Polyphyly of true branching cyanobacteria (Stigonematales)

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Cyanobacteria with true branching are classified in Subsection V (formerly order Stigonematales) in the phylum *Cyanobacteria*. They exhibit a high degree of morphological complexity and are known from particular biotopes. Only a few stigonematalean morphotypes have been cultured, and therefore the high variability of morphotypes found in nature is under-represented in culture. Axenic cultures of *Chlorogloeopsis* and *Fischerella sensu* Rippka *et al.* were, to date, the only representatives of this Subsection in phylogenetic studies. The 16S rDNA sequence analysis data in this report confirm that heterocyst-forming cyanobacteria are a monophyletic group. However, unlike previous studies have suggested, these 16S rDNA data on new Stigonematales strains show that the true branching cyanobacteria are polyphyletic and can be separated into at least two major groups defined by their branching type, the first group being characterized by T-branching and the second group by Y-branching. Cyanobacteria with intercalary heterocysts and either no branching or false-branching also formed separate clusters. In consequence, our phylogenetic data do not correlate with the bacteriological and traditional classifications, which distinguish filamentous heterocystous cyanobacteria with or without true branching (Nostocales/Stigonematales).

INTRODUCTION

Heterocyst-differentiating cyanobacteria correspond to a monophyletic lineage (Giovannoni *et al.*, 1988; Wilmotte & Herdman, 2001) in which two subgroups are traditionally distinguished. The first subgroup consists of filamentous heterocystous cyanobacteria dividing always in a plane at right angles to the long axis of the trichome and therefore uniseriate and lacking true branchings. They are grouped in Subsection IV according to the proposed bacteriological classification (Rippka *et al.*, 1979; Castenholz, 2001) and in the order Nostocales in the traditional classification system (Komárek & Anagnostidis, 1989). The second subgroup consists of filamentous heterocystous cyanobacteria in which longitudinal and oblique cell divisions occur in addition to transverse cell divisions resulting in periodic true branching in all genera and in multiseriate trichomes (two or more rows of cells) in some genera. They are classified in Subsection V (formerly order Stigonematales) (Rippka *et al.*, 1979; Anagnostidis & Komárek, 1990; Castenholz, 2001).

The thallus of the Stigonematales is generally composed of a main creeping axis and secondary erect branches. In some

taxa, a heterotrichous thallus formed by a noticeable difference in the shape and dimensions of the cells of the main axis and the secondary branches may occur. True branching cyanobacteria, characterized by the presence of branch-point cells in contact with three different neighbouring cells, exhibit the highest degree of morphological complexity and differentiation within the cyanobacteria. Three major types of true branchings, named 'T', 'V' and 'Y', can be distinguished (for a review, see Golubíc *et al.*, 1996). T-branching is formed by a lateral, nearly perpendicular branching originating by the change of division plane from transverse to longitudinal. V-branching consists of a dichotomous or pseudodichotomous bifurcation originating from a change in division plane at, or close to, the trichome tip. Y-branching arises from the displacement of an intercalary branch-point cell by meristematic growth around it. False-branching also occurs in some genera. Moreover, the position of the heterocysts varies from terminal to lateral (sessile or pedicellate) and intercalary (see Table 2 in Anagnostidis & Komárek, 1990). Multiplication arises from trichome breakage, hormogonia, hormocysts, akinetes or akinete-like cells, depending on the genus. Based on these morphological criteria, Anagnostidis & Komárek (1990) reorganized the order at the family level and recognized eight families with 48 genera.

The most frequently represented Stigonematales in culture are the genera *Fischerella* (Born. et Flah.) Gom. 1895,

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Hapalosiphon Näg. in Kütz. ex Born. et Flah. 1886 and *Mastigocladus* Cohn ex Kirchner 1898. However, a large number of poorly studied stigonematalean cyanobacteria are known from freshwater and subaerial biotopes, such as slightly acidic, oligotrophic lakes or caves. Only two genera have been characterized from axenic culture strains, *Fischerella* (incl. *Mastigocladus* and *Hapalosiphon*) and *Chlorogloeopsis* Mitra et Pandey 1966 (Rippka *et al.*, 1979; Herdman *et al.*, 1979a, b; Lachance, 1981). Because of the few true branching cyanobacteria studied according to bacteriological standards, only six genera were included in Subsection V of *Bergey's Manual of Systematic Bacteriology* (Hoffmann & Castenholz, 2001).

The analysis of the few available 16S rDNA sequences revealed that true branching cyanobacteria form a monophyletic group arising from an assemblage of filamentous heterocystous cyanobacteria without branching (Giovannoni *et al.*, 1988; Hoffmann & Castenholz, 2001; Fewer *et al.*, 2002; Litvaitis, 2002). In this study, 16 strains allocated to the genera of *Fischerella*, *Hapalosiphon*, *Mastigocladopsis* Iyeng. et Desik. 1946, *Nostochopsis* Wood ex Born. et Flah. 1886, *Stigonema* Ag. ex Born. et Flah. 1886 (reference genus of the Stigonematales), *Symphyonema* Jao 1944, *Symphyonemopsis* Tiwari et Mitra 1969 and *Westiellopsis* Janet 1941

were examined morphologically and genotypically to extend the range of morphotypes represented in phylogenetic trees. In particular, isolates differing by their branching types (Y- vs T-branching) and the position of the heterocysts (intercalary, lateral-sessile, lateral-pedicellate) were represented. The genetic relationship within the true branching cyanobacteria and with the other heterocystous cyanobacteria was inferred by the analysis of 16S rRNA gene sequences.

METHODS

Organisms and growth conditions. A list of branched filamentous heterocystous cyanobacteria used in this study is given in Table 1. Strains 1711, 804-1, 89-45, 1269-1, 1517, VAPOR1, 1590-1, 1590-2, 985-1 and 89-785/4 were isolated from material collected in Papua New Guinea, Corsica and Spain, and identified to the genus level according to algological literature (Table 2). Strains SAG 16.93, SAG 20.27 (=BB98.1), SAG 23.96 and SAG 48.90 were purchased from Sammlung von Algenkulturen at the University of Göttingen, Germany, and strains PCC 6718 and PCC 7414 were obtained from the Institut Pasteur, Paris, France. *Nostochopsis lobatus* BB92.1 (=SAG 2.97) was received from B. Büdel (University of Kaiserslautern, Germany) and the *Mastigocladopsis repens* MORA type strain was from M. Hernandez-Mariné (University of Barcelona, Spain). Clonal,

Table 1. Branched filamentous heterocystous cyanobacteria used in this study

Taxon	Strain*	Origin/date	References†
<i>Chlorogloeopsis fritschii</i>	PCC 6718	Freshwater/1967	3, 10
<i>Chlorogloeopsis</i> sp.	PCC 7518	Hot spring, Iceland/1968	1, 2, 6
<i>Fischerella muscicola</i>	PCC 7414	Hot spring, freshwater, New Zealand/?	3
<i>Fischerella</i> sp.	1711	Soil, Papua New Guinea/1986	This work
<i>Fischerella</i> sp.	SAG 20.27	Rice field, India/?	11, this work
<i>Hapalosiphon welwitschii</i>		Soil, Australia/?	13
<i>Hapalosiphon</i> sp.	804-1	Soil, Papua New Guinea/1986	This work
<i>Mastigocladopsis repens</i>	MORA	Soil, Punta de la Mora, Tarragona, Spain/1991	5, 8
<i>Nostochopsis lobatus</i>	BB92.1	Cryptoendolithic in sandstone, freshwater, South Africa/1990	9
<i>Nostochopsis</i> sp. ' <i>Mastigocladopsis jogensis</i> '	89-45	On submerged stone in freshwater, Corsica, France/1989	4, 7
<i>Stigonema ocellatum</i>	SAG 48.90	<i>Sphagnum</i> bog, Allgäu, Germany/1970	This work
<i>Symphyonema</i> sp.	1269-1	Soil, Papua New Guinea/1986	This work
<i>Symphyonema</i> sp.	1517	Soil, Papua New Guinea/1986	This work
<i>Symphyonemopsis</i> sp.	VAPOR1	Cave El Vapor, Spain/1998	12
<i>Westiellopsis prolifica</i>	SAG 16.93	Soil, Aldabra Atoll, Seychelles/1972	This work
<i>Westiellopsis prolifica</i>	SAG 23.96	Saline soil of pH 9.5, Karnal, India/?	This work
<i>Westiellopsis</i> sp.	1590-1	Soil, Papua New Guinea/1986	This work
<i>Westiellopsis</i> sp.	1590-2	Soil, Papua New Guinea/1986	This work
<i>Westiellopsis</i> sp.	985-1	Soil, Papua New Guinea/1986	This work
<i>Westiellopsis</i> sp.	89-785/4	Soil, Papua New Guinea/1989	This work

*PCC, Institut Pasteur, Paris, France; SAG, Sammlung von Algenkulturen Universität Göttingen, Germany. Other strains are maintained at the Culture Collection of Micro-organisms held at the Environment and Biotechnologies Research Unit of the Public Research Centre – Gabriel Lippmann, Luxembourg. Strains not available for morphological observation are highlighted in bold.

†References: 1, Castenholz (1969); 2, Castenholz (1978); 3, Rippka *et al.* (1979); 4, Hoffmann (1990); 5, Hernandez-Mariné *et al.* (1992); 6, Wilmoth *et al.* (1993); 7, Hoffmann (1994); 8, Merino *et al.* (1994); 9, Weber *et al.* (1996); 10, Turner *et al.* (1999); 11, Fewer *et al.* (2002); 12, Hoffmann *et al.* (2003); 13, D. Hoffmann & D. L. Troelstrup (unpublished).

Table 2. Observation of diacritical morphological features used for the classification of the *Stigonematales*

Morphological observation made on 18 cultures and one unavailable culture (in bold). The latter was based on previously published photomicrographs (Hoffmann & Castenholz, 2001). For each culture, the types of thallus, branching (Irr, irregular; NO, never observed; T, T-branching; Y, Y-branching; FB, false-branching) and multiplication (HG, hormogonia; HY, hormocysts; A, akinetes) are described. In addition, heterotrichy that indicates differences in the shape of the cells of the main and secondary branches [+ , clear differences; (+) small differences, – , no difference; NA, criteria not applicable; U, uniseriate; B, biseriate; M, multiseriate] and the heterocyst position (Tr, terminal; I, intercalary; LS, lateral-sessile; LP, lateral-pedicellate; parentheses indicate infrequent occurrence) are indicated.

Culture	Thallus	Heterotrichy/main axis/branches	Branching	Heterocyst position	Multiplication
<i>Chlorogloeopsis fritschii</i> PCC 6718	Creeping, short filaments	–/NA	Irr	Tr, I	HG
<i>Chlorogloeopsis</i> sp. PCC 7518	Creeping, short filaments	–/NA	NO	Tr*	HG
<i>Fischerella muscicola</i> PCC 7414	Creeping and erect filaments	+ /partly B/U	T	I, (LS)	A, HG
<i>Fischerella</i> sp. 1711	Creeping and erect filaments	+ /U/U	T	I, (LS)	HG
<i>Fischerella</i> sp. SAG 2027	Creeping and erect filaments	+ /B/U	T	I, (LS)	A, HG
<i>Hapalosiphon</i> sp. 804-1	Creeping and erect filaments	–/(B)/U	T	I, (LS)	HG
<i>Mastigocladopsis repens</i> MORA	Creeping and erect filaments	+ /U/U	Y, T	I, (LS, LP)	HG
<i>Nostochopsis lobatus</i> BB92.1	Creeping filaments, abundant mucilage	(+)/U/U	T	I, (LS, LP)	HG
<i>Nostochopsis</i> sp. 89-45	Creeping and partly erect filaments	+ /U/U	T	I, (LS, LP)	HG
<i>Stigonema ocellatum</i> SAG 48.90	Creeping filaments	+ /M/B	T	I, (LS)	HG
<i>Symphyonema</i> sp. 1269-1	Creeping filaments	(+)/U/U	Y (T, FB)	I, (LS)	HG
<i>Symphyonema</i> sp. 1517	Creeping and erect filaments	–/U/U	Y (T, FB)	I, (LS)	HG
<i>Symphyonemopsis</i> sp. VAPOR1	Creeping and erect filaments	+ /U/U	Y (T)	I, (LS)	HY, HG
<i>Westiellopsis prolifica</i> SAG 16.93	Creeping filaments	+ /partly B/U	T	I, (LS)	A, HG
<i>Westiellopsis prolifica</i> SAG 23.96	Creeping filaments	+ /partly B/U	T	I, (LS)	A, HG
<i>Westiellopsis</i> sp. 1590-1	Creeping filaments	+ /partly B/U	T	I, LS	A, HG
<i>Westiellopsis</i> sp. 1590-2	Creeping filaments	+ /partly B/U	T	I, LS	A, HG
<i>Westiellopsis</i> sp. 89-785/4	Creeping filaments	+ /partly B/U	T	I, (LS)	A, HG
<i>Westiellopsis</i> sp. 985-1	Creeping filaments	+ /partly B/U	T	I, LS	A, HG

*Heterocyst differentiation lost in some subcultures (Wilmotte *et al.*, 1993).

uni-cyanobacterial cultures were maintained in BG11 (Rippka *et al.*, 1979) or Z8 media (Kotai, 1972) without nitrogen at a constant temperature (20 °C) and continuous light (20 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$).

Morphology. Morphological observations were made on 2-week-old and 2-month-old cultures, in liquid as well as on solid media. Microphotographs from the strains were taken using a Leica DC 500 video camera with a Leica DMR light microscope. The pictures were then processed with Image Pro Plus. Thallus growth, filament structure, types of branching, position of the heterocysts and multiplication were recorded.

Oligonucleotide primers. 16S rDNA was amplified and sequenced using the eubacterial primer 8F (Lane, 1991) combined with two reverse primers, the heterocystous cyanobacteria-specific primer 1480 (Gugger *et al.*, 2002) and primer 1380R (5'-TAACGACTTCGGGCGTGACC-3') designed in this study. To retrieve the complete sequence in both strands, forward primer 861 (Gugger *et al.*, 2002) and reverse primers 450R (5'-CTGCTGGCAGGAGTTAGC-3') and 920R (5'-TTGTAAGGTTCTTCGCGTTG-3') were used.

PCR amplification. PCR amplification of 16S rDNA was performed in a volume of 50 μl containing 2 μl culture, 200 μM dNTP, 20 μM each primer and 5 μl $10 \times$ LA PCR buffer II (TaKaRa). This template was three times alternatively frozen in liquid nitrogen and boiled prior to the addition of 2.5 U TaKaRa LA *Taq* polymerase. The thermal cycling conditions were 4 min denaturation at 95 °C,

35 cycles of 30 s denaturation at 93 °C, 30 s annealing at 50 °C and 30 s extension at 72 °C, followed by a 7 min elongation at 72 °C. The concentration of the amplified products was checked on 1% agarose gel and purified with the Wizard PCR Preps DNA Purification System (Promega).

Sequencing. The PCR products of 16S rDNA were sequenced directly. DNA sequencing was performed with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 310 Genetic Analyser (Perkin Elmer) according to the manufacturer's instructions. The sequenced fragments were assembled into contigs using GeneDoc version 2.6.002 (www.psc.edu/biomed/genedoc/). The sequences were obtained for both strands independently.

Phylogenetic trees. The sequences were aligned and edited for manual correction using GeneDoc version 2.6.002. Phylogenetic trees were constructed using the neighbour-joining method on Jukes & Cantor (1969) and the Wagner parsimony method of PHYLIP version 3.5c (Felsenstein, 1993). The outgroup was constituted either by distantly related cyanobacteria belonging to *Microcystis* and *Planktothrix* (Otsuka *et al.*, 1998; Lyra *et al.*, 2001; Suda *et al.*, 2002) or by the paralogous group of *Chroococcidiopsis*, as defined by Fewer *et al.* (2002). Bootstrap analysis of 500 resamplings was performed for each consensus tree. Only the bootstrap values above 80% are indicated at the nodes of the trees. The trees were edited using TREEVIEW version 1.6.1 (Page, 1996).

RESULTS AND DISCUSSION

Morphological features of true branching cyanobacteria

Eight morphotypes corresponding to the genera *Fischerella*, *Hapalosiphon*, *Mastigocladopsis*, *Nostochopsis*, *Stigonema*, *Symphyonema*, *Symphyonemopsis* and *Westiellopsis* were distinguished among the studied culture strains (Fig. 1; Table 2). On solid medium, all the stigonematalean isolates had a creeping growth. Old cultures of *Fischerella* sp. 1711 and SAG 20.27, *Nostochopsis* sp. 89-45, *Mastigocladopsis repens* MORA, *Symphyonema* sp. 1517 and *Symphyonemopsis* sp. VAPOR1 also developed erect filaments on solid medium.

The heterotrichous filament morphology (Fig. 1a, b, c; Table 2) was conspicuous for *Fischerella* sp. 1711, PCC 7414 and SAG 20.27, *Nostochopsis* sp. 89-45, *Mastigocladopsis repens* MORA, *Stigonema ocellatum* SAG 48.90, *Symphyonemopsis* sp. VAPOR1, *Westiellopsis prolifica* SAG 16.93 and SAG 23.96, and *Westiellopsis* sp. 1590-1, 1590-2, 985-1 and 89-785/4. Heterotrichy was faint in *Nostochopsis lobatus* BB92.1 (Fig. 1f, g) and *Symphyonema* sp. 1269-1, and absent in old cultures of *Hapalosiphon* sp. 804-1 and *Symphyonema* sp. 1517 (Fig. 1e) and in two *Chlorogloeopsis* strains, PCC 6718 and PCC 7518. The two latter strains formed irregular or no branches. The main axis was uniseriate to biserial in all strains (Fig. 1d) and multiserial in *Stigonema ocellatum* SAG 48.90 (Fig. 1b).

Fig. 1. Photomicrographs of diacritical criteria used in the traditional classification of the Stigonematales. (a) Heterotrichy and young T-branches of *Fischerella* sp. SAG 20.27; (b) multiserial T-branches of *Stigonema ocellatum* SAG 48.90; (c) Heterotrichy and T-branch formation by lateral protuberance in *Westiellopsis* sp. 1590-1; (d) partly biserial main axis in *Westiellopsis prolifica* SAG 23.96; (e) Y-branching formation in *Symphyonema* sp. 1517; (f) lateral pedicellate heterocyst and mature T-branch in *Nostochopsis lobatus* BB92.1; (g) lateral sessile heterocyst and T-branch formation by lens-shaped septation in *Nostochopsis lobatus* BB92.1. Heterocysts are indicated by arrows. Bars, 10 µm.

Mastigocladopsis repens MORA, *Symphyonemopsis* sp. VAPOR1 and *Symphyonema* sp. 1269-1 and 1517 exhibited branches of the Y-type (Fig. 1e), some T-type branches as well as false branches in the two latter isolates. In these four strains, the Y-type branch was initiated by the oblique division of an intercalary cell in a straight portion of the trichome. The resulting branch-point cell became subsequently displaced by elongation or by division of the meristem [respectively observed in *Symphyonema* sp. 1517 in Fig. 1(e) and *Mastigocladopsis repens* MORA].

The 13 remaining strains only formed branches of the T-type (Fig. 1a, b, c, f, g; Table 2). Most of them developed T-branches through three different initial stages: (1) change in division plane by longitudinal septation immediately following a transversal division, (2) branch formation starting with a lateral protuberance or bulge, which is subsequently cut off by longitudinal septation (Fig. 1c), or (3) branch formation starting with lens-shaped septation conspicuous in *Nostochopsis lobatus* BB92.1 (Fig. 1g) and *Nostochopsis* sp. 89-45. In the culture of the latter strain, some Y-type like branches were also observed scarcely. In the description of the field material, this sample was assigned to *Mastigocladopsis jogensis* (Hoffmann, 1990), but the morphological observation of the culture revealed that the ontogeny of branch formation in the isolated strain 89-45 differed from that in the four Y-branching strains. The branches in *Nostochopsis* sp. 89-45 were initiated by typical T-branches. The new branch-point cell elongated, whereas a one-sided cleavage from the opposite side of the T-branch appeared. The cleavage of the cell remained incomplete, leading to a Y-shaped branch-point cell. Although the final appearance of the branch was Y-shaped, its origin is of the T-type.

Except for the two *Chlorogloeopsis* strains, the heterocysts in the stigonematalean isolates were mainly intercalary and less frequently lateral (Fig. 1 b, c, f, g; Table 2). Whereas lateral-sessile heterocysts (Fig. 1g) were observed in almost all strains, at least occasionally, lateral-pedicellate heterocysts (Fig. 1f) were only observed in *Nostochopsis lobatus* BB92.1 and 89-45, and in *Mastigocladopsis repens* MORA. The multiplication strategy of the isolates was mainly by hormogonia, but concomitantly, *Symphyonemopsis* sp. VAPOR1 adopted the differentiation of hormocysts and most of the strains of *Fischerella* and *Westiellopsis* produced akinetes or akinete-like cells.

Primer design

The amplification of the 16S rDNA of the Stigonematales by using the combination of a universal primer and heterocystous-cyanobacteria-specific primer 1480 resulted in poor amplification of the locus from certain strains. The recognition site of primer 1480 is located near a polymorphic region. This region was longer in the 16S rDNA sequence of the Stigonematales compared to that of the Nostocales and thus overlapped the recognition site of the reverse primer. The presence of this polymorphism in

the 3' end of the reverse primer resulted in uneven amplification. Therefore, a new reverse primer, 1380R, hybridizing to the conserved region forward of the polymorphic site was used to retrieve 16S rDNA amplicons. Primer 1380R is specific to the *Cyanobacteria*, but not to heterocystous cyanobacteria and therefore must be used on uni-cyanobacterial or axenic cultures.

Comparison of the 16S rRNA gene sequences of the Stigonematales

The 16 stigonematalean 16S rDNA (1239–1488 bp) sequences obtained were compared to the five Stigonematales sequences available in GenBank. 16S rDNA sequences from the true-branching cyanobacteria shared 89 % or more similarity. Surprisingly, the sequence of *Fischerella* sp. SAG 20.27 from GenBank (AJ344560; Fewer *et al.*, 2002) differed in several sites from the sequence obtained in this study from the purchased strain *Fischerella* sp. SAG 20.27. The divergence between two 16S rDNA sequences from this strain (96.6 % sequence similarity) is high enough to be considered as two taxonomic units. On the other hand, the sequences of *Fischerella* sp. SAG 20.27 from GenBank and of *Nostochopsis lobatus* BB92.1 were identical. It has already been demonstrated that some cyanobacteria contained multiple ribosomal operons (Iteman *et al.*, 2002). However, if strain SAG 20.27 possessed two copies, both of them would be amplified. As *Fischerella* sp. SAG 20.27 (= BB98.1) and *Nostochopsis lobatus* BB92.1 (= SAG 2.97) were both isolated by B. Büdel and deposited in the SAG collection, a possible confusion could explain that the sequence of *Fischerella* sp. SAG 20.27 deposited in GenBank (AJ344560) corresponds to the sequence of *Nostochopsis lobatus* BB92.1.

The sequences of *Nostochopsis lobatus* BB92.1 (and SAG 20.27 from GenBank) and *Nostochopsis* sp. 89-45 differed from that of other Stigonematales by a polymorphic locus of 14 nt contiguous to an insertion of 17 nt in the 5' end of the 16S rRNA gene, and differed from each other by five positions in this polymorphic locus (Fig. 2). This 31 nt region was positioned within helix 6 of the secondary structure model for the 16S rDNA of *Chlorogloeopsis* sp. PCC 7518 (Wilmotte *et al.*, 1993) and may represent a sequence signature for the *Nostochopsis* strains (Fig. 2).

Phylogenetic relationships of true branching cyanobacteria in the 16S rRNA gene tree

To discuss the phylogenetic position of true branching cyanobacteria within the cyanobacterial radiation, the 16S rDNA sequences available for the Stigonematales were compared with those of well identified heterocystous cyanobacteria lacking true branching (Subsection IV, Nostocales), i.e. strains of the genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia*, *Nostoc* and *Scytonema*. The topologies of the trees were similar using as outgroup either distantly related cyanobacteria or the paralogous *Chroococcidiopsis* cluster. Moreover, the distance

Fig. 2. Sequence signature of *Nostochopsis* strains in the 5' end of the 16S rRNA gene. Strains in brackets are representatives of strains showing identical sequences: [BB92.1] represents the sequences of BB92.1 and SAG 20.27 (AJ344560); [SAG 23.96] represents SAG 23.96, 89-785/4, 985-1, 1590-1, 1590-2 and SAG 20.27 (this study); [804-1] represents 804-1 and PCC 7414 (AJ132788); [SAG 48.90] represents SAG 48.90 and VAPOR1; [SAG 16.93] represents SAG 16.93 and *Hapalosiphon welwitschii* (AY034793).

and parsimony trees were congruent. Thus, only the distance tree for the phylogenetic relationships of the Stigonematales with other heterocystous cyanobacteria is presented (Fig. 3).

The group of Stigonematales strains was polyphyletic and separated into four clusters (1, 2, 3 and 5 in Fig. 3) and one separate branch (*Stigonema ocellatum* SAG 48.90). Cluster 1, highly supported by bootstrap values (100/97 %), revealed a tight grouping of T-branching genera. It divided into a single branch with *Fischerella* sp. 1711 and three subclusters, 1a–1c, each highly supported by bootstrap values. Cluster 1a contained the identical sequences of *Westiellopsis prolifica* SAG 23.96, *Westiellopsis* sp. 89-785/4, 985-1, 1590-1 and 1590-2, and *Fischerella* sp. SAG 20.27 (this study). Cluster 1b comprised the closely related sequences of *Westiellopsis prolifica* SAG 16.93 and *Hapalosiphon welwitschii* (98.8 % sequence similarity). Cluster 1c contained the identical sequences of *Nostochopsis lobatus* BB92.1 and *Fischerella* sp. SAG 20.27 from GenBank, and the sequence of *Nostochopsis* sp. 89-45 (97.2 % sequence similarity). According to their high sequence similarity, the strains of *Fischerella*, *Hapalosiphon* and *Westiellopsis* in cluster 1a and 1b are conspecific (Stackebrandt & Goebel, 1994). Moreover, the high variability in morphological characters has already been revealed in *Westiellopsis* (Jeeji-Bai, 1972), suggesting the need for a revision of the genera *Fischerella*, *Hapalosiphon* and *Westiellopsis*. The *Nostochopsis* strains of cluster 1c were distinguished phylogenetically and morphologically from strains of clusters 1a and 1b by their sequence signature, the presence of lateral-pedicellate heterocysts and by a frequent asymmetrical lens-shaped septation of the branch-point cell. Following the classification system of Anagnostidis & Komárek (1990), the strains of cluster 1 are classified into three families (Fischerellaceae, Mastigocladaceae and Nostochopsaceae). This level of classification is not consistent with our sequence data. According to the criterion used for genus definition (95 % 16S rDNA

sequence similarity) of Ludwig *et al.* (1998), the strains of cluster 1 would belong to a single genus.

Clusters 2 and 3 were always basal to cluster 1 (Fig. 3). Cluster 2, highly sustained by bootstrap values (100/97 %), comprised *Fischerella muscicola* PCC 7414 and *Hapalosiphon* sp. 804-1, sharing 95 % sequence similarity. Cluster 3 contained the *Chlorogloeopsis* sp. strains PCC 6718 and PCC 7518, sharing only 91 % sequence similarity. The two *Chlorogloeopsis* strains differed by having irregular or no branching and were the most distantly related to the other branching cyanobacteria (89 % similarity sequence). Moreover, phylogenetic studies based on *nifH* (Zehr *et al.*, 1997) and 16S rRNA genes (Wilmotte *et al.*, 1993) of several *Chlorogloeopsis* isolates suggested that this morphotype probably represents several distinct genera (Wilmotte, 1994). The validity of clusters 2 and 3 must be regarded with caution as they contained long internal branches and low sequence similarities.

Cluster 5 contained the four Y-branching stigonematalean strains *Symphyonemopsis* sp. VAPOR1, *Mastigocladopsis repens* MORA and *Symphyonema* sp. 1269-1 and 1517 (Fig. 3). The two latter strains shared 99.6 % similarity. Despite the low statistical support, these four Y-branching strains were always associated together and separated from the T-branching clusters (clusters 1, 2, 3) in all analyses. The sequences of cluster 5 were equally distant (93 % sequence similarity) with the sequences of the nostocalean strain *Scytonema hofmanni* PCC 7110 and those of cluster 1. Moreover, according to the traditional classification system (Anagnostidis & Komárek, 1990), *Mastigocladopsis repens* would be classified into the family Nostochopsaceae based on the presence of lateral-pedicellate heterocysts, such as found in the *Nostochopsis* strains of cluster 1. The divergence between the *Nostochopsis* and *Mastigocladopsis* strains suggests that the lateral-pedicellate heterocysts are of rather low taxonomic significance.

Fig. 3. Phylogenetic relationships of heterocystous filamentous isolates belonging to the orders Nostocales and Stigonematales based on 16S rDNA sequences (1239–1296 bp). The tree was inferred using the neighbour-joining method based on Jukes & Cantor (1969) distances. Bootstrap values greater than 80% with distance/parsimony methods are indicated on the tree. Sequences from GenBank are indicated by accession numbers. The main clades are indicated by numbers.

The genus *Stigonema* is the type genus of the order Stigonematales. However, the 16S rDNA sequence of *Stigonema ocellatum* SAG 48.90 shared less than 95% similarity with the sequences of the other stigonematalean strains and had an ambiguous position, being equally distant from the T- and Y-clusters (Fig. 3). As *Stigonema* SAG 48.90 was the only example of multiseriate filaments with T-branching among our isolates, no conclusions can be drawn on this morphotype.

The group of Nostocales strains (Subsection IV) was also polyphyletic and separated into two clusters (4 and 6 in Fig. 3). Cluster 4 contained the non-branched heterocystous

cyanobacteria distributed in four distinct subclusters, 4a–4d, containing, respectively, *Nodularia*, *Anabaena*/*Aphanozomenon*, *Nostoc* and *Cylindrospermopsis*. The sequence similarity within each of these clusters exceeded 97.5%. Cluster 6 contained the false-branching *Scytonema* strains, which shared only 95.2% sequence similarity. This cluster was always basal in the phylogenetic analyses of heterocystous cyanobacteria.

Taxonomy of heterocystous cyanobacteria

Representatives of Subsections IV (Nostocales) and V (Stigonematales), currently circumscribed on the basis of

the presence or absence of true branching, were inter-mixed in all our phylogenetic inferences. Subsequently, the traditional distinction of each Subsection (Desikachary, 1959; Rippka *et al.*, 1979; Bourrelly, 1985; Komárek & Anagnostidis, 1989; Anagnostidis & Komárek, 1990; Castenholz, 2001) was not supported by this study.

The phylogenetic analyses of our dataset supported the monophyletic lineage of heterocyst-forming cyanobacteria as described previously on the basis of 16S rDNA sequences (Giovannoni *et al.*, 1988; Turner, 1997; Wilmotte & Herdman, 2001; Fewer *et al.*, 2002; Litvaitis, 2002) as well as *nifH* gene sequences (Zehr *et al.*, 1997). This implies that, unlike the filamentous non-heterocystous or unicellular morphotypes, all the cyanobacteria with heterocysts studied so far evolved from a common ancestor. Consequently, a single Subsection formed of the actual Nostocales and Stigonematales members, and united upon the synapomorphic character of the heterocyst would better fit their monophyly and be in agreement with the Heterocystaceae described by Bornet & Flahault (1886).

Several monophyletic groups, such as *Prochlorococcus*/marine *Synechococcus* (Urbach *et al.*, 1998; Roca *et al.*, 2002), *Euhalothece* (Garcia-Pichel *et al.*, 1998) and *Planktothrix* (Suda *et al.*, 2002), were recently revealed and correlated with ecological and/or physiological characteristics. Within the heterocystous cyanobacteria, only morphological features seem so far to be used to delineate the main monophyletic assemblages. The combination of presence or absence of branching and branching type may represent an important diacritical character for defining monophyletic lineages. Thus, clusters 1, 4, 5 and 6 can be defined as containing, respectively, genera with true branching of the T-type, without branching, with true branching of the Y-type and with false-branching. Furthermore, a great similarity is observed in the ontogeny of false- and Y-branching, as in both types the development of a branch is associated with the activation of an intercalary meristem. Y-branching, as well as geminate false-branching, are frequently observed in at least some strains of cluster 5 (especially in the *Symphyonema* strains). Moreover, the sequence of *Symphyonemopsis* sp. VAPOR1 was as distant from the other Y-branching strains as from the false-branching *Scytonema hofmanni* PCC 7110. New isolates are needed to study further the possible evolution of the Y-branching cyanobacteria from false-branching heterocystous cyanobacteria.

In conclusion, the 16 new stigonematalean 16S rDNA sequences presented in this study demonstrated that the true branching cyanobacteria are polyphyletic and can be separated into at least two major groups that are well defined by their branching types. So far, only 9 out of the 48 true branching cyanobacterial genera (Anagnostidis & Komárek, 1990) are represented in the 16S rRNA gene tree. Several morphotypes, especially pseudoparenchymatous taxa (e.g. *Pulvinularia*) or dichotomous branching taxa (e.g. *Loriella*), as well as representative taxa of the

Borzinemataceae and the Capsosiraceae, are lacking in cultures and in phylogenetic analyses. Furthermore, it remains to be investigated whether non-heterocystous true branching cyanobacteria (such as *Doliocattella*, *Geitleria* and *Umezakia*) originated from filamentous non-heterocystous cyanobacteria or from the heterocystous cyanobacteria.

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