriction between the cells. **a** *Phormidium* sp. OL 05 as an example of a strain with thin filaments (less then 2 µm and cylindrical cells with pronounced constrictions between them, **b** *Phormidium foveolarum* SAG 1462-1 with filaments of intermediate diameter (2–4 µm) and approximately isodiametric cells, **c** *Phormidium autumnale* CCAP 1462/10 as an example of broad trichomes (>4.5 µm) with disc-shaped cells lacking cross-wall constrictions, and **d** *Phormidium* sp. OL 75 as an example of a strain with shallow cross-wall constrictions. The *arrow* in (**c**) indicates the calyptra

Phormidium unicatum SAG 81.79 (no. 30, cluster 11) were shorter than wide to disc-shaped (Table 4).

Moreover, in one strain, *Phormidium* sp. AA (no. 8, cluster 3), cell shape was quite variable (length/diameter <1 to>1, Table 4). Constrictions between the cells might be missing or be present to different degrees. Distinct constrictions were found in the strains of clusters 2, 3, 5, and 7 and in *Leptolyngbya* sp. PCC 8936 (no. 17). Strains of clusters 1 and 4, and three of the four strains of cluster 8, had very shallow constrictions, only clearly visible in some filaments or parts of the same filament (cf. Fig. 4d). Strains of clusters 9–11 had no constrictions at all (cf. Fig. 4c). A calyptra, a thick-ended cap on the outer cell wall of an apical cell, was only found with one strain, *Phormidium autumnale* CCAP 1462/10 (no. 25, cluster 10) (Figs. 4c, 5h).

Ultrastructure

In our TEM investigation we focused on the number and orientation of thylakoid membranes, which are the most conspicuous structures within the cells. The results are compiled in Table 4. In the majority of strains (clusters 1–8), the thylakoids were arranged peripherally, running parallel to the cytoplasmic membrane (Fig. 5a, c). In *Leptolyngbya* sp. CCALA 094 (no. 16, cluster 6) and *Phormidium uncinatum* SAG 81.79 (no. 30, cluster 11), the thylakoids were undulating at the periphery of the cells, giving rise to a more irregular pattern (Fig. 5b). The number of thylakoids in strains where they were arranged peripherally was usually no more than six. Only in *Phormidium* sp. OL S6 and OL 75 (no. 21 and 22, cluster 8), *Phormidium inundatum* SAG 79.79 (no. 27, cluster 10) and *Phormidium unicatum* SAG 81.79 (no. 30, cluster 11) thylakoid number was higher, between 8 and 12 (Table 4).

Highly irregular thylakoid arrangements were found only in a few strains. *Phormidium tergestinum* CCALA 155 (no. 28, cluster 11) and *Phormidium* cf. *nigrum* CCALA 147 (no. 29, cluster 11) showed a fascicular thylakoid pattern (sensu Casamatta et al. 2005) where the thylakoids are arranged in fascicles running parallel to the longitudinal axis of the cell (Fig. 5d, f). In *Phormidium animale* CCALA 140 (no. 24, cluster 9) the thylakoids also ran parallel to the longitudinal cell axis but were arranged perpendicularly to the cell wall, giving rise to a radial pattern in transversal sections (Fig. 5e). In *Phormidium autumnale* CCAP 1462/10 (no. 25, cluster 10) the thylakoid membranes formed stacks lying irregularly within the cells (Fig. 5g). The number of thylakoids in these strains could not be determined exactly, but it was significantly higher than

Table 4 Morphological, ultrastructual and pigment characteristics of the examined cyanobacterial strains

No.	Taxon	Strain	Cell diameter (μm)[a]	Cell length/ diameter	Degree of constrict[b]	No. of thylakoids	Thylakoids arrangement	PE[c]
Cluster 1								
1	*Phormidium fragile*	OL 03	1.8 ± 1.4	≥ 1	+	3	Peripheral	–
Cluster 2								
2	*Phormidium* sp.	OL M10	0.7 ± 0.18	>1	++	2–3	Peripheral	–
3	*Phormidium* sp.	OL S3	0.7 ± 0.18	>1	++	4–5	Peripheral	–
4	*Phormidium* sp.	OL05	0.7 ± 0.18	>1	++	4	Peripheral	–
5	*Phormidium* sp.	OL S5	0.7 ± 0.12	>1	++	2–3	Peripheral	–
6	*Phormidium* sp.	OL 32	0.7 ± 0.18	≥ 1	++	4	Peripheral	–
7	*Phormidium persicinum*	SAG 80.79	1.1 ± 1.1	>1	++	4	Peripheral	+
Cluster 3								
8	*Phormidium* sp.	AA	0.8 ± 1.4	<1 to >1	++	4	Peripheral	–
9	*Oscillatoria* sp.	CCMEE 416	0.8 ± 0.18	>1	++	4	Peripheral	–
Cluster 4								
10	*Phormidium* sp.	SAG 37.90	0.8 ± 0.16	>1	+	3	Peripheral	–
Cluster 5								
11	*Phormidium* sp.	CCMEE 327	1.4 ± 0.18	>1	++	5	Peripheral	–
12	*Leptolyngbya foveolarum*	CCALA 081	1.6 ± 1.0	≈1	++	5	Peripheral	–
13	Cyanobacterium (Oscillatoriaceae)	CCMEE 315	0.9 ± 0.18	≥ 1	++	5	Peripheral	–
14	*Phormidium foveolarum*	SAG 1462–1	1.6 ± 0.18	≈1	++	6	Peripheral	–
15	*Leptolyngbya* sp.	PCC 73110	1.8 ± 1.0	>1	++	5	Peripheral	–
Cluster 6								
16	*Leptolyngbya* sp. without cluster assignment	CCALA 094	2.4 ± 1.8	<1	–	6	Peripheral undulating	+[d]
17	*Leptolyngbya* sp.	PCC 8936	1.7 ± 1.2	>1	++	4	Peripheral	+
Cluster 7								
18	*Phormidium* sp.	OL S4	1.1 ± 0.14	>1	++	4–6	Peripheral	–
19	*Phormidium* sp.	OL 81	1.0 ± 0.8	≥ 1	++	5	Peripheral	–
Cluster 8								
20	*Phormidium* sp.	OL S12	2.1 ± 1.4	>1	–	4–6	Peripheral	–
21	*Phormidium* sp.	OL S6	2.0 ± 1.1	≥ 1	+	≤ 12	Peripheral	–
22	*Phormidium* sp.	OL 75	2.2 ± 1.4	≥ 1	+	9	Peripheral	–
23	*Phormidium* sp.	OL "sphere"	2.7 ± 1.6	≥ 1	+	4	Peripheral	–
Cluster 9								
24	*Phormidium animale*	CCALA 140	2.4 ± 2.0	Mostly <1	–	>30	Radial	–
Cluster 10								
25	*Phormidium autumnale*	CCAP 1462/10	6.3 ± 2.4	<1	–	>20	Stacked	+[c]
26	*Oscillatoria* sp.	PCC 6407	4.5 ± 2.4	<1	–	ND	ND	–
27	*Phormidium inundatum*	SAG 79.79	4.5 ± 0.24	≈1	–	11	Peripheral	–
Cluster 11								
28	*Phormidium tergestinum*	CCALA 155	3.4 ± 1.2	<1	–	>20	Fascicular	–
29	*Phormidium* cf. *nigrum*	CCALA 147	4.4 ± 1.8	<1	–	>30	Fascicular	–
30	*Phormidium unicatum*	SAG 81.79	2.3 ± 0.1	<1	–	8	Peripheral undulating	+

[a] Mean value ± standard deviation, $n = 100$

[b] ++ pronounced constrictions; + shallow constrictions; − no constrictions

[c] Presence/absence of phycoerythrin

[d] Phycoerythrin/phycocyanin ratio enhanced under green light

in cyanobacteria with peripheral thylakoids. All strains with irregular thylakoid patterns are members of clusters 9, 10 and 11.

Presence of phycoerythrin

Phycoerythrin was found only in five strains investigated (Table 4). It was a dominating pigment in *Phormidium persicinum* SAG 80.79 (no. 7, cluster 2), a strain that is colored bright red. In the other four strains it was a minor pigment only detectable in the absorbance spectrum. In two strains, *Leptolyngbya* sp. CCALA 094 (no, 16, cluster 6) and *Phormidium autumnale* CCAP 1462/10 (no. 25, cluster 10), the phycoerythrin/phycocyanin ratio was enhanced under green light (Table 4) which enhances the expression of

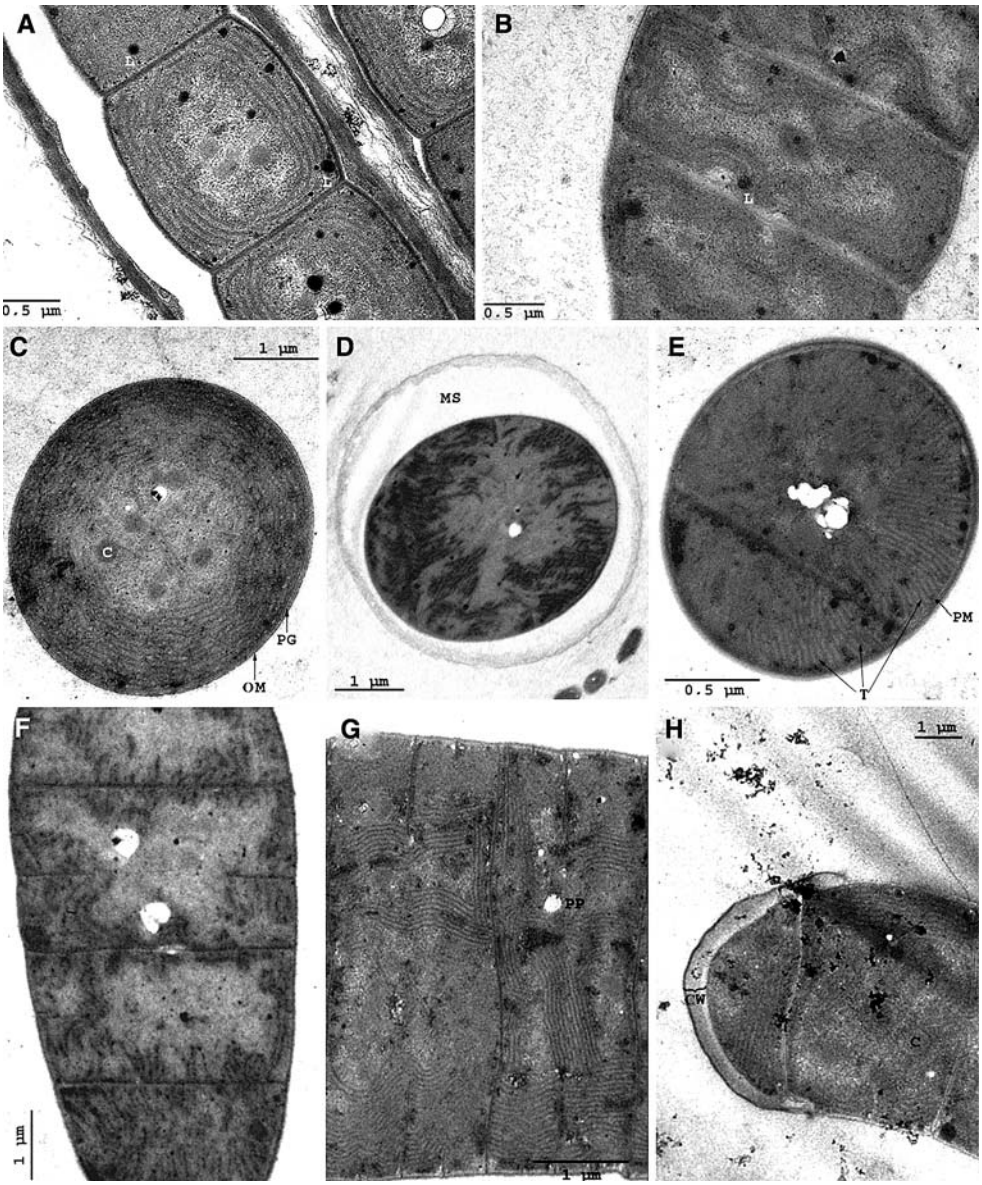

Fig. 5 Transmission electron micrographs of some of the examined cyanobacteria. *Leptolyngbya foveolarum* CCALA 081 (**a**) and *Leptolyngbya* sp. CCALA 094 (**b**) in longitudinal sections, *Phormidium inundatum* SAG 79.79 (**c**), *Phormidium tergestinum* CCALA 155 (**d**) and *Phormidium animale* CCALA 140 (**e**) in cross-section (in *Phormidium autumnale* not exactly perpendicular to the longitudinal axis), *Phormidium* cf. *nigrum* CCALA 147 (**f**) and *Phormidium autumnale* CCAP 1462/10 (**g**, **h**) in longitudinal section. **a** and **c** show two views of peripheral thylakoids, in **b** the thylakoids are peripheral, but undulating. **d** and **f** show fascicular thylakoids in cross and longitudinal section. **g** demonstrate the stacked thylakoids and **h** the calyptra of *Phormidium autumnale*. *C* carboxysome, *CW* cell wall, *L* lipid granulum, *MS* mucilaginous sheath, *OM* outer membrane, *PG* peptidoglycan layer, *PM* plasma membrane, *Pp* polyphosphate granulum, *T* thylakoid

phycoerythrin in the course of complementary chromatic adaptation (Tandeau de Marsac 1977).

Discussion

The strains here classified as *Phormidium* sensu Geitler 1932 were found to be members of several different phylogenetic clusters, which also contain strains, assigned to the genera *Leptolyngbya, Oscillatoria, Geitlerinema, Microcoleus, Pseudanabaena* or *Plectonema* (Figs. 1, 2). These seemingly surprising relationships are largely the result of the divergent generic nomenclature employed by different authors, or culture collection curators, even for morphologically similar filamentous non-heterocystous cyanobacteria. This is the consequence of the simultaneous use of different taxonomic guides for identification of the

organisms. Taxonomic treatment of cyanobacteria, including formal description of new taxa, can presently be carried out under the aegis of either the Botanical or Bacteriological Codes of Nomenclature (Castenholz 1989; Palinska et al. 2006). However, the rules of these two codes are quite different and, when applied to the same group of organisms, confusions are unavoidable (Oren 2004). Furthermore, choice of inappropriate generic or specific assignments may result from naming or renaming organisms after they have been maintained in culture, where certain properties of determinative value may no longer be expressed, or have been lost due to mutations (Palinska et al. 1996; Otsuka et al. 2001; Lyra et al. 2001). Two strains assigned to the same nomenspecies, *Phormidium autumnale* CCAP 1462/10 (no. 25, cluster 10) and *Phormidium autumnale* UTEX 1580 (cluster 11), were found in different phylogenetic clusters (clusters 10 and 11, respectively). This suggests that morphologically similar strains may differ genetically. Alternatively, a genuine misidentification or strain-mixup cannot be excluded. Such a possibility was discovered for *Pseudanabaena tremula* UTCC 471 (cluster 4) which previously was classified as *Phormidium autumnale* (and is still found under this name in the UTCC catalog). The incorrect classification of the strain was noticed by Casamatta et al. (2005), who renamed the strain based on morphological characteristics and 16S rDNA sequence analyses. Further investigations with additional *Phormidium autumnale* strains are required to explore the genetic diversity of this taxon in more detail.

Phormidium fragile OL O3, and *Phormidium* sp. OL S4 and OL 81 (clusters 1 and 7, respectively) were found to group with two different strains of *Synechococcus* (Figs. 1, 2). The affiliation of these, and some other, unicellular cyanobacteria with thin oscillatorian representatives was already noticed by other authors (e.g. Turner 1997; Honda et al. 1999; Robertson et al. 2001) Ishida et al. 2001; Wilmotte and Herdman 2001; Taton et al. 2003; Casamatta et al. 2005). These relationships, as suggested by Honda et al. (1999), probably reflect the convergent evolution of cellular organization. Furthermore, they demonstrate that neither the Chroococcales nor the Oscillatoriales are monophyletic.

The phylogenetic clustering of strains assigned to the genus *Phormidium* with other members of the Oscillatoriales, including strains assigned to the genera *Leptolyngbya* and *Oscillatoria* has also been shown previously (Turner 1997; Ishida et al. 2001; Lee and Bae 2001; Litvaitis 2002; Ceschi-Berrini et al. 2004). Similarly, the high level of genetic diversity within the genus *Phormidium* was demonstrated (Baker et al. 2001) by analysis of the *rpoC1* gene, in agreement with Teneva et al. (2005), who examined the cpB-IGS-cpA locus. In the phylogenetic tree generated by the latter study, *Phormidium* strains even clustered together with members of the Nostocales. However, this conclusion is contradicted by all other reports demonstrating that heterocystous cyanobacteria are monophyletic (e.g. Wilmotte 1994; Wilmotte and Herdman 2001; Lyra et al. 2001; Henson et al. 2004). In our study, the heterocystous species *Anabaena cylindrica, Nostoc commune* and *Fischerella muscicola* form a well-supported (89% bootstrap support) separate cluster.

A 16S rRNA sequence similarity of 95% has been suggested as a threshold for a congeneric bacterial genus (Stackebrandt and Goebel 1994). In this study, this criterion was met for strains of clusters 3, 4, 5, 7, 8 and 9 (Table 3) and the subcluster *Plectonema* sp.-*Synechococcus* sp. PCC 7335-*Phormidium fragile* OL 03 of cluster 1, the subclusters *Phormidium* sp. strains: OL M10, OL S3, OL 05, OL S5, OL 32-*Phormidium* sp. [BD061304] and *Leptolyngbya ectocarpi* PCC 7375-*Phormidium persicinum* SAG 80.79 of cluster 2, the subclusters *Microcoleus vaginatus* PCC 9802-*Phormidium autumnale* CCAP 1462/10-*Oscillatoria* sp. [AF263333] and *Oscillatoria* sp. PCC 6407-*Phormidium inundatum* SAG 79.79 of cluster 10, and the subcluster *Phormidium autumnale* UTEX 1580-*Phormidium tergestinum* CCALA 155 of cluster 11. Therefore the *Phormidium* strains analyzed are representatives of at least 10 different genera.

Cyanobacterial intergenic transcribed spacer (ITS) regions investigated earlier vary in size from 354 to 545 nucleotides (Iteman et al. 2000; Otsuka et al. 2001; Boyer et al. 2002; Laamanen et al. 2002). However, Laloui et al (2002) and Rocap et al. (2003) reported longer ITS, up to more than 1,000 bp. The 30 cyanobacteria examined here contain ITS regions of different length (442–694 nt, Table 3), but length distinctions do not correlate with specific clusters or subclusters inferred from 16S rDNA sequences. For example, the ITS regions of strain *Leptolyngbya* PCC 73110 (no. 15, cluster 5) and most of the strains of cluster 2 (Table 3) shared the same length. Some of the strains (see clusters 5, 9 and 11, Table 3) were shown to have at least two ITS that differed in length and tRNA content, demonstrating the heterogeneity of their *rrn* operons. Multiple ITS regions with different lengths were also found in *Nostoc* PCC 7120, where the longer and shorter ITS regions differed in the presence and absence, respectively, of the two tRNA genes (Iteman et al. 2000). In contrast, the two *rrn* operons of *Synechocystis* PCC 6803 contain ITS regions of identical

length and tRNA content, as can be deduced from the genome sequence (Kaneko et al. 1996). Although in parts conserved, some regions in the ITS are highly variable. This makes a proper alignment and the deduction of phylogenetic relationships difficult for strains that are not closely related, and thus was not been attempted here. However, for some strains with identical or nearly identical 16S rDNA sequences, a higher resolution of discrimination within some of the clusters (see Table 3) was achieved based on ITS sequence analyses.

Our results show that the presence of phycoerythrin is not correlated with the strains' position in the phylogenetic trees. Phycoerythrin-producing representatives occur in different clusters together with strains that lack phycoerythrin (Table 3). The deeply red-colored *Phormidium persicinum* SAG 80.79 (no. 7, cluster 2) and the phycoerythrin-rich *Leptolyngbya ectocarpi* PCC 7375 are on the same branch (Figs. 1, 2) and share a very high 16S rDNA similarity (99.7% identity). Consequently, they may in fact represent the same taxon kept in different culture collections under different names. With the latter exception where the high phycoerythrin content may be a characteristic feature of a specific genotype (Table 3), the presence of phycoerythrin appears unsuitable as a systematic marker. Similar conclusions were drawn by Otsuka et al. (2001) for phycoerythrin-containing strains of *Microcystis*. As for phycoerythrin, there is no correlation between the clustering of the strains and their geographic origin (see Table 1). The strains of cluster 3, for instance, came from Israel and Antarctica, those of cluster 5 from the European Alps, Australia or India, and *Oscillatoria* sp. PCC 6407 and *Phormidium inundatum* SAG 79.79 (nos. 26 and 27, cluster 10) with almost identical 16S rDNA sequence came from California and France, respectively. This is in agreement with findings of a cosmopolitan distribution of many cyanobacterial species (e.g. Mullins et al. 1995; Wilmotte et al. 1997; Garcia-Pichel et al. 1996).

On the other hand, it is noticeable that strains identical at the 16S rRNA loci were repeatedly isolated from the same geographic region: of the 12 strains from the Oldenburg collection isolated at the North Sea coast of northwestern Germany, 11 were assignable to only three genotypes, two of which (clusters 2 and 8, Table 3) are represented by 4–5 independent isolates. These *Phormidium* types might be numerically dominant in this area or, more likely, are easily selected for by the growth conditions employed.

In contrast to geographic origin, there is a good correlation between the original environment of the strains and their groupings/subgroupings in the phylogenetic trees, though organisms from similar habitats may also occur in different phylogenetic clusters. For example, all strains of cluster 5 are from terrestrial origin, and the OL strains of cluster 1, 2, 7 and 8, as well as their relatives, originate from marine or highly saline habitats. The OL strains show a wide range of salt tolerance with a growth optimum between 16 and 34 psu, i.e. in brackish water. The other clusters contain either terrestrial organisms or a mixture of terrestrial and freshwater strains. Terrestrial habitats can vary greatly in the amount of available water; e.g. a rock might be moistened regularly by stream or rainwater. An example of a strain from an extreme terrestrial habitat is *Phormidium* sp. AA (no. 8, cluster 3) that comes from the particularly dry Nizzara desert. At first glance, it may seem surprising that this strain clusters together with *Oscillatoria* sp. CCMEE 416 (no. 9) from Antarctica. However, the ice-free regions of Antarctica are deserts as well. A similar close phylogenetic relationship was found by Casamatta et al. (2005) for Antarctic *Microcoleus acremannii* and *Microcoleus vaginatus* from desert soil. These authors point out that both organisms share a similar habitat that is characterized by long periods of desiccation and high levels of ultraviolet radiation.

In agreement with their 16S rDNA sequence similarity, strains within a given phylogenetic cluster are morphologically rather homogeneous. However, similar morpho- and ecotypes may also occur in different clusters (e.g. clusters 2 and 7), demonstrating that even the combination of morphological and ecological data does not allow precise identification.

Although the number of thylakoids varied (Table 4), all strains of clusters 1–8 share peripheral thylakoids and thus can not be distinguished based on their cellular ultrastructure. A radial thylakoid arrangement, considered typical for members of the genus *Phormidium* sensu Anagnostidis and Komarek (1988), was observed in only one strain, *Phormidium animale* CCALA 140 (no. 24, cluster 9). If this type of thylakoid arrangement is a reliable taxonomic feature, one would predict that the close relative of this strain, *Oscillatoria pristleyi* UTCC 4762 (Figs. 1, 2), should also have radial thylakoids. Casamatta et al. (2005) found congruence between inferred phylogenetic relationships among strains and their thylakoid arrangement. In our study this is only partly true, since clusters 10 and 11 contain members with both peripheral and irregularly arranged thylakoids (Table 4). However, these clusters are not very well supported (75 and 71% bootstrap support, respectively).

Our results reemphasize the polyphyletic nature of the Oscillatoriales. They show in particular that, even if

ultrastructural features are included, the exclusive use of phenotypic traits does not permit confident identification of oscillatorian cyanobacteria and their arrangements in a hierarchical order. Based on our phylogenetic analyses, the strains of *Phormidium* examined are representatives of more than 10 generic entities. Furthermore, some of the phylogenetic subclusters undoubtedly represent distinct species, for which we could propose new specific epithets. However, Fox et al. (1992) found that *Bacillus* strains with more than 99% 16S rRNA similarity had less than 70% DNA–DNA hybridization values, the most commonly accepted benchmark used to distinguish between bacterial species (Stackebrandt and Goebel 1994). Furthermore, the nomenclatural problems highlighted in this study evidently diminish the value of the phylogenetic trees and the possibilities of deducing meaningful systematic and evolutionary relationships. Therefore, we feel that nomenclatural changes and drastic taxonomic revisions should await acquisition of further knowledge. More phenotypic and molecular data from additional organisms and multiple genes are needed to confirm and refine the systematic relationships so far revealed within this group of cyanobacteria. Finally, strain discrimination should be attempted using sequence data of genes other than that of the small ribosomal subunit, which would provide greater phylogenetic resolution among closely related species.

Our phylogenetic trees emphasize the polyphyletic nature of the Oscillatoriales and the doubtful identification of many strains existing in culture collections.

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