Phenotypic variability and phylogenetic relationships of the genera *Tolypothrix* and *Calothrix* (Nostocales, Cyanobacteria) from running water

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The taxonomy of heterocystous cyanobacteria belonging to the genera *Calothrix* and *Tolypothrix* has long been a matter of debate, but their phylogenetic relationships are still not well understood. Our aim was to compare the phylogeny and morphology of members of these genera, which exhibit basal–apical polarity. A phylogeny was reconstructed on the basis of 16S rRNA gene sequences and compared with the morphological characterization of new isolates and environmental samples. Strains isolated from several rivers and streams showed a high degree of tapering when they were cultured in a nutrient-rich medium. However, clear differences were apparent when they were transferred to a nutrient-poor medium. Some strains showed a low degree of tapering and other morphological features corresponding to the genus *Tolypothrix*, such as false branching, whereas others maintained the morphological characteristics of the genus *Calothrix*. Phylogenetic analysis was congruent with the phenotypic characterization, in which the strains and environmental samples of the *Tolypothrix* and *Calothrix* morphotypes could be clearly separated. Isolates with a low degree of tapering and natural samples of *Tolypothrix distorta* were grouped in the same cluster, but strains of the genus *Calothrix* fell into well separated clades. Results from this study showed that representatives of the genus *Tolypothrix* share most morphological and developmental properties and a high degree of 16S rRNA gene sequence similarity. However, although similar and sometimes overlapping morphologies may occur in isolates of the genus *Calothrix*, these morphotypes may be distinguished on the basis of their clear genetic divergence.

INTRODUCTION

Cyanobacteria are the simplest and oldest oxyphototrophic micro-organisms, comprising a single taxonomic and phylogenetic group. They are important since they can be used as experimental and model strains for studying the diversification of prokaryotic cells and the physiological processes occurring within the cell. The correct determination of cyanobacteria species or strains is one of the main challenges of experimental work. However, the extreme phenotypic flexibility of many species of cyanobacteria in culture produces atypical or anomalous morphologies that differ from those that are characteristic in nature. As a result, identification and taxonomy based on morphology are often

difficult. A serious problem in experimental studies is the arbitrarily selected names for strains. The number of incorrectly identified strains in collections is surprisingly high (Komárek, 1994; Wilmotte, 1994). Attempts to identify cyanobacteria in culture by using a field-based system of classification, combined with the placement of cyanobacteria under the rules of the Bacteriological Code (Stanier *et al.*, 1978), have added more ambiguities, but the establishment of two parallel and competing systems has been avoided. Oren (2004) stressed the need for botanical and bacteriological taxonomists to use unified rules to describe new taxa and to adopt a single taxonomic classification scheme. Traditionally, descriptive accounts of natural communities have been based on botanical species, whereas experimental research carried out on cultures often uses names based on the bacteriological approach that refer solely to the number of the culture collection and that avoid specific names (Whitton, 2002). In some cases, the questionable names of certain strains are even more of a problem, because they have been used for molecular studies

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining; MP, maximum-parsimony.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequence data reported in this paper are HM751842–HM751856.

Supplementary figures are available with the online version of this paper.

and the subsequent assessment of similarity between strains, and the information arising therefrom has sometimes been used to inform nomenclatural decisions (Whitton, 2009). Many problems remain unresolved concerning the taxonomy of cyanobacteria.

The taxonomic status of the genera *Calothrix* and *Tolypothrix* has been widely discussed (Herdman *et al.*, 2001; Komárek & Anagnostidis, 1989; Rippka *et al.*, 1979, 2001a, b; Whitton, 1989; Wilmotte & Herdman, 2001). The genus *Calothrix* is classified in the family Rivulariaceae in the Botanical Code (Geitler, 1932; Komárek & Anagnostidis, 1989) and in Subsection IV.II according to the proposed bacteriological classification (Rippka *et al.*, 2001b). The genus *Tolypothrix* was assigned to the botanical family Scytonemataceae (Geitler, 1932), but was later transferred to the family Microchaetaceae (Komárek & Anagnostidis, 1989), and is included in the current bacteriological approach in Subsection IV.II together with the family Rivulariaceae (Rippka *et al.*, 2001b). Field populations of members of the genus *Calothrix* are characterized by single or small bundles of filaments, which are often united to form distinct colonies that can form mats or crusts. Trichomes are tapered and are arranged more or less parallel to one another. They are mostly erect, unbranched or with occasional false branches, and surrounded by a firm sheath in the lower part of the trichome (Geitler, 1932; Whitton, 2002). The thallus of members of the genus *Tolypothrix* is generally composed of filaments with a firm, thick or thin sheath that is sometimes colourless but frequently yellow to deep brown. There is a single trichome in each sheath, which is false-branched, the branches being single and mostly subtended by a heterocyst. Growth usually occurs apically, and apices are often broad with shorter cells (Geitler, 1932; Whitton, 2002). However, the genera *Calothrix* and *Tolypothrix* present similar morphologies in culture, which has led some authors to include all heterocyst-forming cyanobacteria with a low or high degree of tapering in the genus *Calothrix*, irrespective of their ecology (Rippka *et al.*, 1979). Subsequently, Rippka & Herdman (1992) reassigned various freshwater strains with a low degree of tapering from the genus *Calothrix* to the genus *Tolypothrix*. Members of the genus *Tolypothrix* share most structural and developmental properties with members of the family Rivulariaceae, except the degree of tapering, and these were included in the same subsection IV.II of the current edition of *Bergey's Manual of Systematic Bacteriology* (Rippka *et al.*, 2001a). A distinctive feature of the genus *Tolypothrix* is that its mature trichomes exhibit a low degree of tapering, whereas those of the genus *Calothrix* have a distinct basal-apical polarity and display a high degree of tapering (Rippka *et al.*, 2001a). Phylogenetic analysis of 16S rRNA gene sequences and DNA–DNA hybridization studies (Lachance, 1981) of axenic cultures of the Pasteur Culture Collection (PCC) have revealed great genetic diversity in the genera *Calothrix* and *Tolypothrix* (Herdman *et al.*, 2001; Rippka *et al.*, 2001b; Wilmotte & Herdman, 2001). Hongmei *et al.* (2005) showed that a single morphotype of *Calothrix* in

thermophilic cyanobacterial mats contained five different 16S rRNA genotypes. In addition, Sihvonen *et al.* (2007) observed great variability in strains of *Calothrix* from the Baltic Sea, suggesting that they belonged to at least five genera. They also found that their *Tolypothrix* sequences did not form a monophyletic group. However, only a limited number of strains have been analysed genetically, and, considering the great genetic diversity found, analyses of new isolates in conjunction with natural populations seemed necessary. In this study, we have taken a polyphasic approach to examine samples of natural freshwater (rivers or streams) habitats and isolated strains of tapered cyanobacteria (genera *Calothrix* and *Tolypothrix*). We examined the samples by microscopy, culturing the isolates in different media, and analysed the 16S rRNA gene sequences to assess genotypic diversity in environmental samples and isolated strains.

METHODS

Strain isolation and cultivation. The locations of the sampling sites from which samples were collected are shown in Table 1. The Tejada and Cereal streams are located in the central region of Spain near the city of Madrid. These streams characteristically flow through siliceous substrates. However, the Amir, Muga and Matarraña Rivers are located in the Mediterranean region of Spain and are characterized by highly calcareous waters. Several cobbles or pebbles were collected from a submerged part of the riverbank. The epilithon was removed from these stones by brushing and was then resuspended in culture medium. Aliquots were used for cyanobacterial culturing on Petri dishes (1.5 % agar) in order to isolate species. The cells were spread out in the nitrogen-free medium described by Mateo *et al.* (1986), BG11₀ (Rippka *et al.*, 1979) and a modification of CHU No. 10 medium (Chu, 1942) to give a final phosphorus concentration of 0.2 mg l⁻¹. This enrichment was allowed to grow and was then examined under the microscope to distinguish the different morphotypes. Cultures were obtained by picking material from the edge of discrete colonies that had been growing for approximately 4 weeks on solid medium. Clonal isolates were obtained by twice subculturing a single filament originating from the same colony (Rippka *et al.*, 1979). Cultures were grown in light: dark periods of 16:8 h at a temperature of 18 °C. The intensity of light during the light period was 20 µmol photon m⁻² s⁻¹. The strains (Table 1) were named after the river or stream from which they originated. Macroscopic colonies of members of the genus *Tolypothrix* were collected from the Muga River. Samples for genetic analyses were frozen in liquid nitrogen in the field and aliquots for microscopic examination were fixed with formaldehyde at a final concentration of 4 % (w/v).

Morphological characterization. Morphological observations of environmental *Tolypothrix* colonies and isolated strains were made under a dissecting microscope (Leica; Leica Microsystems) and a photomicroscope (BH2-RFCA; Olympus) equipped with phase-contrast and video camera systems (Leica DC Camera; Leica Microsystems). The key morphological features considered for identifying and differentiating natural samples and isolates included the type of colony, arrangement of trichomes, heteropolarity, length and width of cells, morphology and colour of sheaths, type and frequency of false branching, presence or absence of heterocysts, and the length and width of heterocysts. In addition, we measured the width of the thickest portion of the basal cells and the thinnest portion of the apical trichome. The traditional taxonomic works (Geitler, 1932; Komárek & Anagnostidis, 1989; Whitton, 2002) as well as descriptions

Table 1. Morphological characteristics and source of samples analysed in this studyMeasurements are expressed as μm .

Samples	Filament		Trichome		Cell length		Heterocyst		Basal–apical ratio		Geographical origin
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	
Natural environment samples											
<i>Tolypothrix distorta</i> var. <i>penicillata</i>	15.8	10.1–20.0	11.4	9.0–14.0	9.3	5.0–14.0	11.2 × 14.6	9.0–14.0 × 10.0–20.0	1.1	1.0–1.1	Muga River, Girona, north-east Spain
Tolypothrix strains											
MA11 UAM 357	6.8	6.0–7.5	6.3	5.0–7.0	8.8	7.0–10.0	7.0 × 7.3	6.0–8.0 × 6.0–8.0	1.2	1.0–1.5	Matarraña River, Teruel, east Spain
CR1 UAM 332	9.4	6.0–10.6	7.7	4.8–10.0	5.2	4.0–7.0	8.1 × 4.7	7.0–9.0 × 3.3–7.0	1.2	1.0–1.6	Cereal Stream, Madrid, central Spain
TJ1 UAM 333	8.4	5.0–10.4	7.2	6.0–8.0	5.5	3.0–8.2	6.2 × 4.6	5.0–7.0 × 3.6–5.7	1.3	1.1–1.5	Tejada Stream, Madrid, central Spain
TJ6 UAM 334	6.8	5.1–9.0	5.8	4.4–7.0	4.8	3.0–5.7	5.5 × 4.7	3.6–7.7 × 3.0–6.0	1.3	1.0–1.7	Tejada Stream, Madrid, central Spain
TJ7 UAM 335	9.5	9.0–10.0	6.0	6.0	5.0	4.0–6.0	5.0 × 4.0	4.0–6.0 × 4.0	1.1	1.0–1.2	Tejada Stream, Madrid, central Spain
TJ10 UAM 337	7.4	6.4–8.3	6.3	5.4–7.0	5.3	3.0–6.7	5.8 × 5.1	4.9–7.0 × 4.0–6.0	1.4	1.1–1.6	Tejada Stream, Madrid, central Spain
TJ11 UAM 371	7.7	6.4–9.0	6.6	5.7–7.5	5.7	3.0–7.6	6.2 × 5.4	4.8–8.0 × 3.5–8.0	1.3	1.0–2.0	Tejada Stream, Madrid, central Spain
TJ13 UAM 340	7.4	6.3–9.0	6.1	5.0–7.5	5.1	4.0–6.6	6.1 × 4.6	5.0–7.0 × 4.0–5.4	1.1	0.8–1.2	Tejada Stream, Madrid, central Spain
Calothrix strains											
TJ9 UAM 336	6.4	5.0–8.0	5.6	4.0–7.0	5.1	4.0–7.0	5.8 × 5.9	4.0–8.0 × 4.6–8.0	2.3	2.0–2.9	Tejada Stream, Madrid, central Spain
TJ12 UAM 372	10.8	8.0–17.0	8.4	5.0–11.5	4.9	3.0–7.0	7.8 × 5.3	6.0–13.0 × 4.0–7.0	2.1	1.5–2.9	Tejada Stream, Madrid, central Spain
TJ14 UAM 352	7.6	6.5–9.0	6.0	5.0–8.0	5.5	4.0–9.0	5.9 × 4.9	5.0–7.0 × 3.0–7.0	2.4	2.0–3.5	Tejada Stream, Madrid, central Spain
TJ15 UAM 373	13.8	12.0–16.0	10.3	9.0–12.0	7.4	6.0–10.0	8.4 × 3.7	6.0–10.0 × 2.0–6.0	2.3	1.8–2.9	Tejada Stream, Madrid, central Spain
TJ16 UAM 374	12.0	8.0–17.5	10.1	7.0–13.0	3.6	2.0–5.0	8.8 × 5.2	5.0–12.0 × 4.0–6.0	2.6	2.0–3.5	Tejada Stream, Madrid, central Spain
A1 UAM 342	15.9	11.0–19.0	12.6	9.4–15.0	8.2	5.0–11.0	11.0 × 9.5	8.0–16.0 × 5.0–16.0	2.5	2.1–2.7	Amir River, Murcia, south-east Spain
A2 UAM 345	10.9	9.0–13.0	8.3	6.0–10.5	5.8	4.0–8.0	7.4 × 4.8	5.0–10.0 × 4.0–6.0	2.4	2.0–2.6	Amir River, Murcia, south-east Spain

from *Bergey's Manual of Systematic Bacteriology* (Herdman *et al.*, 2001; Rippka *et al.*, 2001a, b) were used as reference texts.

DNA extraction, PCR amplifications and sequencing. Genomic DNA from isolated cultures or frozen field samples was extracted following a modification of a technique for isolating DNA from fresh plant tissue, using cetyltrimethylammonium bromide (CTAB) (Doyle & Doyle, 1990), as described by Berrendero *et al.* (2008). PCR amplification of a cyanobacterial 16S rRNA gene was carried out using primers pA (Edwards *et al.*, 1989) and cyanobacteria-specific B23S (Lepère *et al.*, 2000), which produced amplifications of about 1400 bp, and the conditions described by Gkelis *et al.* (2005). The 16S rRNA gene was sequenced with Big Dye Terminator v3.1 Cycle Sequencing kit and ABI Prism 3730 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions, using primers pA (Edwards *et al.*, 1989) and cyanobacteria-specific CYA781R(a) (Nübel *et al.*, 1997), 16S684F (5'-GTGTAGCGGTGAAATGCGTAGA-3') and 16S1494R (Wilmutte *et al.*, 2002) or pLG2.1 (Urbach *et al.*, 1992). The sequences were obtained for both strands independently.

Phylogenetic analysis. Nucleotide sequences obtained from DNA sequencing were compared with sequence information available in the National Center for Biotechnology Information database using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). Multiple-sequence alignment was performed using CLUSTAL W (Thompson *et al.*, 1994) from the current version of the BioEdit program (Hall, 1999). The alignment was later visually checked and corrected with BioEdit. The Mallard program (Ashelford *et al.*, 2006) was used to identify anomalous 16S rRNA gene sequences within multiple sequence alignments. Trees based on the 16S rRNA gene were constructed using the neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-parsimony (MP) and maximum-likelihood (ML) methods. NJ and MP trees were constructed using the MEGA 4 program (Tamura *et al.*, 2007), while ML was inferred using PhyML software (<http://atgc-montpellier.fr/phyml/>) (Guindon & Gascuel, 2003). Distances for the NJ tree were estimated by using the algorithm of the Tajima & Nei (1984) model.

Nucleotide positions containing gaps and missing data were initially retained for all such sites in the analyses and then excluded as necessary in the pairwise distance estimation (pairwise deletion option). MP analysis was performed with the close-neighbour-interchange search algorithm with random tree addition; missing information and alignment gap sites were treated as missing data in the calculation of tree length. Bootstrap analysis of 1000 replicates was performed for NJ and MP trees. For ML, the general time-reversible model (GTR) was selected, assuming a discrete gamma distribution with four categories of site-to-site variability of change (gamma shape parameter: 0.219) with the nearest-neighbour-interchange algorithm, using 100 bootstrap samples. Complete sequences (1478–1542 bp) were determined (on both strands) for the majority of samples analysed. However, because we also generated 16S rRNA gene fragments and the length of the sequences obtained from the GenBank/EMBL/DBJ was very variable (from about 700 to 1400 bp), we constructed several trees with partial as well as complete sequences. Since similar clustering was obtained by the three methods using just the long sequences and using a larger taxon set which included long and partial sequences, we present the ML tree including our sequences and genetically closely related but partial sequences from GenBank/EMBL/DBJ.

RESULTS

Morphological features of samples

Field *Tolypothrix* colonies and isolates from calcareous and siliceous rivers were evaluated macroscopically and microscopically to characterize them morphologically (Table 1, Figs 1 and 2). Field *Tolypothrix* colonies collected from the calcareous Muga River were identified according to traditional morphological criteria (Geitler, 1932; Whitton, 2002) as *Tolypothrix distorta* var. *penicillata* [Agardh] Lemmermann. They showed the typical characteristics of

Fig. 1. Microphotographs of natural samples collected from the Muga River and strain MA11 isolated from the Matarraña River. (a, b) Field *Tolypothrix* filaments showing false branching. (c) *Calothrix* filament observed in the epilithic biofilm from the Muga River. (d, e) Strain MA11. (c) Bright light micrograph. (d) Autofluorescence micrograph. Bars, 10 µm (b, c, d) and 50 µm (a, e). Arrows show false branching; ht, heterocyst.

Fig. 2. Morphology of representatives of isolated strains cultured in BG11₀ medium. (a) TJ13; (b) A2; (c) TJ1; (d) TJ12; (e) CR1; (f) TJ16; (g) TJ7. Bars, 10 µm; ht, heterocyst; ho, hormogonium.

this taxon, such as penicillate cushion or tuft colonies, sometimes calcified, and were attached to rocks in fast-flowing waters. The filaments showed false branches, mostly subtended by a heterocyst (Fig. 1a, b). The sheaths were thin, colourless and close to the trichome in young filaments, whereas in mature trichomes the sheaths were thick, yellow to deep brown in colour, and separated from the trichome

by a width of up to 20 µm. The cells were 9–14 µm wide and 5–14 µm long and the heterocysts were usually single and intercalary, with width of 9–14 µm and length 10–20 µm. Strain MA11, isolated from the calcareous Matarraña River, fitted the previous description, although the cellular dimensions were smaller, and could be identified as *Tolypothrix distorta* var. *penicillata*.

Some single *Calothrix* filaments were observed among the samples of epilithic biofilm of the Muga River (Fig. 1c) and were identified as *Calothrix parietina* [Thuret] Bornet *et* Flahault, according to traditional taxonomic criteria (Geitler, 1932; Whitton, 2002).

Strains isolated from the calcareous Amir River and the siliceous Tejada and Cereal streams cultured under laboratory conditions were phenotypically very similar, showing morphological characteristics corresponding to the genus *Calothrix* (Fig. 2), with distinct basal–apical polarity. In a previous study, we found that the morphology varied widely depending on the culture medium employed (Berrendero *et al.*, 2008), so strains were transferred to a medium with few nutrients (liquid CHU10-modified medium; see Methods). Fig. 3 shows the morphological differences between the strains analysed, allowing two morphotypes to be distinguished corresponding to the genera *Calothrix* and *Tolypothrix*. Some isolated strains (CR1, TJ1, TJ6, TJ7, TJ10,

TJ11, TJ13) showed morphological characteristics of the genus *Tolypothrix* when the culture conditions were changed: false branches that were mostly subtended by a heterocyst (Fig. 3a–d), and a low basal–apical polarity of trichomes, with a ratio between 1.1 and 1.4 (Fig. 3a–g, Table 1), were observed. However, other isolates (A1, A2, TJ9, TJ12, TJ14, TJ15 and TJ16) maintained the morphological characteristics of the genus *Calothrix* when the culture conditions were changed (Fig. 3h–k). These strains displayed pronounced tapering with an apical–basal polarity of 2.1–2.6 (Fig. 3h–k, Table 1).

Phylogenetic analysis of the 16S rRNA gene

To determine the phylogenetic relationships of the cyanobacterial isolates and field samples from running waters, the 16S rRNA genes obtained were compared with the database sequences of tapering cyanobacteria belonging to

Fig. 3. Morphology of representatives of isolated strains cultured in the nutrient-poor CHU10-modified medium. (a) TJ13; (b) TJ11; (c) CR1; (d) TJ13; (e) TJ11; (f) CR1; (g) TJ1; (h) TJ16; (i) TJ12; (j) TJ12; (k) A2. Bars, 10 µm. Arrows indicate false branching; ht, heterocyst; ho, hormogonium.

the genera *Calothrix*, *Rivularia* and *Tolypothrix* from this habitat. The three approaches used, NJ, ML and MP, produced similar clustering. Therefore, only the ML tree, with bootstrap values indicated, is presented in Fig. 4. The *Tolypothrix* sequences from this study formed one cluster (group I) and were distinct from the other cyanobacteria with high bootstrap estimates. This group was separated into two subclusters. The first subcluster contained seven sequences, corresponding to isolates from the siliceous Cereal and Tejada streams (CR1, TJ1, TJ6, TJ7, TJ10, TJ11 and TJ13) with 97–100% 16S rRNA gene sequence similarity. The second subcluster harboured the two sequences of *Tolypothrix* from calcareous rivers from this study identified as *T. distorta* var. *penicillata* (field *Tolypothrix* colonies from the Muga River and strain MA11), which had high sequence similarity (99.3%).

In contrast, strains with morphological characteristics of *Calothrix* were very heterogeneous, so their 16S rRNA gene sequences were distributed in different groups in the phylogenetic tree intermixed with *Rivularia* (Fig. 4).

Following the generation of these trees, we ran phylogenetic analyses with greater taxon sampling. We included sequences from databases of other genera assigned to the botanical families Microchaetaceae and Rivulariaceae, and from different biotopes (e.g. desert soil, freshwater, brackish water) as well as other false-branching genera, such as representatives of the genera *Scytonema* and *Brasilonema*, belonging to the botanical family Scytonemataceae. Since the three methods gave congruent results for the major branching patterns of the trees, only the ML tree is presented here (Fig. 5); the NJ and MP analyses can be found in Supplementary Figs S1 and S2, respectively (available at IJSEM Online). Similar results of previous trees (Fig. 4) were found whereby all *Tolypothrix* sequences in this study and other *Tolypothrix* sequences from databases clustered together in the same branch of the tree (cluster A), although this cluster also contained other members of the family Microchaetaceae and some *Gloeotrichia* spp. (family Rivulariaceae). This group included characteristic false-branched cyanobacteria. However, as in the previous trees, *Calothrix* spp. were distributed in different branches. A sister

Fig. 4. Maximum-likelihood tree based on analysis of 16S rRNA gene of tapering cyanobacteria from running waters showing the position of the sequences obtained in the present study (in bold). Numbers at nodes indicate bootstrap values $\geq 50\%$ for ML, NJ and MP analyses, respectively. Bar, 0.02 substitutions per nucleotide position.

Fig. 5. Maximum-likelihood tree based on analysis of 16S rRNA gene of representatives of the families Microchaetaceae and Rivulariaceae (Subsection IV.II of the current bacteriological approach) showing the position of the sequences obtained in the present study (in bold). The family Scytonemataceae (Subsection IV.I) was used as outgroup. Numbers at nodes indicate bootstrap values $\geq 50\%$. Bar, 0.01 substitutions per nucleotide position.

cluster (B) of cluster A grouped two *Calothrix* sequences from this study, *Calothrix* A1 and *Calothrix* TJ16, which had 94% sequence similarity. The highly supported cluster C contained sequences corresponding to the genus *Rivularia*, previously described by Berrendero *et al.* (2008), and *Calothrix* sequences, including those from *Calothrix* TJ15 from this study and previous *Calothrix* isolates from the Tejada stream and the Muga River, as well as others from databases with 91–100% rRNA gene sequence similarity. Cluster D included strains *Calothrix* TJ14 and *Calothrix* TJ9, which were isolated in this study from the siliceous Tejada stream, as well as sequences of *Calothrix* from the databases, such as five isolates from the Baltic Sea, *Calothrix* PCC 8909 and the freshwater strain CAL3361. The sequence similarity within cluster D ranged from 97 to 100%. Cluster E was composed of four *Calothrix* isolates from the Baltic Sea, with 97.8–99.8% 16S rRNA gene sequence similarity. Cluster F contained sequences of the genera *Calothrix* and *Rivularia* intermixed, including *Calothrix* A2 from this study, four sequences from *Calothrix* strains from the database sequences isolated from diverse habitats such as hot spring effluent in Yellowstone National Park, USA (*Calothrix* CCME 5085) (Dillon & Castenholz, 2003), a sphagnum bog (*Calothrix* PCC 7507) (Rippka *et al.*, 2001b), Antarctic lakes (*Calothrix* ANT.PROGRESS2.4) (Taton *et al.*, 2006) and ten sequences of the genus *Rivularia*. Some of these *Rivularia* sequences were from natural samples: for example, *Rivularia biasolettiana* from the calcareous Alharabe River (Berrendero *et al.*, 2008), *Rivularia atra* from the Baltic Sea (Sihvonen *et al.*, 2007), and others were isolated strains, such as the marine *Rivularia* PCC 7116 (Sihvonen *et al.*, 2007). The sequence similarity within cluster F ranged from 92 to 99.9%.

DISCUSSION

Our results indicate that the phylogenetic molecular data were consistent with the morphological characterization, enabling us clearly to separate the *Tolypothrix* and *Calothrix* morphotypes. Field *Tolypothrix* colonies collected from the Muga River, identified as *T. distorta* var. *penicillata*, had characteristics typical of this taxon and were similar to those found in *Tolypothrix* strain MA11 isolated from a calcareous river with similar characteristics. This species occurs on rocks in rivers with fast-flowing and hard water, large streams and the upstream regions of small rivers (Whitton, 2002) and conspicuous growths of *T. distorta* have been recorded from several calcareous Spanish rivers (Aboal *et al.*, 2002, 2005) and from the Muga River (Mateo *et al.*, 2010). The results of the 16S rRNA gene analyses of *Tolypothrix* strain MA11 and field

Tolypothrix colonies were consistent with the morphological characterization, revealing a close gene sequence similarity of 99.3%.

Tapering strains isolated from the Cereal and Tejada streams and the Amir River cultured under laboratory conditions in nutrient-rich media all had morphological characteristics of the genus *Calothrix*. However, cultures transferred to nutrient-poor medium showed differences between strains. Some isolates displayed *Tolypothrix* morphological characteristics when the culture conditions were changed, including a low degree of trichome tapering and of false branching development, but others retained the morphological characteristics of the genus *Calothrix*. As has been pointed out by several authors (Whitton, 1992; Whitton & Potts, 2000; Berrendero *et al.*, 2008), the choice of laboratory culture medium is very important in taxonomic studies because the culture conditions may affect the development of morphological features (Gugger *et al.*, 2002; Lehtimäki *et al.*, 2000), and even these can be lost genetically during the long-term cultivation of strains (Whitton, 2002). As previously mentioned, Komárek & Anagnostidis (1989) estimated that more than half the strains in culture collections were poorly identified. In fact, *Tolypothrix* PCC 7610 was originally identified as *Fremyella diplosiphon*, subsequently as *Calothrix* PCC 7610, and most recently as *Tolypothrix* PCC 7610 (Mazel *et al.*, 1988).

The degree of tapering of the trichomes has been used to separate the genera *Tolypothrix* and *Calothrix*. Cyanobacteria with a ratio of basal to apical cell width of less than 1.5 were assigned to the genus *Tolypothrix* (Herdman *et al.*, 2001), while strains with a tapering range between 3 and 5 were retained in the genus *Calothrix* (Rippka *et al.*, 2001a, b). In this study, strains with *Calothrix* characteristics had a basal–apical ratio between 2.1 and 2.6. These values were slightly below the range established in axenic cultures of *Calothrix* from the Pasteur Collection (Rippka *et al.*, 2001a, b), although similar results were obtained in *Calothrix* strains from the Baltic Sea (Sihvonen *et al.*, 2007). On the other hand, strains that showed *Tolypothrix* morphological characteristics had a basal–apical ratio of less than 1.5, coinciding with the range established for this genus by Herdman *et al.* (2001), and confirming the assignment of these strains to the genus *Tolypothrix*. However, Sihvonen *et al.* (2007) found two strains (TOL328 and BECID4) with low attenuation (<1.5), which were assigned to different genera (TOL328 to the genus *Tolypothrix*; BECID4 to the genus *Calothrix*) after phylogenetic analysis of 16S rRNA gene sequences. They suggested that the apical–basal relationship alone was not sufficient for identifying strains. In contrast, our phylogenetic analysis was congruent with the phenotypic

characterization, in that the strains and environmental samples of the *Tolypothrix* and *Calothrix* morphotypes could be clearly separated. 16S rRNA gene sequences of isolates, field *Tolypothrix* colonies and *Tolypothrix* sequences from databases were grouped in the same cluster (cluster A), while *Calothrix* morphotypes were clearly distributed in distinct branches. Cluster A could be subdivided into subgroups A₁, A₂ and A₃, although group A₂ was not highly supported in the ML and MP trees, despite achieving 80 % support in the NJ tree. Group A₁ contained our field samples and isolates from calcareous waters identified as *T. distorta* var. *penicillata*, together with other *Tolypothrix* from the databases, and other genera of the family Microchaetaceae (*Coleodesmium*, *Hassallia*, *Rexia* and *Spirirestis*). Interestingly, all the genera included in this subcluster are morphologically very similar, with repeated false branching at heterocysts and a high degree of 16S rRNA gene sequence similarity (>95 %). The overall structures are like a form of *Tolypothrix* but with different branching processes or other morphological variations. The genus *Coleodesmium* has basically the same structure as *Tolypothrix* but with multiple trichomes in the sheath (Whitton, 2002; Taton *et al.*, 2006). This genus was described by Geitler (1932) as *Desmonema*, and was changed to *Coleodesmium* by Komárek & Watanabe (1990). Taton *et al.* (2006) isolated one Antarctic strain belonging to the genus *Coleodesmium* whose sequence was grouped in their phylogenetic tree with *T. distorta* (97.7 % similarity), and which exhibited a maximum of 96.9 % similarity with the other sequences of the genus *Coleodesmium* available in the databases. The taxonomic delimitation of the genus *Hassallia* has been widely discussed (Hoffmann & Demoulin, 1985; Komárek & Anagnostidis, 1989; Hoffmann, 1993). The genus *Hassallia* was first included under *Tolypothrix* by Geitler (1932), but later separated (Geitler, 1932) to include only terrestrial forms with only single false branches arising beside single-pored heterocysts. The subsequent taxonomic treatment by Desikachary (1959) questioned the value of the latter character for separating members of the genus *Hassallia* from those of the genus *Tolypothrix*. Hoffmann & Demoulin (1985) stated that the only differences between the genera *Tolypothrix* and *Hassallia* are the constantly short cells and subaerial habitat of the latter. The genera *Rexia* and *Spirirestis* have both been described more recently (Flechtner *et al.*, 2002; Casamatta *et al.*, 2006). The genus *Rexia* differed from all genera in the family Microchaetaceae by the presence of hormogonia, one to few celled, capable of undergoing division in two planes. Its filaments are falsely branching, with one or more trichomes per filament (Casamatta *et al.*, 2006). The genus *Spirirestis* shares morphological characters with the genera *Tolypothrix* and *Scytonema*, principally heterocyst formation, false branching, and presence of the sheath, but most trichomes are tightly spiralled (Flechtner *et al.*, 2002). The phylogenies based on 16S rRNA sequence placed the genus *Rexia* close to the genus *Coleodesmium* (96.02 % similarity), but also sharing 96.1 % similarity with both *Tolypothrix distorta*

and *Spirirestis rafaensis* obtained from desert soils (Casamatta *et al.*, 2006). 16S rRNA gene sequence similarity among *Tolypothrix distorta* and *Spirirestis rafaensis* was 99.02 % (Casamatta *et al.*, 2006). Overall sequence similarity values (ranges) have been used in the assignment of defined taxa. There is extensive documented evidence that two strains sharing less than 97 % 16S rRNA gene sequence similarity are not members of the same species (see Tindall *et al.*, 2010). There have been suggestions that this value should be set higher. For instance, Stackebrandt & Ebers (2006) recommended a 16S rRNA gene sequence similarity threshold range of 98.7 to 99 %. However, less attention has been paid to other taxonomic ranks, such as the genus (Tindall *et al.*, 2010). Ludwig *et al.* (1998) suggested that 16S rRNA gene sequence similarities of around 95 % would be a practicable border zone for genus definition. Rosselló-Mora & Amann (2001) and Tindall *et al.* (2010) stated that a genus could be defined by species with 95 % sequence similarity, a threshold that has been used as a circumscription limit in cyanobacterial taxonomic analysis (e.g. Rajaniemi *et al.*, 2005; Rajaniemi-Wacklin *et al.*, 2006; Palinska *et al.*, 2006). Therefore, if the boundary value of 95 % sequence similarity is applied to representatives of cluster A₁, these results would suggest generic-level identity. These genera are phenotypically similar, but show some morphological differences. The distinction of new genera on the basis of morphological differences, despite candidates exhibiting a genetic similarity greater than 95 %, continues to be controversial. More research using both approaches is required to resolve this matter satisfactorily.

Group A₂ contained *Tolypothrix* sequences obtained in this study corresponding to isolates from the siliceous rivers, which exhibited a *Tolypothrix* morphotype when transferred to a low nutrient concentration medium, as well as sequences from other databases. Herdman *et al.* (2001) in *Bergey's Manual of Systematic Bacteriology* divided the genus *Tolypothrix* into two major clusters on the basis of results from DNA–DNA hybridization studies (Lachance, 1981). Cluster 1 included six PCC strains of *Tolypothrix* that were relatively highly related genetically, although they were further divided into four subclusters. Cluster 2 contained three other strains from PCC, *Tolypothrix* PCC 7415 being the reference strain in this cluster (Herdman *et al.*, 2001). In our study, *Tolypothrix* PCC 7415 was included in subcluster A₂ along with our isolates from siliceous rivers and some *Tolypothrix* strains from subcluster 1 of the tree of Herdman *et al.* (2001). Therefore, similar clustering can be inferred.

Group A₃ comprised the sequences of the genera *Gloeotrichia*. This genus is easily recognized from environmental samples in that the tapered trichomes with a basal heterocyst are arranged in a radial more or less parallel disposition, and often with false branches, showing a spherical colony, belonging to the botanical family Rivulariaceae (Whitton, 2002). Its taxonomic position has been discussed by several authors (Rippka *et al.*, 2001c;

Sihvonen *et al.*, 2007). Our results showed that these sequences were phylogenetically close to the sequences of the family Microchaetaceae and distantly related to sequences of other members of the family Rivulariaceae. Sihvonen *et al.* (2007) also indicated that the genus *Gloeotrichia* is distantly related to the genus *Calothrix*. Further sequences of phylogenetically and morphologically similar cyanobacteria might help to determine whether the grouping of the family Microchaetaceae and the genus *Gloeotrichia* represents artefacts or true relationships.

We found great heterogeneity among the genus *Calothrix*, as has previously been noted (Berrendero *et al.*, 2008; Sihvonen *et al.*, 2007; Thomazeau *et al.*, 2010). *Calothrix* strains isolated from the calcareous Amir River were separated in different clusters in the phylogenetic tree. *Calothrix* A1 and strain TJ16 from the siliceous Tejada stream formed cluster B, although with only 94 % sequence similarity, suggesting that they were probably grouped together because no other closely related sequences were available. Strain A2 clustered with other 16S rRNA gene sequences from the genera *Calothrix* and *Rivularia* of the databases (97.3–99.3 % similarity) in cluster F. Strains TJ9 and TJ14 clustered with the *Calothrix* strains isolated from the Baltic Sea in cluster D, which matched the description of the botanical species *C. scopulorum*, which is a very commonly recorded species in the Baltic Sea (Hällfors, 1984; Sihvonen *et al.*, 2007). With respect to the other *Calothrix* sequences in their study, Sihvonen *et al.* (2007) concluded from the sequence divergence of this group that the strains belonging to the genotype corresponding to *C. scopulorum* were probably a new taxon, related to, but not belonging to, the genus *Calothrix*.

The TJ12 and TJ15 strains were clustered (in group C) with other published *Calothrix* sequences of widely different geographical origin and from diverse habitats such as desert sand and freshwater, including thermal springs, ponds and lakes, as well as other sequences of the genus *Rivularia* similar to that previously described by Berrendero *et al.* (2008).

The results of this study show that the phenotypically defined genera *Calothrix* and *Rivularia* cannot be reliably separated from each other on the basis of small-subunit rRNA gene sequence phylogenetic analysis. However, it should be noted that there is great genetic divergence between the two genera (Sihvonen *et al.*, 2007; Berrendero *et al.*, 2008). The genus *Rivularia* is easily recognized in the field by its characteristic development in gelatinous, hemispherical or subspherical colonies containing a large number of filaments, arranged radially or sometimes parallel to each other in part of the colony. On the other hand, filaments of members of the genus *Calothrix* usually occur singly or in small bundles, running parallel to each other in more cushion-shaped colonies or intermingled in flatter colonies (Geitler, 1932; Komárek & Anagnostidis, 1989; Whitton, 2002). Isolated cultures of the genus *Rivularia* show great morphological variability and often

do not exhibit features typical of the genus (Rippka *et al.*, 2001c). However, when growth conditions are similar to those found in nature, some characteristics of the genus can be observed (Berrendero *et al.*, 2008). In addition, ecophysiological differences between these genera can be noted in running waters. For this reason, *Rivularia* could be considered as a bioindicator of clean waters, since it is absent from contaminated waters (Whitton, 1987; Mateo *et al.*, 2010).

Our results indicate that although similar and sometimes overlapping morphologies may occur in isolates of the genus *Calothrix*, these morphotypes may be distinguished on the basis of their clear genetic divergence. The genotypes of *Calothrix* were clearly differentiated in distinct clusters in phylogenetic trees, exhibiting <95 % similarity in the sequence of the 16S rRNA gene. These results support the findings of Sihvonen *et al.* (2007) concerning the great genetic diversity within the *Calothrix* morphotype and their proposed revision of this genus.

We conclude that this polyphasic taxonomic examination integrating phenotypic and genotypic characterizations has been useful for distinguishing the genera *Tolypothrix* and *Calothrix*. However, new isolates are needed to study further the possible evolution of false-branching cyanobacteria. Therefore, more representatives of the families Microchaetaceae and Rivulariaceae need to be analysed to confirm their phylogenetic positions.

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