Genetic and morphological characterization of *Rivularia* and *Calothrix* (Nostocales, Cyanobacteria) from running water

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In this study, a polyphasic approach was adopted to investigate natural freshwater (river and stream) samples of *Rivularia* colonies and isolated strains of cyanobacteria with a high degree of trichome tapering (genera *Rivularia* and *Calothrix*). Analysis of the phycocyanin (PC) operon and the intervening intergenic spacer (*cpcBA*-IGS) and 16S rRNA gene sequences were used for genetic characterization. In addition, a molecular fingerprinting method, temperature-gradient gel electrophoresis, which allows sequence-dependent separation of PCR products, was used to assess genotypic diversity in environmental samples and isolated strains. The results showed a high variability of the PC-IGS among the genotypes that was not associated with the morphologies observed. This study underlines the importance of choosing a low-nutrient-content culture medium, especially one with a low phosphorus concentration, for studying typical morphological features of *Rivularia* for taxonomic purposes. Molecular fingerprinting methods and morphological analyses confirmed the diversity in *Rivularia* colonial structure and trichome features corresponding to genetic diversity within a single colony. Phylogenetic analysis of *cpcBA*-IGS was largely consistent with that obtained from 16S rRNA gene sequence analysis and confirmed the high level of divergence between genotypes. The sequences of *Rivularia* and *Calothrix* from this study and database sequences showed great heterogeneity and were clearly not monophyletic. The results of this genetic and morphological study of field samples and fresh isolates indicated that the current classification of these genera needs to be revised.

INTRODUCTION

Heterocystous cyanobacteria correspond to a monophyletic lineage (Wilmotte & Herdman, 2001) that contains the orders Nostocales and Stigonematales (subsections IV and V) (Rippka *et al.*, 1979; Castenholz, 2001). The genera *Rivularia* and *Calothrix* belong to the order Nostocales and the family Rivulariaceae according to traditional classification (Geitler, 1932; Komárek & Anagnostidis, 1989; Whitton, 2002) and to subsection IV by bacteriological classification (Rippka *et al.*, 2001a). The Rivulariaceae are among the most morphologically complex cyanobacteria (Whitton, 1987). They are characterized by tapered trichomes, apart from short phases of hormogonium

formation. The mature trichome has a terminal heterocyst, although some species also have intercalary heterocysts, and cell division is largely localized to a region near the heterocyst (Whitton, 2002). Traditional taxonomy (Geitler, 1932) included 12 genera, some of which are widespread and others that have rarely been recorded (Whitton, 1987). Thus, *Calothrix, Rivularia* and *Gloeotrichia* can be considered the most representative genera of this group and are the most thoroughly studied. The genus *Rivularia* Agardh is easily distinguishable in the field by its characteristic development as gelatinous, hemispherical or subspherical colonies containing a large number of filaments, arranged radially or sometimes parallel to each other in part of the colony (Geitler, 1932; Komárek & Anagnostidis, 1989; Whitton, 2002). However, isolates from natural colonies identified as *Rivularia* often do not produce gelatinous colonies in culture and as such are indistinguishable from the genus *Calothrix*. Thus, Rippka *et al.* (1979) included in the genus *Calothrix* all heterocystous cyanobacteria with a low or high degree of tapering, irrespective of their ecology, on the grounds that colony appearance observed in feral samples was a taxonomic trait of dubious value (Rippka *et al.*, 2001c).

However, Komárek & Anagnostidis (1989) pointed out that the genus *Rivularia* differs also by other morphological features such as the sheath morphology, which has repercussions for the development of meristematic zones where divided trichomes and young filaments persist within common old sheaths. DNA–DNA hybridization studies (Lachance, 1981) also showed that freshwater and soil isolates of *Calothrix* were unrelated to the marine representatives. Consequently, Rippka *et al.* (2001b, c) assigned the marine members to the genus *Rivularia*. In a recent study (Sihvonen *et al.*, 2007), analysis of sequences of cyanobacterial cultures and environmental samples belonging to the genera *Calothrix*, *Rivularia* and *Gloeotrichia* from the Baltic Sea revealed a high level of genetic diversity and overlapping morphologies among phylogenetic groups. The authors pointed out that the *Calothrix* morphotype was also a highly diverse group genetically, and one in need of revision (Sihvonen *et al.*, 2007). Therefore, it remains a difficult task to establish an accurate taxonomy of traditional Rivulariaceae.

Many authors have stressed that traditional morphological characteristics must be integrated with genetic characterization (polyphasic approach), and interpreted both within and between taxa, to resolve taxonomic issues (Wilmotte, 1994; Komárek & Kaštovský, 2003; Garcia-Pichel *et al.*, 2001; Castenholz & Norris, 2005; Taton *et al.*, 2006). Molecular genetic approaches have been developed that have the potential to resolve relationships between closely related cyanobacteria. The sequences of the rRNA genes

have been used successfully to aid morphological taxonomy. However, at the intrageneric level the rRNA (16S rRNA) gene sequences have demonstrated little clearly interpretable variation among several strains of cyanobacteria (Fox *et al.*, 1992; Moore *et al.*, 1998). An alternative to the use of conserved 16S rRNA genes is to examine intergenic spacer regions which usually show considerably more variation, for example the intervening intergenic spacer of the c-phycocyanin (PC) genes *cpcA* and *cpcB* (*cpcBA*-IGS) (Neilan *et al.*, 1995; Bolch *et al.*, 1996).

In this work we have adopted a polyphasic approach to examine natural freshwater (rivers or streams) samples of *Rivularia* colonies and isolated strains of cyanobacteria with a high degree of tapering (genera *Rivularia* and *Calothrix*). We examined the samples by microscopy and analysed the 16S rRNA gene sequences and the PC operon and the intervening intergenic spacer (*cpcBA*-IGS). In addition, a molecular fingerprinting method, temperature-gradient gel electrophoresis (TGGE), which allows sequence-dependent separation of PCR products (Muyzer & Smalla, 1998), was used to assess genotypic diversity in environmental samples and isolated strains.

METHODS

Sampling. The locations of sampling sites from which samples were collected are shown in Table 1. The Muga, Matarraña, Endrinales, Blanco and Alharabe rivers are located in the Mediterranean region of Spain. These rivers are characterized by highly calcareous waters. Red

Table 1. Isolated strains and field *Rivularia* colonies examined in this study

Sample	Culture collection no.*	Geographical origin
Natural environment samples		
Rivularia colonies Alharabe	−	Alharabe river, Murcia, south-east Spain
Rivularia colonies Blanco	−	Blanco river, Teruel, east Spain
Rivularia colonies Endrinales	−	Endrinales river, Albacete, south-east Spain
Rivularia colonies Matarraña	−	Matarraña river, Teruel, east Spain
Rivularia colonies Muga	−	Muga river, Girona, north-east Spain
Rivularia colonies Red	−	Red Sike, Upper Teesdale, northern England, UK
Isolated strains		
BL1	UAM 346	Blanco river, Teruel, east Spain
BL2	UAM 347	Blanco river, Teruel, east Spain
E1	UAM 302	Endrinales river, Albacete, south-east Spain
E7	UAM 313	Endrinales river, Albacete, south-east Spain
E17	UAM 358	Endrinales river, Albacete, south-east Spain
MA8	UAM 355	Matarraña river, Teruel, east Spain
MA14†	−	Matarraña river, Teruel, east Spain
MU15	UAM 369	Muga river, Girona, north-east Spain
MU24	UAM 305	Muga river, Girona, north-east Spain
MU27	UAM 315	Muga river, Girona, north-east Spain
MU28	UAM 341	Muga river, Girona, north-east Spain
MU41	UAM 370	Muga river, Girona, north-east Spain
TJ12	UAM 372	Tejada stream, Madrid, central Spain

*UAM, UAM Culture Collection, Universidad Autónoma de Madrid, Spain.
†Strain MA14 was only cultivable for a few months.

Sike is a stream in the Upper Teesdale National Nature Reserve, an upland area of northern England, which is also highly calcareous. However, Tejada stream, located in central Spain, near Madrid, flows through siliceous substrates.

Rivularia colonies were collected from calcareous rivers, where they were dominant. In addition, 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), Allen and Arnon's medium (AA) (Allen & Arnon, 1955), BG11$_0$ (Rippka *et al.*, 1979) and a modification of CHU No. 10 (CHU10) medium (Chu, 1942) to give a final phosphorus concentration of 0.2 mg l^{-1}. This enrichment was allowed to grow, and was examined under the microscope to distinguish 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 subculturing a single filament originating from the same colony twice (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.

Morphological characterization. Morphological observations of environmental *Rivularia* colonies and isolated strains were made using a dissecting microscope (Leica; Leica Microsystems) and an Olympus BH2-RFCA photomicroscope equipped with phase-contrast and video camera systems (Leica DC Camera; Leica Microsystems). The key features taken into consideration for the identification and differentiation of natural samples and isolates were: the environment (freshwater or marine and brackish water), the colony or thallus (hemispherical colonies, solitary trichomes or their groups), calcification (abundant, moderate or slight, absent), texture (hard, soft or gelatinous), section of colony (marked zonation, little or no zonation), the filaments (arrangement of trichomes, heteropolarity, hairs, width of trichomes), morphology of sheaths (colour and lamellations), type and frequency of false branching and development of falsely branched filaments (divided trichomes and young filaments within common old sheaths or not, thus secondary trichomes remaining or not remaining in the 'mother' sheath), according to traditional criteria (Geitler, 1932; Whitton, 1987, 2002; Komárek & Anagnostidis, 1989).

Isolation of genomic DNA. Total 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). Cells were harvested by centrifugation after the addition of about 150 μl of sterile glass beads (212–300 μm; Sigma) and resuspended in 400 μl extraction buffer [100 mM Tris/HCl, pH 8.0, 20 mM EDTA, 2.5 % (w/v) CTAB, 1.4 M NaCl, 0.2 % (v/v) 2-mercaptoethanol]. Samples were frozen in liquid nitrogen and then homogenized in extraction buffer using a hand-operated homogenizer (Bosch, CSB-850-2RET). Subsequently, they were incubated at 60 °C for 30 min, followed by two extractions with chloroform. DNA-containing phases were placed in new tubes and an equal volume of 2-propanol was added. The samples were centrifuged at 12 000 *g* for 5 min. The pellet was resuspended in 400 μl sterile water and DNA was precipitated again by addition of 0.1 volumes of 3 M sodium acetate and two volumes of ethanol before being stored at –20 °C in 15 μl sterile water.

PCR amplification and cloning of the PC-IGS region: design of specific primers. PCR amplifications were performed with a Perkin-Elmer GeneAmp 2400 PCR system. The reaction volume was 25 μl and contained 6 μl genomic DNA, 10 pmol of each primer, 200 μM dNTP, 1 μg BSA, 1.5 mM MgCl$_2$, 2.5 μl 10 × polymerase buffer, 5 μl 5 × Eppendorf Taqmaster PCR-enhancer and 0.75 U Ultratools DNA polymerase (Biotools). Primers PCβ (forward primer) and PCα (reverse primer) were adopted initially using the conditions described by Neilan *et al.* (1995). As amplification of the DNA was not successful, and not enough DNA for direct sequencing was obtained, PCR products were cloned using a Qiagen PCR Cloning^{plus} kit. Recombinant clones carrying the correct-sized insert were sequenced in both strands. Subsequently, the primers RivF (5′-TGGAAATCATCTTGCGCTATGT-3′) and RivR (5′-CACCAG-CAACTAAACAGTA-3′) were deduced from the alignment of the sequences obtained and were used to amplify this region. PCR consisted of initial denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The concentration of the amplified products was checked on a 1.5 % agarose gel and the products were purified using the Real Clean Spin kit (Real).

PCR amplification of the 16S rRNA gene. To amplify the 16S rRNA gene segments, two sets of cyanobacteria-specific primers were used, as described by Nübel *et al.* (1997) and Wilmotte *et al.* (2002). This produced fragments of about 700 and 1400 bp, respectively. The reaction conditions used were those described by Nübel *et al.* (1997). The PCR product was quantified as described above for the PC-IGS region.

Sequencing. DNA sequencing was performed and analysed by using the chain-termination method with the DyeDeoxy terminator cycle sequencing kit protocol, following the manufacturer's instructions (Applied Biosystems). The sequences were obtained for both strands independently.

TGGE analysis of PC-IGS PCR products. TGGE Maxi System (Biometra) was used for sequence-specific separation of PCR products. A 23-nucleotide GC-rich sequence was attached to the 5′-end of the forward primer to improve the detection of sequence variation in the amplified DNA fragments by subsequent TGGE (Sheffield *et al.*, 1989). Electrophoresis was performed by loading 6 μl of PCR product in a gel containing 5 % acrylamide/bisacrylamide, 8 M urea, 2 % glycerol, 20 % formamide and 1 % TAE, as described previously (Rodríguez *et al.*, 2007), with a gradient ranging from 40 to 51 °C. The gels were then stained following a routine silver-staining protocol (Sanguinetti *et al.*, 1994) and photographed using a Nikon Coolpix 995 digital camera.

Analysis of sequence data. 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 done with all partial and complete sequences using the CLUSTAL_X package (Thompson *et al.*, 1997). Alignments were corrected manually using GeneDoc version 2.6.002 (Nicholas & Nicholas, 1997). Trees based on the *cpcBA*-IGS and 16S rRNA gene were constructed using the neighbour-joining (NJ) and maximum-parsimony (MP) algorithms (Saitou & Nei, 1987) in MEGA 3.1 (Kumar *et al.*, 2004). Distances for the NJ tree were estimated by using the algorithm of Tajima & Nei (1984) and nucleotide positions containing gaps and missing data were considered. The parameters (base frequencies, substitution rate matrix of types and shape of gamma distribution) were estimated from the data. Bootstrap analysis of 1000 replications was performed for each consensus tree (Felsenstein, 1985). Similar clustering was obtained with the NJ and MP algorithms, so we have chosen to represent the PC sequence relationships with the neighbour-joining tree (696 bp). In the case of the ribosomal operon, complete sequences (*Escherichia coli*

positions 27–1494) were determined (on both strands) for at least one representative genotype. However, because we also generated 16S rRNA gene fragments from about 700 to 1400 bp (except one of about 400 bp), and there are many short sequences in GenBank/ EMBL/DDBJ, we constructed several trees with partial as well as complete sequences. Since similar clustering was obtained by using both NJ and MP methods with our complete and database complete sequences, we represented the NJ tree (461 bp, *E. coli* positions 362– 823) in order to include genetically closely related but partial *Calothrix* sequences from GenBank/EMBL/DDBJ.

RESULTS AND DISCUSSION

Morphological observations and *cpcBA*-IGS sequence analysis

Field *Rivularia* colonies and fresh rivulariacean-type isolates from calcareous rivers (Table 1) were evaluated macroscopically and microscopically to characterize them morphologically (Fig. 1). Some field-collected *Rivularia* colonies could be identified according to traditional morphological criteria (Geitler, 1932; Komárek & Anagnostidis, 1989; Whitton, 2002) as *Rivularia biasolettiana* [Ag.] Born. *et* Flah., showing the typical characteristics of this taxon, such as hemispherical colonies, colour from blue-green, dark olive-green to golden-brown, with calcite crystals deposited inside mucilage, but the extent varying markedly, sheaths wide, lamellated, splayed out at the apical end, some yellow-brown, though usually very pale ones, present (Fig. 1a–c). Other colonies that were highly calcified and whose sections showed obvious zonation were identified as *Rivularia haematites* [Men.] Born. *et* Flah. (Fig. 1d–f). Similar occurrence of both types of *Rivularia* on rocks in calcareous springs and streams has been described previously (Whitton, 2002), and are dominant in the majority of the Spanish rivers studied (Aboal *et al.*, 2002, 2005). However, when the rivulariacean-type strains isolated from these calcareous rivers (Table 1), where *Rivularia* was dominant, were cultured under laboratory conditions and examined morphologically, all of them were very similar phenotypically (Fig. 1g, h). The strains all showed *Calothrix*-like morphological characteristics and did not exhibit features typical of the genus *Rivularia*. Previous reports featured isolates from natural colonies that, although they were identified as *Rivularia,* did not produce gelatinous colonies in culture and therefore were indistinguishable from *Calothrix* (Rippka *et al.*, 1979).

In order to distinguish between related isolates and to compare them with natural environment samples the PC operon was used as a molecular marker. The *cpcBA*-IGS genetic analysis showed that all the sequences of environmental *Rivularia* colonies and rivulariacean-type isolates from calcareous rivers (confirmed on both strands) fell into one of three distinct classes of genotype (Table 2). These genotypes were designated PC-IGS type I, type II and type III. All three genotypes shared the same length for coding regions, but differed in the IGS region, so that the length of PC fragment varied from 688 to 696 nt (Table 2).

Sequences within a genotype class were identical or had a high percentage of similarity to each other. Alignment of the sequences revealed 100 % similarity of some isolated strains with the sequences found in some field *Rivularia* colonies, suggesting that they might belong to the genus *Rivularia* (data not shown). When considered by genotype, fragments exhibited a maximum pairwise divergence of 1 % in type I, 0.3 % in type II and 0.6 % in type III for the calcareous rivulariacean-type. When we analysed a *Calothrix*-like strain (TJ12) isolated from the siliceous Tejada stream, where *Rivularia* is unable to grow, we found that the sequence clearly fell into the genotype II class although it showed more variability (2.7 %). Genotype I was found in natural *Rivularia* colonies from the Endrinales and Matarraña rivers, and in the isolated strains BL1, BL2, E1, E7, MA8, MU15 and MU24. Genotype II was found only in isolated cultures E17, MU27, MU28, MU41 and TJ12. Genotype III was found in natural *Rivularia* colonies from Muga river, Alharabe river and Red Sike, and in strain MA14. This strain could only be maintained in culture for a few months and, therefore, although PC operon sequence analysis was performed when the strain was first isolated, no subsequent analyses could be made.

Sequence divergence between genotype types ranged from 13.7 to 31.9 % (Table 3). Sequence type III was the most divergent, with very similar percentages of nucleotide substitution along the entire fragment, and a variation of 20–30 % in the coding regions, and greater than 60 % in IGS. Differences between sequences of genotypes I and II were 7.0 % in *cpcB*, 38.6 % in IGS and 11.7 % in *cpcA*. (Table 3). Multiple alignment of the sequences of this IGS region of samples from this study and database entries indicate that the nucleotide sequence is highly conserved within each genotype, but clearly differs among them. This information is available as Supplementary Fig. S1 in IJSEM Online. Thus, the large sequence divergence in the PC-IGS among the genotypes found indicated a clear taxonomic separation. Sequence analysis within the genera *Microcystis* and *Nodularia* revealed intrageneric sequence variation across the *cpcBA*-IGS of 6.4 and 6.7 %, respectively. The percentages of nucleotide substitutions were 6.9 % for *cpcB*, 13.7 % for *cpcA* and 7.6 % for IGS for the genus *Microcystis* (Bittencourt-Oliveira *et al.*, 2001), and 8.2 and 8.4 % for the coding regions and 11.7 % for IGS for the genus *Nodularia* (Bolch *et al.*, 1999). Manen & Falquet (2002) found 5 % variable positions within the PC-IGS locus in the genus *Arthrospira*, and Barker *et al.* (2000) recorded a value of 5.4 % in *Aphanizomenon*. In contrast, Teneva *et al.* (2005) showed a greater divergence (16.5 %) in *Phormidium*. Robertson *et al.* (2001) also found a high level of sequence divergence in the genus *Synechococcus*. However, based on sequences from a large number of isolates for both the 16S rRNA gene and *cpcBA*-IGS, the authors suggested that the genus *Synechococcus* should be revised extensively. Thus, the high degree of variability among the three genotypes appeared to indicate that they belong to different genera.
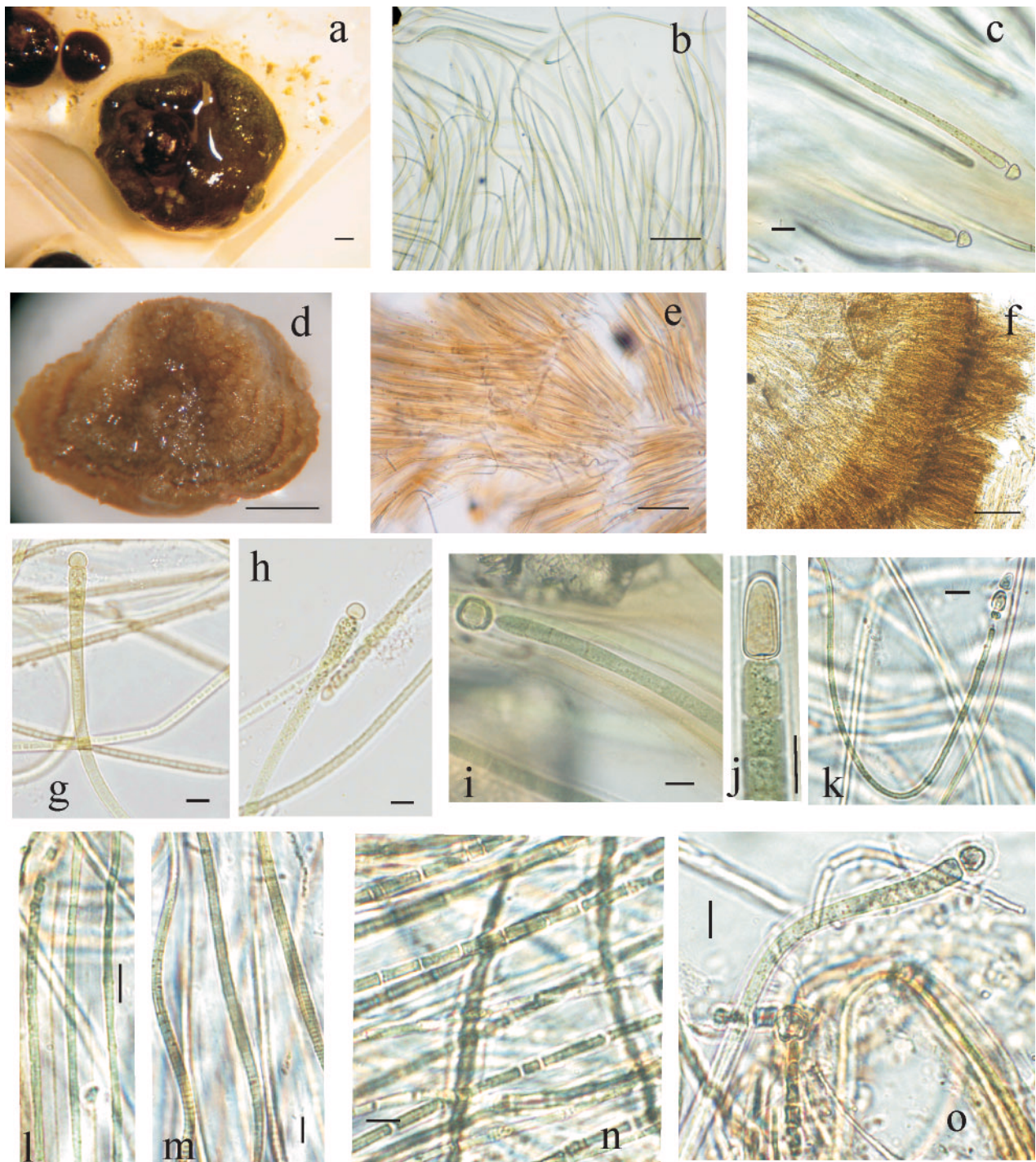
Fig. 1. Microphotographs of field *Rivularia* colonies and isolated strains showing morphological variability. (a–c) *Rivularia* colony from Alharabe river, *R. biasolettiana* type; (d–f) *Rivularia* colony from Endrinales river, *R. haematites* type; (g) isolated strain MU27; (h) isolated strain MU24; (i and j) different trichomes found in a single *Rivularia* colony from Red Sike; (k–n) different trichomes found in a single *Rivularia* colony from Matarraña river; (o) *Calothrix* filament found in a *Rivularia* colony from Muga river. Bars, 1 mm (a, d), 100 μm (b, e and f) and 10 μm (c, g–o).

However, this high variability of the PC-IGS among the three genotypes was not associated with the morphology observed, as all isolates had typical *Calothrix*-like features.

In order to study the morphological variability of our rivulariacean-type isolates in detail and, as the initial isolates were obtained using nutrient-rich media, the

Table 2. Summary of the length of the *cpcBA*-IGS locus of genotypes I, II and III, percentage similarity, and environmental colonies or isolated cultures in which these genotypes were found

Genotype	PC fragment (nt)	*cpcB* (nt)	*cpcA* (nt)	IGS (nt)	Percentage similarity	Strains/colonies
I	688	283	314	91	99–100	*Rivularia* Endrinales *Rivularia* Matarraña BL1, BL2 E1, E7 MA8 MU15, MU24
II	696	283	314	99	97.3–100	E17 MU27, MU28, MU41 TJ12
III	693	283	314	96	99.4–100	*Rivularia* Muga *Rivularia* Alharabe *Rivularia* Red MA14

cultures were transferred to a medium with a low level of nutrients that more closely approximated the characteristics of the rivers with *Rivularia* colonies (liquid CHU10-modified medium – see Methods). Fig. 2 shows that isolated strains that presented as genotype I were able to adopt *Rivularia*-like morphological characteristics when the culture conditions were changed, including secondary trichomes remaining in the 'mother' sheath (Fig. 2a), lamellated sheath (Fig. 2b) and confluent trichomes (Fig. 2c, d).
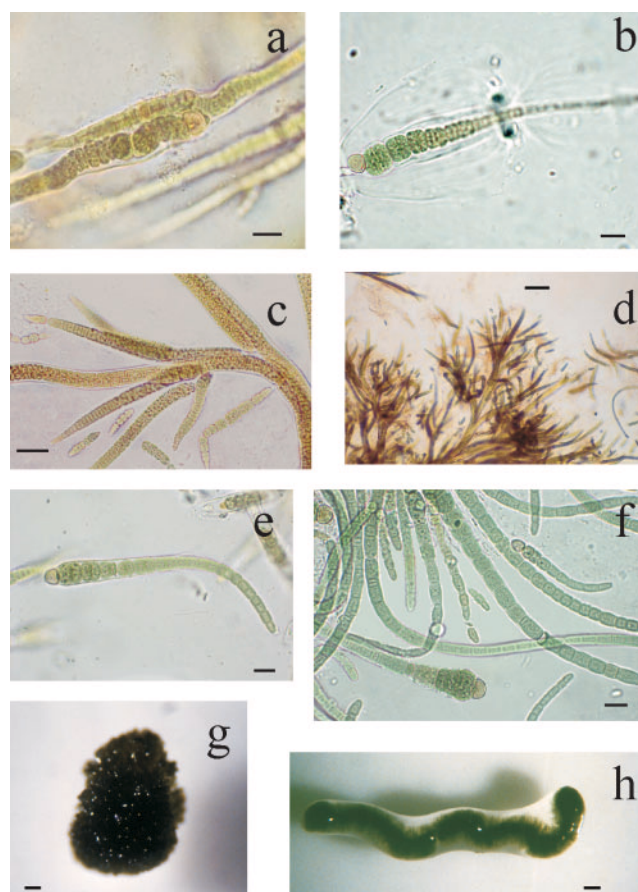
However, isolated strains that presented as genotype II maintained *Calothrix*-like morphological characteristics when the culture conditions were changed (Fig. 2e, f). In addition, colonies of strains corresponding to genotype I tended to be spherical whereas strains corresponding to genotype II formed groups of filaments or irregularly shaped trichome bundles in liquid culture (Fig. 2g, h).

A more detailed study was carried out to assess the influence of the medium on morphological variability of *Rivularia* on agar plates. Three different types of media were selected in order to have a wide range of nutrient concentrations: CHU10-modified was selected as a very low nutrient and salt content medium (0.2 mg phosphorus l^{-1}), $BG11_0$ (~5 mg phosphorus l^{-1}) and AA as a very high

Fig. 2. Morphology of representatives of isolated strains cultured in the low-nutrient content CHU10-modified medium (liquid). (a) Strain E1; (b) strain MU24; (c and d) strain MU15; (e) strain E17; (f) strain MU41; (g) strain MU24; and (h) strain MU27. Bars, 1 mm (g and h), 100 μm (d) and 10 μm (a–c, e and f).
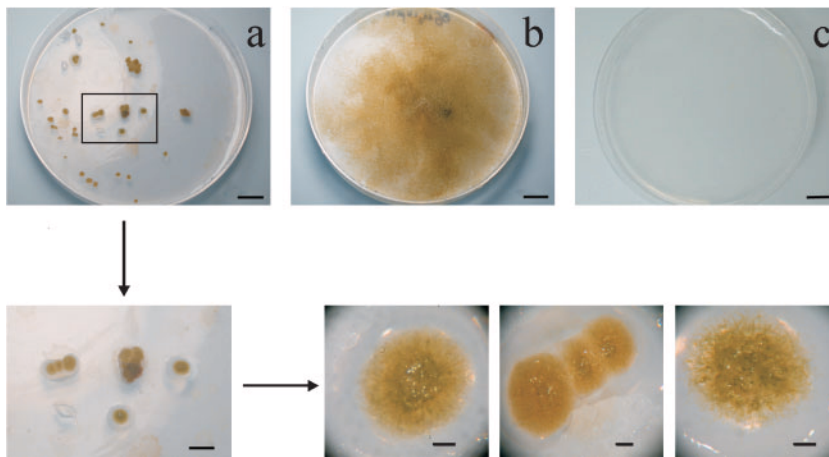
Table 3. Variable substitutions determined in the nucleotide sequences of genotypes I, II and III

Values given are percentage divergence.

Genotype	PC fragment	*cpcB*	*cpcA*	IGS
I and II	13.7	7	11.7	38.6
I and III	31.5	21.9	30.5	62.2
II and III	31.9	21.2	30.5	66

Fig. 3. Effect of different culture media on *Rivularia* colonies on agar plates. (a) CHU10-modified medium; (b) BG11$_0$ medium; and (c) AA medium. Bars, 10 mm (a–c); insets: 5 mm (left) and 500 μm (three right panels).

nutrient concentration medium (~60 mg phosphorus l^{-1}). When a single colony of *Rivularia* was homogenized and aliquots were spread on agar plates of these media, differences in growth and morphology became apparent (Fig. 3). In CHU10 low-phosphorus medium, typical hemispherical colonies grew after about 2 months (Fig. 3a). In BG11$_0$, filaments were spread all over the surface, similar to *Calothrix* cultures (Fig. 3b). Finally, *Rivularia* was unable to grow in the AA medium with very high nutrient concentrations (Fig. 3c).

The importance of the choice of laboratory culture medium has been emphasized for a long time (Whitton, 1992; Whitton & Potts, 2000). Whitton (2002) pointed out that, whereas most researchers are aware of the need to grow an organism in nitrogen-free medium if it is a nitrogen-fixer, organisms have been grown much less frequently under phosphorus limitation and only very rarely so in taxonomic studies, and that major taxonomic studies and routine subculturing of culture collection strains have often been conducted in a medium containing too much phosphate. This means that morphological features that are influenced by phosphorus limitation are never seen and, in the case of some strains in culture collections, may have been lost genetically (Whitton, 2002). Many authors agree that the extreme phenotypic flexibility of many species of cyanobacteria in culture produces atypical or anomalous morphologies that are different from those typically found in nature (Golubic, 1979; Komárek & Anagnostidis, 1999; Pomati *et al.*, 2000). It has been suggested that the low correlation of phenotypic features and genotypic characterization based on PC-IGS (Barker *et al.*, 1999; Bittencourt-Oliveira *et al.*, 2001) is a result of culture-induced variation. BG11$_0$ and, to an even greater extent, AA are media with higher phosphorus concentrations than those found in the rivers, and this phosphorus enrichment could be responsible for the morphological variations and differential growth observed in our study. Fogg (1969) reported that even quite low concentrations of inorganic phosphate in artificial medium appear to be inhibitory for some genera, including

Gloeotrichia. A number of subsequent studies have indicated that the phosphorus status of the environment may be a particularly important factor in the growth of Rivulariaceae (Whitton, 1987).

All these results appeared to indicate that isolated strains BL1, BL2, E1, E7, MA8, MU15 and MU24 belong to the traditional *Rivularia* genus, and that strains E17, MU27, MU28, MU41 and TJ12 belong to the traditional *Calothrix* genus. However, it has been reported that some cyanobacteria possess two or three PC operons (Mazel *et al.*, 1988; Dubbs & Bryant, 1993; Golden, 1995) that are differentially expressed under different growth conditions. In this way, it is possible that our strains possess two or three operons, only one of which we have sequenced. This could give rise to misleading conclusions about the taxonomy.

Molecular fingerprinting analysis – TGGE

In order to evaluate the possibility suggested above, TGGE was carried out on the basis that PCR fragments of the same size but different sequence could be separated (Muyzer & Smalla, 1998). For the TGGE analysis, we cloned specific sequences from strains or colonies (confirmed by sequencing) as markers corresponding to the three genotypes found. PCR products for each genotype were cloned in commercial vectors to ensure that, as only one fragment could be inserted in the vector, only one copy would be resolved on the gel. The high sequence variability among the three genotypes gave rise to three clearly separated bands that allowed us to characterize each genotype by its corresponding fingerprint (Fig. 4a, b). TGGE analysis of the isolated strains revealed only one band for each culture, and this band was consistent with the corresponding fingerprint (Fig. 4a).

For TGGE of natural *Rivularia* colonies, different samples from the rivers studied were examined morphologically beforehand (Fig. 1). Large colonies were collected from the Alharabe river. They had a very soft texture and consisted entirely of the *R. biasolettiana* type (Fig. 1a–c). We also
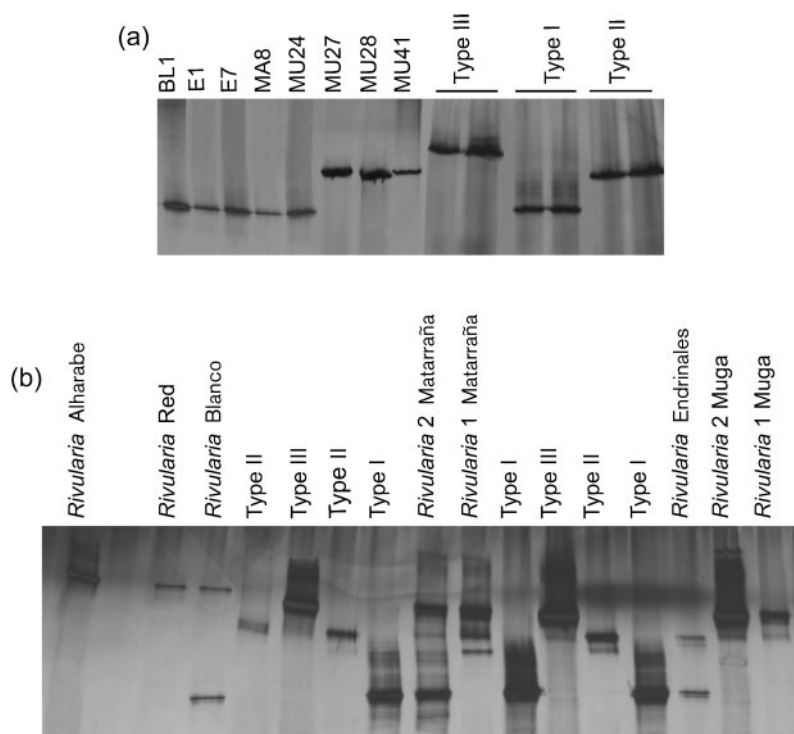
Fig. 4. TGGE fingerprinting analysis of PC-IGS PCR amplification of representative strains isolated from calcareous rivers (a) and field *Rivularia* colonies from the rivers of this study (b). Types I, II and III correspond to PCR products of cloned PC-IGS genotypes I, II and III, respectively, sequenced previously.

examined small hemispherical highly calcified colonies exhibiting obvious zonation (Fig. 1d–f) from Endrinales river, which were of the *R. haematites* type. However, some colonies had an ambiguous macroscopic and microscopic morphology, and some heterogeneity was found between trichomes of *Rivularia*, and even within trichomes of single colonies (Fig. 1i–o). Similar observations were described by Sinclair & Whitton (1977), who found that three different morphological types of Rivulariaceae were released during incubation of pond *Rivularia* colonies and that the trichomes released from stream *Rivularia* colonies appeared to be of *Calothrix*.

This heterogeneity in colonial structure and trichome features is mirrored by a corresponding genetic diversity in TGGE analysis. In many samples, the colonies presented at least two distinct bands (Fig. 4b). Colonies collected from Red Sike and the Alharabe river, which were clearly of the *R. biasolettiana* type, presented a profile corresponding to genotype III. Colonies from the Endrinales river, which were typical *R. haematites* with very high calcification and with distinct zones inside, mainly displayed the band pattern corresponding to genotype I, although another band corresponding to genotype II could also be found. Colonies from the river Blanco presented two clear bands corresponding to genotypes I and III, similar to a colony from Matarraña river, but the other colony from the same river displayed a different profile with genotypes II and III. Two colonies from the river Muga shared a similar pattern that corresponded to genotype III, although a blurred band corresponding to genotype II could be observed (Fig. 4b). These results indicate that there may be genetic diversity

even within a colony. In addition, we cannot rule out the possibility that other trichomes that are sometimes found in the colony, such as those for *Calothrix* (Fig. 1o), can be amplified by PCR of colonies. This would correspond to genotype II, which appears as blurred bands on gels (Fig. 4b).

Phylogenetic analysis of the 16S rRNA gene and *cpcBA*-IGS sequences

Similar results were found in the phylogenetic analysis of the 16S rRNA gene and PC operon sequences, whereby there was a distinct clustering of the three genotypes (Figs 5 and 6). To determine the phylogenetic position of the rivulariacean-type within the cyanobacterial radiation, the 16S rRNA gene sequences obtained in this study in conjunction with the recently published sequences of *Rivularia* and other *Calothrix* from GenBank/EMBL/DDBJ were compared with those of representatives of other genera of Nostocales, i.e. strains of the genera *Tolypothrix*, *Nostoc*, *Nodularia*, *Cylindrospermopsis*, *Anabaena* and *Aphanizomenon*, and also some distantly related cyanobacteria, such as strains of *Chroococcidiopsis* and *Oscillatoria* (Fig. 5). The rivulariacean-type sequences from this study and those from the database (except some *Calothrix* strains from the database) formed a cluster, separate from other cyanobacteria. This cluster was divided into three subclusters (A, B, C) in which genotypes II, I and III, respectively, were included. Clusters D, E, F and G contained, respectively, the genera *Tolypothrix*, *Nostoc*, *Nodularia* and *Cylindrospermopsis*. *Anabaena* and
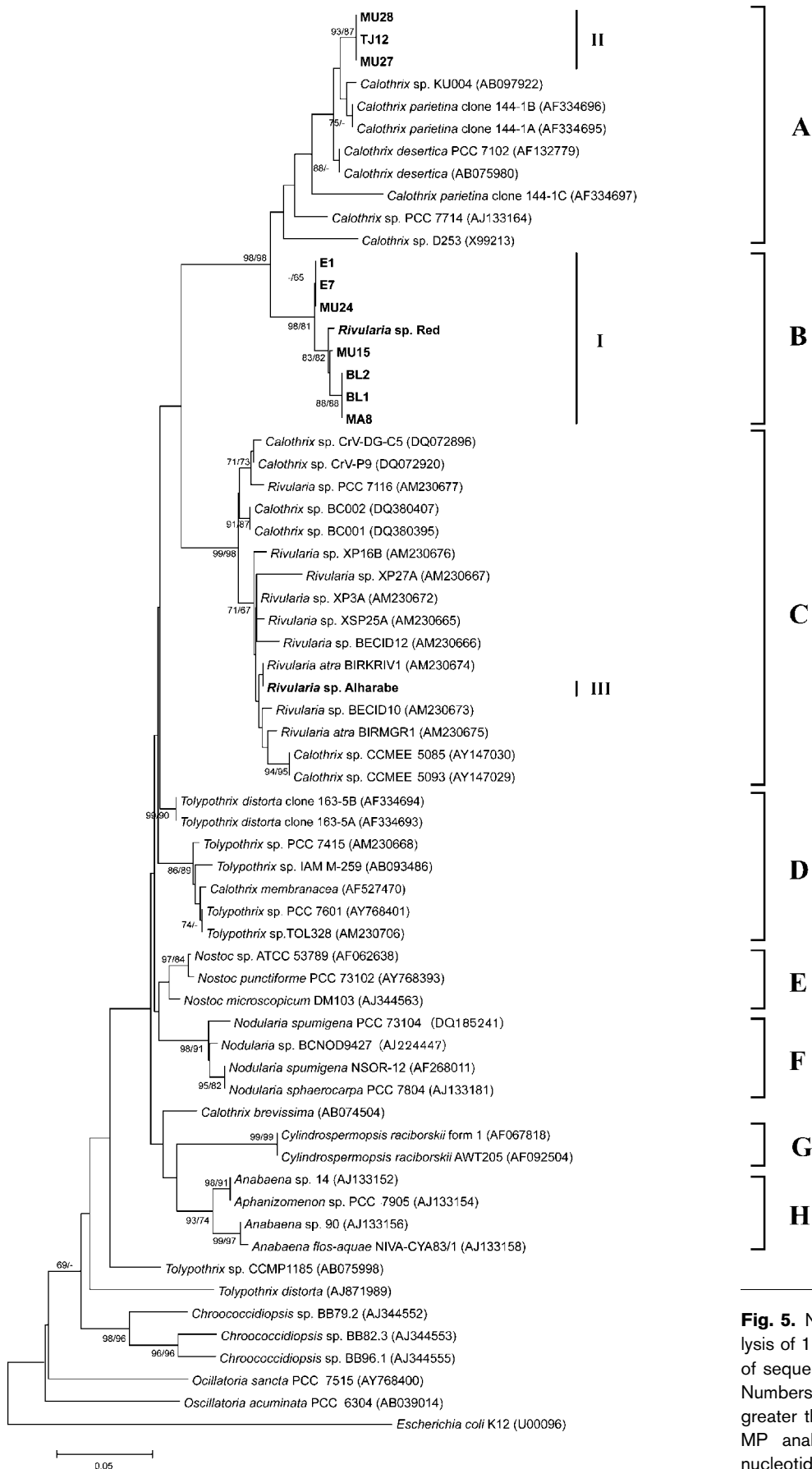
Fig. 5. Neighbour-joining tree based on analysis of 16S rRNA genes showing the position of sequences obtained in this study (in bold). Numbers at nodes indicate bootstrap values greater than or equal to 65 % for the NJ and MP analyses. Bar, 0.05 substitutions per nucleotide position.

Aphanizomenon were grouped together in cluster H, as found previously (Gugger & Hoffmann, 2004; Rajaniemi *et al.*, 2005).

Cluster A comprised the three identical sequences corresponding to genotype II from this study: two strains (MU27 and MU28) from the calcareous Muga river, and a strain (TJ12) from the siliceous Tejada stream, as well as other sequences of *Calothrix* from the database. These include two sequences of *C. parietina*, which showed 98.8 % similarity to strains of genotype II, two strains of *C. desertica* (98 % similarity), *Calothrix* D253, originally classified as *C. vigueri*, and *Calothrix* PCC 7714, originally classified as *C. marchica* (96 and 95.6 % similarity, respectively). A third clone of *C. parietina* was also included in the cluster but only had 93.5 % similarity. DNA–DNA hybridization studies have been used to measure the degree of relatedness between organisms with high 16S rRNA sequence similarity. Organisms with more than 70 % DNA–DNA relatedness, which is the boundary value for recognition of a species (Wayne *et al.*, 1987), often share more than 97 % 16S rRNA gene similarity (Stackebrandt & Goebel, 1994). Thus, when strains have <97.5 % similarity, the sequence data provide strong evidence that the strains represent separate species. However, it has been cautioned that, for strains with >97.5 % similarity, the molecular data may not be adequate to enable decisions to be made at the species level (Casamatta *et al.*, 2005). Here, strains corresponding to genotype II had 98.8 % similarity with *C. parietina* from the database, a species whose trichomes were frequently observed in microscopic analysis of our environmental samples, raising the possibility that genotype II corresponds to traditional *C. parietina*. DNA–DNA hybridiza-

tion studies with *C. parietina* field populations could confirm species identity.

The highly supported cluster B contained sequences of strains, and a field *Rivularia* colony from Red Sike, from this study, corresponding to genotype I, which shared high 16S rRNA gene sequence similarity (98.8–100 %). These findings suggest that cluster B represents a novel evolutionary branch which has not been characterized previously.

Cluster C comprised sequences of the genera *Calothrix* and *Rivularia*, and included the sequence of a *R. biasolettiana* type from the river Alharabe corresponding to genotype III of this study. Our *Rivularia* sequence showed a maximum sequence similarity of 99.9 % (1384 bp) with *Rivularia atra* BIRKRIVI, but 99.2 % with the other *R. atra* sequence (strain BIRMGR1), and a minimum of 97.3 % similarity with *Rivularia* PCC 7116. The most divergent sequence in cluster C was that of *Rivularia* PCC 7116, ranging from 96 to 97.5 % similarity.

In the *cpcBA*-IGS tree (Fig. 6), the nine sequences from natural *Rivularia* colonies and isolated strains from calcareous rivers corresponding to genotype I formed one cluster (eight of them are shown in Fig. 6), the five sequences from isolated strains from calcareous and siliceous rivers corresponding to genotype II formed a second cluster (three of them are shown in Fig. 6), and the four sequences from *Rivularia* colonies and a single isolated strain from calcareous rivers formed a third cluster (Fig. 6). Genotypes I and II clustered together in one group that was well supported by high bootstrap values. However genotype III was distant, consistent with the highest recorded percentage variability.
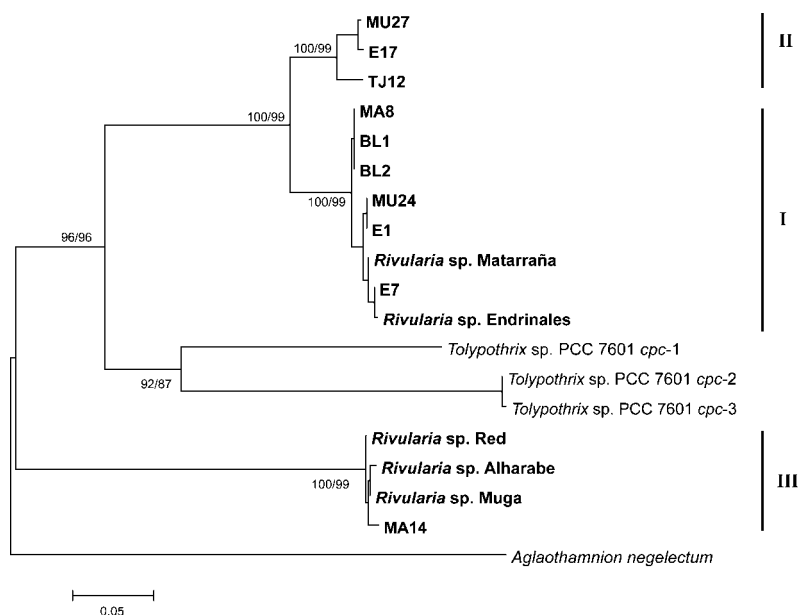

Fig. 6. Neighbour-joining tree based on analysis of sequences of the *cpcBA*-IGS and flanking regions from 16 representative strains and field *Rivularia* colonies obtained during the present study (in bold) and three operons of *Tolypothrix* PCC 7601 as the only tapered strain from the database. Strains MU28 and MU41 had identical sequences to that of strain MU27, strain BL2 had an identical sequence to that of strain BL1, and strain MU15 had an identical sequence to that of strain MU24. Numbers at nodes indicate bootstrap values greater than or equal to 65 % for the NJ and MP analyses. Bar, 0.05 substitutions per nucleotide position.

Strain and field sample sequences corresponding to genotypes I and II from this study always clustered together, regardless of the method used to generate the tree and the gene analysed, suggesting that they were closely related. In contrast, PC operon analysis appeared to indicate that they belong to different genera. However, it has been pointed out that the phylogeny of the PC-IGS locus must be treated with care when attempting to deduce organismal phylogenies because the locus displays a complex evolutionary pattern (Manen & Falquet, 2002; Janson & Granéli, 2002). In our study, 16S rRNA gene and *cpcBA*-IGS data are clearly in agreement. Sequence similarity between genotypes I and II ranged from 93.3 to 94.3 % in the 16S rRNA gene phylogenetic analysis. A threshold of 95 % 16S rRNA gene sequence similarity has been suggested for the definition of a genus (Ludwig *et al.*, 1998). Based on this definition, the evolutionary distances of this study confirmed the genotypes to represent separate genera, as the PC operon analysis had previously suggested. Taken together the morphological, phylogenetic and fingerprinting results, we can conclude that genotype I corresponds to the traditional genus *Rivularia* and genotype II to the traditional genus *Calothrix*.

The taxonomic status of the genus *Calothrix* has been discussed widely (Whitton, 1989; Komárek & Anagnostidis, 1989; Wilmotte & Herdman, 2001; Rippka *et al.*, 2001b). Phylogenetic analysis of 16S rRNA gene sequences, and relationships between strains of the genus *Calothrix* deduced from DNA–DNA hybridization results (Lachance, 1981) demonstrated a high genetic diversity in this genus (Wilmotte & Herdman, 2001; Rippka *et al.*, 2001b). Recently, great sequence variability of the 16S rRNA gene has been found among *Calothrix* strains from the Baltic Sea (Sihvonen *et al.*, 2007), suggesting that they belong to at least five different genera. Hongmei *et al.* (2005) showed that a single morphotype of *Calothrix* in thermophilic cyanobacterial mats comprised five different 16S rRNA genotypes. The tree topology obtained from phylogenetic analysis of cyanobacterial 16S rRNA gene sequences in our study was in agreement with that of Sihvonen *et al.* (2007) and supported the idea that members of the genus *Calothrix* are polyphyletic and should be divided into several different genera.

Regarding the genus *Rivularia*, the sequences from this study and those from GenBank/EMBL/DDBJ showed great variability and were clearly not monophyletic. Morphological, sequencing and TGGE fingerprinting analysis showed that environmental *Rivularia* colonies were phenotypically and genotypically heterogeneous. It has been discussed whether *R. haematites* and *R. biasolettiana* are ecoforms of the same species or are different taxa (Kann, 1977). In contrast, Zehnder (1985), who worked with *Rivularia* cultures, concluded that *R. biasolettiana*, isolated from Lake Erken in Sweden, and *R. haematites*, isolated from a small creek in Switzerland, represented two distinct species. Whitton (2002) pointed out that *R. haematites* merges with *R. biasolettiana*, from

which it differs only in the very high calcification and distinct zones.

Our results suggest that genotype I corresponds to traditional *R. haematites* and genotype III to traditional *R. biasolettiana*, and that sometimes clear traditional phenotypic types can been found that correspond to these genotypes. However, an ambiguous morphology can often be observed, which probably corresponds to the genetic diversity found within a single colony, as suggested previously by Whitton (1987). It has been pointed out that the colonies of some genera, such as *Rivularia*, are typically formed by the aggregation of a number of hormogonia (Whitton, 1987; Whitton & Potts, 2000). Darley (1967), during a laboratory study of marine Rivulariaceae, found that hormogonia would only develop when a large quantity was inoculated simultaneously. In our study, morphological and genetic analysis suggested that representatives of both genotypes could be found in the same place. Thus, if the hormogonia involved in the aggregation are genetically homogeneous, the resulting colony will be dominated by one genotype, such as genotype III in the *R. biasolettiana* type, or genotype I in the *R. haematites* type. However, colonies formed by aggregation of genetically heterogeneous hormogonia will develop the genetic diversity found in this study. This also explains why phenotypically similar colonies collected at the same time from the same place had different genotypes (e.g. *Rivularia* colonies from Red Sike showing genotype III in the PC operon tree but genotype I in the 16S rRNA gene sequence analysis), and why both genotypes I and III could be found in a single colony in the fingerprinting analysis (e.g. colonies from the Blanco and Matarraña rivers), whereas others only showed one band, which corresponded to genotype III (e.g. another colony from Matarraña river). These results cast doubt on the validity of using colony morphology to identify and differentiate these species of *Rivularia* and suggest that classification clearly requires the use of distinction criteria other than morphology.

On the other hand, it must be emphasized that, although a similar and sometimes overlapping morphology has been observed in environmental *Rivularia* colonies, a clear genetic divergence has been demonstrated. Genotypes I and III were clearly distinct in the two phylogenetic trees. Only a 90.6–91.1 % 16S rRNA gene sequence similarity was found between genotypes I and III, indicating that the *Rivularia* sequences studied may actually represent two different genera. Rippka *et al.* (2001a, b, c) in the current taxonomic classification of *Bergey's Manual of Systematic Bacteriology* only include strains isolated from saline environments (intertidal zone, supralittoral shore, seawater aquarium) in the genus *Rivularia*, thus separating it from *Calothrix* by its higher salt requirement or tolerance. Sihvonen *et al.* (2007) found that, in their *Rivularia* isolates, only the truly marine *Rivularia* PCC 7116 was able to grow in elevated salt concentrations, but that the Baltic Sea *Rivularia* strains did not require elevated salt as the brackish water of the Gulf of Finland, where most of the strains were isolated, has a

salinity of about 5 ‰. *Calothrix* CCMEE 5085 and *Calothrix* CCMEE 5093, which were also included in cluster C along with all *Rivularia* sequences, were isolated from a siliceous substrate of shallow, tepid (<35 °C) hot spring effluents in Yellowstone National Park with a salinity of about 1‰ (Dillon & Castenholz, 2003). Our sequence in this cluster corresponds to a *Rivularia* from freshwater although, as it only occurs in calcareous rivers, elevated concentrations of Ca^{2+} could be required for its growth. It is also possible that they are euryhaline since a tolerance to desiccation has been observed. Therefore, if we maintain genotype III as *Rivularia*, a new genus should be invoked for the *Rivularia* representatives of genotype I in cluster B of our study.

These results suggest that the cyanobacteria classified in the traditional genus *Rivularia* diversified through evolution. A possible explanation is that the *Rivularia*-like morphology represents an ancestral morphology type from which other morphologies, such as *Calothrix*-like, have evolved. Another possibility is that filament tapering may have evolved more than once during the diversification of heterocyst-forming cyanobacteria, as suggested previously (Sihvonen *et al.*, 2007).

In conclusion, the genera *Rivularia* and *Calothrix* showed great heterogeneity. Rivulariacean-type isolates from this study showed considerable genetic divergence but overlapping morphologies. However, the use of a culture medium with a low level of nutrients, more closely approximating the characteristics of the rivers with *Rivularia* colonies, allowed us to observe *Rivularia*-like characteristics under laboratory conditions. All the sequences of environmental *Rivularia* colonies and rivulariacean-type isolates analysed fell into one of three distinct genotypes, denominated types I, II and III. On the basis of 16S rRNA gene and PC genetic analysis, these three genotypes were polyphyletic and, according to the genetic data, may actually represent three different genera. On the other hand, morphological and fingerprinting analysis showed that trichomes within single *Rivularia* colonies could be phenotypically and genetically different, which makes the taxonomic assessment of *Rivularia* even more difficult. Representatives of the genera *Rivularia* and *Calothrix* in our study and in GenBank/ EMBL/DDBJ were intermixed in phylogenetic inferences and showed great genetic divergence. This supports the hypothesis that the traditional family of Rivulariaceae is one of the most diverse cyanobacterial lineages, with the implication that a revision of their taxonomy is needed.

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