

## Phylogenetic and molecular clock inferences of cyanobacterial strains within *Rivulariaceae* from distant environments

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### Keywords

cyanobacteria; *Nostocales*; *Rivulariaceae*; molecular clock; phylogeny.

### Introduction

Cyanobacteria have evolved to become one of the most diverse groups of bacteria (Waterbury, 1991; Whitton & Potts, 2000; Castenholz, 2001). They contribute significantly to global primary production via photosynthesis and some contribute considerably to the nitrogen cycle via dinitrogen (N<sub>2</sub>) fixation. Genome-scale analyses suggest that oxygenic photosynthesis evolved early in the cyanobacterial radiation (Swingley *et al.*, 2008). The capacity to use water as an electron donor in oxygenic photosynthesis, with its consequent generation of molecular oxygen, most likely appeared by 2700 million years ago (MYA) or earlier (Falcón *et al.*, 2010).

Nitrogen fixation is restricted to Bacteria and Archaea, and is present throughout the cyanobacteria (albeit not in all species), that are among the ecologically most important

### Abstract

Heterocyst-forming cyanobacteria are important players at both evolutionary and ecological scales, but to date it has been difficult to establish their phylogenetic affiliations. We present data from a phylogenetic and molecular clock analysis of heterocystous cyanobacteria within the family *Rivulariaceae*, including the genera *Calothrix*, *Rivularia*, *Gloeotrichia* and *Tolypothrix*. The strains were isolated from distant geographic regions including fresh and brackish water bodies, microbial mats from beach rock, microbialites, pebble beaches, plus PCC strains 7103 and 7504. Phylogenetic inferences (distance, likelihood and Bayesian) suggested the monophyly of genera *Calothrix* and *Rivularia*. Molecular clock estimates indicate that *Calothrix* and *Rivularia* originated ~1500 million years ago (MYA) ago and species date back to 400–300 MYA while *Tolypothrix* and *Gloeotrichia* are younger genera (600–400 MYA).

nitrogen fixers (Capone *et al.*, 1997; Raymond *et al.*, 2004). In contrast to photosynthesis, the capacity to fix nitrogen is a paraphyletic event within the cyanobacterial radiation (Swingley *et al.*, 2008). The ‘patchy’ distribution of nitrogen fixation in cyanobacteria has been inferred to be a result of lateral gene transfer and/or gene duplication (Swingley *et al.*, 2008). The origin of nitrogen fixation among cyanobacteria is dated at 3000–2500 MYA (Shi & Falkowski, 2008; Falcón *et al.*, 2010), and probably appeared three times independently (Swingley *et al.*, 2008).

Taxonomic classification has divided Cyanobacteria in five subsections/groups: (1) Order *Chroococcales* includes unicellular cells with binary reproduction; (2) Order *Pleurocapsales* includes unicellular cells with reproduction by multiple bipartition; (3) Order *Oscillatoriales* includes filamentous colonies without heterocysts and cell division in one plane; (4) Order *Nostocales* includes filamentous

colonies that divide in one plane and include heterocysts; (5) Order *Stigonematales* includes filamentous colonies with heterocysts that divide in more than one plane (Rippka *et al.*, 1979; Waterbury, 1991; Castenholz, 2001). Nitrogen-fixing cyanobacteria separate their oxygenic photosynthesis and oxygen-intolerant nitrogenase activity via temporal and spatial strategies (Bergman *et al.*, 1997; Sandh *et al.*, 2009; Berman-Frank *et al.*, 2001). Heterocystous cyanobacteria including *Nostocales* and *Stigonematales* (true branching) separate CO<sub>2</sub> and N<sub>2</sub> fixation spatially. Heterocysts are terminal, intercalary or both, differentiated cells specialized for nitrogen fixation, which lack the oxygen-producing photosystem II and have thick cell walls that are less permeable to gases, efficiently protecting the oxygen-sensitive nitrogenase and allowing nitrogen fixation to occur during the daytime (Haselkorn, 2007).

Morphological and molecular-based classifications verify that heterocyst-forming cyanobacteria constitute a monophyletic group (Honda *et al.*, 1998; Tomitani *et al.*, 2006; Gupta & Mathews, 2010). Cyanobacterial orders that form heterocysts are usually intermingled in terms of their genealogies, and it has been difficult to precisely establish their phylogenetic affiliations (Rajaniemi *et al.*, 2005; Sihvonen *et al.*, 2007; Berrendero *et al.*, 2008).

Tomitani *et al.* (2006) suggested, based on genetic distances and fossil calibrations, that heterocyst-forming cyanobacteria arose within the age range of 2450–2100 MYA. Later, molecular clock dating confirmed the age of the appearance of heterocystous cyanobacteria to 2211–2057 MYA (Falcón *et al.*, 2010). These time frames coincide with the Great Oxidation Event (~2450 MYA), the time period when free oxygen starts to be traced in the fossil record (Holland, 2002).

Although heterocyst-forming cyanobacteria are important players at an evolutionary and an ecological scale, our knowledge is also scant with regard to their natural history and phylogenetic affiliations. Attempts have been made to unravel life history patterns of certain heterocystous cyanobacteria, including those pertaining to the multigenera Order *Nostocales* (*Anabaena*, *Aphanizomenon*, *Aulosira*, *Trichormus*, *Nostoc*, *Nodularia*, *Mojavia*, *Calothrix*, *Gloeotrichia*, *Tolypothrix*, *Rivularia*, *Sacconema*, *Isactis*, *Dichothrix*, *Gardnerula*, *Microchaete*, *Cylindrospermopsis* and *Raphidiopsis*) (Lehtimäki *et al.*, 2000; Castenholz, 2001; Henson *et al.*, 2004; Lyra *et al.*, 2005; Rajaniemi *et al.*, 2005; Sihvonen *et al.*, 2007; Berrendero *et al.*, 2008; Lukesová *et al.*, 2009; Stucken *et al.*, 2010; Thomazeau *et al.*, 2010). Nevertheless, sequences available for the *Rivulariaceae* 16S rDNA gene are restricted to the four genera *Rivularia*, *Calothrix*, *Gloeotrichia*, and *Tolypothrix* (Narayan *et al.*, 2006; Tomitani *et al.*, 2006; Sihvonen *et al.*, 2007; Berrendero *et al.*, 2008), which has hindered the advancement of our knowledge with regard to their evolutionary relationships.

The aim of this study was to advance our knowledge on the phylogenetic affiliations of heterocyst-forming cyanobacteria within the *Rivulariaceae* (order *Nostocales*), specifically including representatives of the genera *Calothrix*, *Rivularia*, *Gloeotrichia* and *Tolypothrix* collected from different environments. These genera form nitrogen-fixing, globally widespread benthic communities, primarily in aquatic but also in terrestrial ecosystems, and have potential ecological significance. Recently, the importance of *Calothrix rhizosoleniae* has been acknowledged as open ocean symbionts in a variety of diatoms (Foster *et al.*, 2010). Nevertheless, to date no estimate of the overall influence in the C and N cycles of the genera within *Rivulariaceae* has been attempted and questions remain open regarding their phylogenetic organization.

## Materials and methods

Strains examined in this study were isolated from natural populations such as microbial mats, microbialites and rocky shore biofilms, summarized in Table 1. Unicyanobacterial cultures were obtained from enrichment cultures, and individual tapering filaments with heterocysts were picked using light microscopy (Axioscope 40, Carl Zeiss, Germany). Individual cultures were grown in 50- or 100-mL flasks in an incubation chamber at an average temperature of 29 °C, 14/10 light/dark cycles (Pozas Azules), 18 °C, 12/12 light/dark cycles (Askö) and 28 °C, 12/12 light/dark cycles (Heron Island). All cultures were grown in 50–100 µE m<sup>-2</sup> s<sup>-1</sup>. Cultures were transferred to new media lacking reduced forms of nitrogen every 3 weeks.

## Nucleic acid extraction

DNA was extracted from individual cultures (approximately 500 µL) that were incubated overnight at 50 °C with 10 × extraction buffer (20 mM Tris-HCl, pH 7.5–8.2, 50 mM EDTA, 20 mM NaCl) and proteinase K (final concentration 0.25 mg mL<sup>-1</sup>). Proteins and lipids were separated with two

**Table 1.** Isolated cyanobacterial strains used in this study indicating geographical origin, growth medium and culture collection

Strains	Medium	Culture collection
Baltic Sea (Askö, Sweden): 3, 14, 16, 12	Z8	Department of Botany, SU (Sweden)
Great Barrier Reef (Heron Island): 14, 15	SN-	Department of Botany, SU (Sweden)
Pozas Azules I (Mexico): 1PA1, 1PA3, 1PA4, 1PA5, 1PA9, 1PA10, 1PA12, 1PA17, 1PA18, 1PA19, 1PA20, 1PA21, 1PA22, 1PA23, 5PA13, 5PA11, 7PA3, 7PA4, 7PA6, 7PA9, 7PA14	SN-	Institute of Ecology, UNAM (Mexico)

SU, Stockholm University; UNAM, Universidad Nacional Autónoma de México.

phenol and one chloroform extraction and DNA was precipitated with sodium acetate (3 M) and absolute ethanol, followed by a 45-min incubation at  $-20^{\circ}\text{C}$ . DNA pellets were stained with GlycoBlue<sup>TM</sup> (Ambion, Austin, TX) and resuspended in water.

### 16S rRNA gene amplification and analysis

A fragment consisting of almost the complete 16S rRNA gene, the intergenic transcribed spacers and part of the 23S rRNA gene was amplified from all strains using universal primer 27F (5'AGA GTT AGA GTT TGA TCM TGG CTC AG 3') (Lane, 1991) and cyanobacteria-specific B23S (5'CT T CGC CTC TGT GTG CCT AGG T 3') (Gkelis *et al.*, 2005). The amplification reaction had a final volume of 50  $\mu\text{L}$  with  $1 \times$  reaction buffer, 2.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTPs, 0.6  $\mu\text{M}$  of each primer and 5 U Taq DNA polymerase. The thermal cycle included an initial denaturalization at  $94^{\circ}\text{C}$  for 2 min, followed by 25 cycles of  $94^{\circ}\text{C}$  for 45 s;  $54^{\circ}\text{C}$  for 45 s;  $68^{\circ}\text{C}$  for 2 min and a final extension of 30 min at  $68^{\circ}\text{C}$ . The PCR products obtained (approximately 1800 bp) were gel-extracted (Qiagen, Austin, TX) and sequenced. Sequences were obtained on a capillary sequencer (Applied Biosystems Avant-100) with five reactions including primers 27F, 1492R (5'TAC GGY TAC CTT GTT ACG ACT T 3') (Lane, 1991) and B23S (Gkelis *et al.*, 2005).

Sequences were assembled and aligned with SEQUENCHER 3.1.1 (Gene Codes Corporation, Ann Arbor, MI), and identified with the Greengenes dataset (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>) with BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST). The 27 sequences obtained from the different strains analyzed in this study (plus sequences for PCC 7103 and PCC 7504) were aligned to 56 cyanobacterial sequences from Greengenes with a final consensus of 1290 bp (Table 2). The alignment was verified with MACCLADE 4.033 PCC software (Sinauer Associates Inc., Sunderland, MA) and phylogenetic analysis were run with PAUP\* 4.0b10 (Swofford, 2002). Maximum-likelihood (ML) reconstruction considered the Akaike Information Criterion as a model of nucleotidic evolution after a MODEL TEST analysis (Posada & Crandall, 1998). The model with the best fit was GTR+I+G, where  $I=0.3894$  (proportion of invariable sites) and  $G=0.5246$  (gamma distribution). Topologies were also inferred with neighbor-joining (NJ) (Kimura 2 Parameters) and maximum parsimony (MP). Bootstrap considered 500 (ML, NJ) and 1000 (MP) replicates, respectively. *Crocospaera watsonii*, a unicellular nitrogen-fixing cyanobacteria, was included as the outgroup.

### Maximum a posteriori (MAP) topology

Molecular clock estimates were inferred from a MAP topology calculated from a Bayesian phylogenetic analysis with MRBAYES v3.1.2 (Huelsenbeck & Ronquist 2001) using

the model with best fit to the data set. Bayesian analysis consisted of two independent Markov Chain Monte Carlo runs, performed by four differentially heated chains of  $5 \times 10^6$  generations. Phylograms with a topology identical to the MAP topology were recovered with PAUP\* 4.0b10 and 100 were chosen to conduct age estimates. The timing of phylogenetic divergence was calculated with r8s v1.71 (Sanderson, 2006) with penalized likelihood (Sanderson, 2002). The node defining Cyanobacteria was fixed at 2700 MYA and a minimum age for the heterocystous cyanobacteria was defined at 1618 MYA (Falcón *et al.*, 2010). The outgroup was *Chloroflexus aurantiacus*, a green nonsulfur bacterium.

Sequences generated in this study are deposited in the NCBI database with accession numbers: FJ660972–FJ661026. Sequences FJ660972–FJ660992 correspond to isolates from microbialites in Pozas Azules I, a desert pond in Cuatro Ciénegas, México; FJ660993 and FJ660994 are from a microbial mat on a beach rock in Heron Island at the Great Barrier Reef, Australia; FJ660995–FJ661005 and FJ66101–FJ661021 are from separate isolates obtained from type cultures of *Tolypothrix* sp. PCC 7504 and *Calothrix* sp. PCC 7103 maintained in culture at the Department of Botany at Stockholm University, Sweden; and FJ661006–FJ661009 correspond to isolates from the shore line of a rocky islet outside the Stockholm University Marine Research Station at Askö in the Baltic Sea, Sweden.

## Results and discussion

### Phylogenetic ordination of *Calothrix*, *Tolypothrix* and *Rivularia*

Phylogenetic differentiation was well sustained, suggesting three natural groups pertaining to *Calothrix* from Askö (Sweden), also including the strain PCC 7103, *Rivularia* from strains in Pozas Azules I (Mexico) and *Tolypothrix* including the strain PCC 7504 (Fig. 1). These genera were earlier defined based on molecular identities (Rajaniemi *et al.*, 2005; Taton *et al.*, 2006; Sihvonen *et al.*, 2007).

Topologies of inferred genealogies with NJ (Kimura 2-parameters), ML and MP were congruent, and bootstrap values for NJ and MP are shown in the ML topology (Fig. 1). Sequences of nonheterocyst-forming unicellular and filamentous cyanobacteria of groups I, II and III were used as outgroups.

The 16S rRNA genealogy revealed four clades. Clade I was formed by the unicellular genera *Synechococcus*, *Prochlorococcus* and the filamentous genus *Phormidium*; clade II contained all cyanobacterial sequences originating from Pozas Azules, a desert pond in northern Mexico, plus three sequences assigned to *Rivularia* from the Baltic Sea

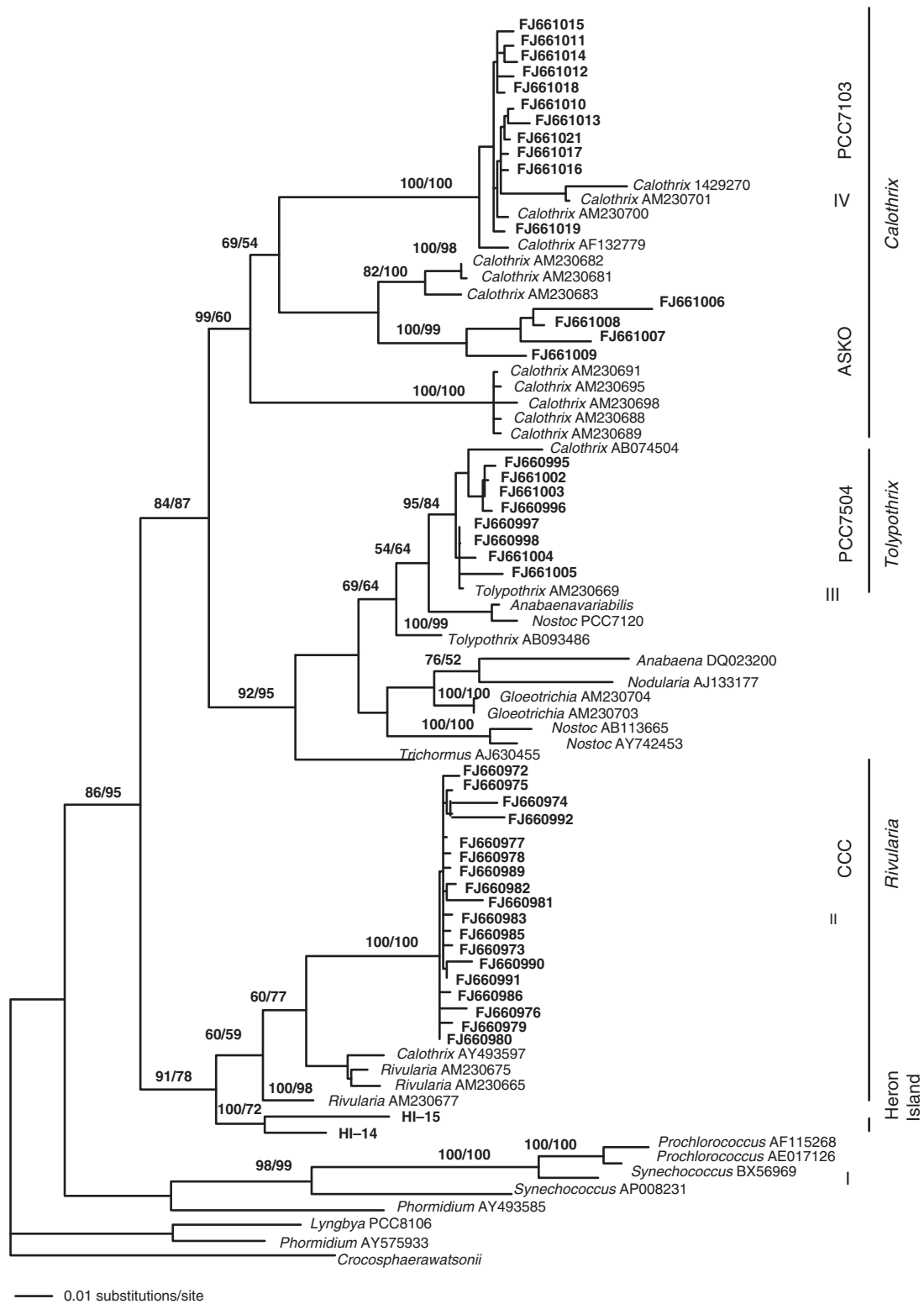
**Table 2.** Cyanobacterial strains included in this study (*Nostocales*), indicating code, affiliation, geographic origin, habitat and 16S rDNA gene sequence accession numbers (GenBank)

Strain code	Phylogenetic affiliation	Geographical origin	Habitat	Reference	Accession number
<b>PCC 7103</b>	<b><i>Calothrix</i> sp.</b>	Unknown	Unknown	Rippka <i>et al.</i> (2001)	AM230700
MU27 UAM 315	<i>Calothrix</i> sp.	Muga River, Spain	Epilithic Freshwater	Berrendero <i>et al.</i> (2008)	EU009152.1
TJ12 UAM 371	<i>Calothrix</i> sp.	Tejada Streaan, Spain	Epilithic Freshwater	Berrendero <i>et al.</i> (2008)	EU009154.1
<b>PCC7102</b>	<b><i>Calothrix desertica</i></b>	Antofagasta, Chile	Sand	Rippka & Herdman (1992)	AF132779
MU24 UAM305	<i>Calothrix</i> sp. (proposed in this study)	Muga River, Spain	Epilithic Freshwater	Berrendero <i>et al.</i> (2008)	EU009149.1
BECID6	<i>Calothrix</i> sp.	Baltic Sea, Finland	Periphytic Brackish	Sihvonen <i>et al.</i> (2007)	AM230691
XP11C	<i>Calothrix</i> sp.	Baltic Sea, Finland	Sediment Brackish	Sihvonen <i>et al.</i> (2007)	AM230698
BECID26	<i>Calothrix</i> sp.	Baltic Sea, Finland	Epilithic	Sihvonen <i>et al.</i> (2007)	AM230695
PCC 8909	<i>Calothrix</i> sp.	Baltic Sea	Brackish Unknown	Sihvonen <i>et al.</i> (2007)	AM230693
XP2B	<i>Calothrix</i> sp.	Baltic Sea, Finland	Brackish Periphytic	Sihvonen <i>et al.</i> (2007)	AM230689
BECID9	<i>Calothrix</i> sp.	Baltic Sea, Finland	Epilithic Brackish	Sihvonen <i>et al.</i> (2007)	AM230688
Askö 3	<i>Calothrix</i> sp.	Baltic Sea, Sweden	Epilithic Brackish	This study	FJ661007
Askö 14	<i>Calothrix</i> sp.	Baltic Sea, Sweden	Epilithic Brackish	This study	FJ661006
Askö 16	<i>Calothrix</i> sp.	Baltic Sea, Sweden	Epilithic Brackish	This study	FJ661009
Askö 12	<i>Calothrix</i> sp.	Baltic Sea, Sweden	Epilithic Brackish	This study	FJ661008
UKK3412	<i>Calothrix</i> sp.	Baltic Sea, Finland	Unknown Brackish	Sihvonen <i>et al.</i> (2007)	AM230681
BECID16	<i>Calothrix</i> sp.	Baltic Sea, Finland	Epilithic Brackish	Sihvonen <i>et al.</i> (2007)	AM230682
BECID33	<i>Calothrix</i> sp.	Baltic Sea, Finland	Epilithic Brackish	Sihvonen <i>et al.</i> (2007)	AM230683
IAM M-249 Mk1-C1	<i>Calothrix brevissima</i> IAM M-249 <i>Calothrix</i> sp. Mk1-C1	Palau island, Palau Mikurajima island, Japan	Soil Liquen	Ichimura & Itoh (1977) Miura & Yokota (2006)	AB074504 AB275345.1
<b>PCC 7504</b>	<b><i>Tolypothrix</i> sp.</b>	Sweden	Freshwater aquarium	Rippka <i>et al.</i> (2001)	AM230706
<b>PCC 7415</b>	<b><i>Tolypothrix</i> sp.</b>	Sweden	Green house soil	Sihvonen <i>et al.</i> (2007)	AM230668
IAM M-259 240292	<i>Tolypothrix</i> sp. IAM M-259 <i>Anabaena variabilis</i> ATCC 29413	Unknown Lake Tankanyika, Africa	Unknown Plankton	Seo & Yokota (2003) West, 1907	AB093486 AY584512.1
BC Ana 0025	<i>Anabaena solitaria</i> sp. BC Ana 0025	Cotswold Water Park, UK	Plankton	N. A. El Semary & P. K. Hayes (unpublished data)	DQ023200
<b>PCC 7120</b>	<b><i>Nostoc</i> sp. PCC 7120</b>	Unknown	Unknown	Rippka <i>et al.</i> (2001)	AF317631.1
PYH14	<i>Gloeotrichia echinulata</i> PYH14	Lake Pyhäjärvi, Säskylä, Finland	Freshwater	Sihvonen <i>et al.</i> (2007)	AM230704
PYH6	<i>Gloeotrichia echinulata</i> PYH6	Lake Pyhäjärvi, Säskylä, Finland	Freshwater	Sihvonen <i>et al.</i> (2007)	AM230703
URA3	<i>Gloeotrichia echinulata</i> URA3	Lake Pyhäjärvi, Säskylä, Finland	Freshwater	Sihvonen <i>et al.</i> (2007)	AM230705
BY1	<i>Nodularia baltica</i>	Baltic Sea	Plankton	Lehtimäki <i>et al.</i> (2000)	AJ133177
Huebel 1987/311	<i>Nodularia spumigena</i>	Baltic Sea	Plankton	Lyra <i>et al.</i> (2005)	AJ781133
<i>T. doliolum</i> 1	<i>Trichormus doliolum</i> str. <i>doliolum</i> 1	Unknown	Unknown	Rajaniemi <i>et al.</i> (2005)	AJ630455
7PA4	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660989
7PA14	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660992

**Table 2.** Continued.

Strain code	Phylogenetic affiliation	Geographical origin	Habitat	Reference	Accession number
1PA5	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660982
1PA3	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660973
1PA17	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660974
1PA20	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660977
1PA1	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660972
1PA9	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660983
1PA4	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660981
1PA21	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660978
1PA12	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660985
1PA10	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660984
7PA3	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660988
7PA9	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660991
7PA6	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660990
1PA19	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660976
1PA23	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660980
1PA22	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660979
5PA13	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660986
5PA11	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660987
1PA18	<i>Rivularia</i> sp.	Pozas Azules I, Mexico	Microbialite Freshwater	This study	FJ660975
ERIVALH2	<i>Rivularia</i> sp. uncultured	Alharabe river, Spain	Epilithic Freshwater	Berrendero <i>et al.</i> (2008)	EU009142
BIR KRIV1	<i>Rivularia atra</i>	Baltic Sea, Finland	Periphytic Brackish	Sihvonen <i>et al.</i> (2007)	AM230674
XP16B	<i>Rivularia</i> sp. XP16B	Baltic Sea, Finland	Epilithic Brackish	Sihvonen <i>et al.</i> (2007)	AM230676
BIR MGR1	<i>Rivularia</i> sp. BIR MGR1	Baltic Sea, Finland	Epilithic Brackish	Sihvonen <i>et al.</i> (2007)	AM230675
XP27A	<i>Rivularia</i> sp. XP27A	Baltic Sea, Finland	Epilithic Brackish	Sihvonen <i>et al.</i> (2007)	AM230667
<b>PCC 7116</b>	<b><i>Rivularia</i> sp.</b>	Baja California, Mexico	Epilithic Marine	Rippka <i>et al.</i> (2001)	AM230677
HI15	Unknown	Heron island, Australia	Microbial mat Marine	This study	FJ660993
HI14	Unknown	Heron island, Australia	Microbial mat Marine	This study	FJ660994

Strains given in bold represent strains maintained in the Pasteur Culture Collection (PCC).



**Fig. 1.** Phylogram (1290 bp) of cyanobacterial gene fragments generated with ML, including part of the 16S rRNA gene, intergenic transcribed spacers and the 23S rRNA gene. Strains given in bold represent those isolates for which new sequences were obtained in the course of the present study. Bootstrap values for 1000 replicates are shown at branch nodes (NJ/MP). Vertical labels indicate geographic sites for analyzed isolates.

(AM230665, AM230675), Baja, Mexico (AM230677) and one sequence (AY493597) assigned to *Calothrix* from Antarctica, which we propose belongs to the genus *Rivularia*. Clade III grouped the sequences of *Tolypothrix* PCC 7504 originating from the Baltic Sea, *Tolypothrix* AB093486, *Calothrix* AB074504, from Palau island, which we propose to be a *Tolypothrix*, *Anabaena variabilis* and *Nostoc* PCC 7120. Clade IV was a *Calothrix* clade, and included all sequences from the Baltic Sea and the strain PCC 7103. The cyanobacterial sequences from Heron Island (Australia) grouped more closely to *Rivularia*, although they showed enough genetic distance to be considered as a separate clade.

Recent molecular-based analysis has attempted to disentangle the evolutionary relationships between *Calothrix* and closely related genera (Hongmei et al., 2005; Sihvonen et al., 2007; Berrendero et al., 2008). Using a region of the 16S rRNA gene, strains morphologically identified as *Calothrix* were found to be representatives of *Gloeotrichia* and *Tolypothrix* (Sihvonen et al., 2007). Further, the work of Berrendero et al. (2008) suggest a phylogenetic analysis that strains from calcareous rivers and streams attributed based on morphological traits to *Calothrix* actually pertain to *Rivularia*, a genus that has been proposed to be extremely abundant in calcareous freshwater habitats (Pentecost & Whitton, 2000). Nevertheless, due to differences between morphologic and phylogenetic classifications, Sihvonen et al. (2007) and Berrendero et al. (2008) supported the idea that the genus *Calothrix* is polyphyletic and suggested that it should be divided into different genera. Berrendero et al. (2008) also suggested that *Rivularia* is not monophyletic.

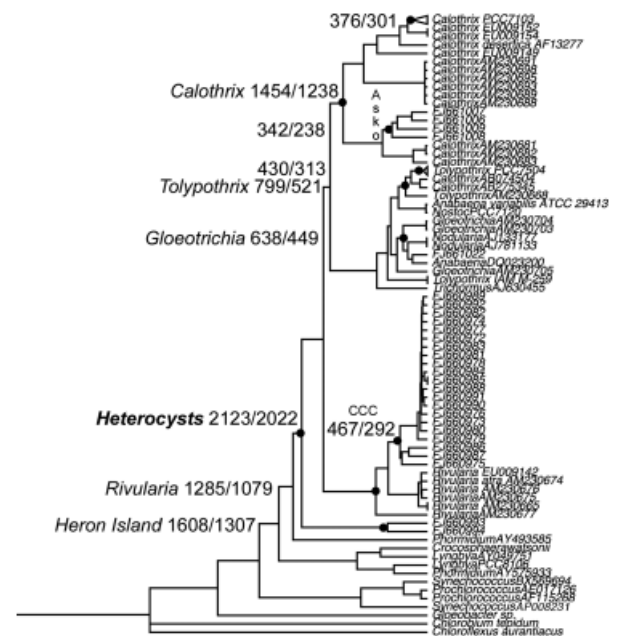
In contrast to the above, our Bayesian phylogenetic inference analyses showed a robust separation of *Calothrix* and *Rivularia*, suggesting that they represent monophyletic genera (Figs 1 and 2). The sequences obtained in the present study for the strains *Calothrix* PCC 7103 and *Tolypothrix* PCC 7504 were found to be heterogenous (Fig. 1), and are clearly monophyletic, showing the interspecific divergence of these strains. It is also clear from our data that *Tolypothrix* and *Gloeotrichia* constitute phylogenetic groups with imprecise demarcations according to existing sequences in public databases. Both of these clades were intermingled with various genera in the MAP topology (Fig. 2).

It has been stated that approximately 50% of deposited strains in major cyanobacterial collections are misidentified (Komárek & Anagnostidis, 1989), causing confusion in the literature. Here we propose based on MAP, NJ, MP and ML topologies that *Calothrix* AB074504 pertains to *Tolypothrix* and that sequence EU009149 pertains to *Calothrix*. We also conclude, like Stucken et al. (2010), that morphologic characteristics do not suffice for detailed classification of filamentous, heterocystous cyanobacteria, whereas robust phylogenetic analysis can clarify phylogenetic affiliations.

## Ancestry of *Rivulariaceae* genera and species

Molecular clock estimates of the 27 strains of *Rivulariaceae* examined here revealed interesting features. The heterocystous clade dated at  $2061 \pm 38$  MYA, which coincides with recent molecular clock estimates of the origin for this group (Falcón et al., 2010), as well as with previous estimates based on genetic distance and fossil calibrations (Tomitani et al., 2006). The monophyly of the heterocyst-forming cyanobacteria is reflected in this and other studies based on 16S rRNA gene sequences as well as with other phylogenetically informative regions (*nifH* and *hetR*) (Honda et al., 1998; Marquardt & Palinska, 2006; Tomitani et al., 2006).

The robust MAP topology was used to date times of separation between genera and species within the *Rivulariaceae* strains included in our study (Fig. 2). The molecular clock estimated that dates for the appearance of both genera *Calothrix* ( $1346 \pm 108$  MYA) and *Rivularia* ( $1132 \pm 53$  MYA) fell within the same time span. The time of appearance of the strains *Calothrix* PCC 7103 ( $338 \pm 37$  MYA), *Tolypothrix* PCC 7504 ( $372 \pm 58$  MYA) and *Rivularia* spp. from Pozas Azules I in México ( $380 \pm 88$  MYA) and *Calothrix* from Askö in the Baltic Sea in Sweden ( $290 \pm 52$  MYA) also coincided. In contrast, the clade representing the strains from the subtropical Great Barrier Reef (Heron Island) appeared about the same time as the genera *Calothrix* and *Rivularia* ( $1458 \pm 151$  MYA), and together with the genetic



**Fig. 2.** Bayesian phylogenetic analysis showing the MAP between the three heterocystous genera *Rivularia*, *Tolypothrix* and *Calothrix*. Divergence time estimate intervals for the appearance of each clade are shown in parentheses. The tree was calibrated fixing the cyanobacterial clade at 2700 MYA.

distance that separates this clade from the others, suggests they may constitute one genus. The molecular clock-estimated dates for the appearance of *Tolypothrix* ( $610 \pm 89$  MYA) and *Gloeotrichia* ( $494 \pm 46$  MYA) suggest that these genera are much younger than *Calothrix*, *Rivularia* and the strains from Heron Island (Australia). The above is the first suggestion that not all the genera of cyanobacteria may have appeared during a single evolutionary explosion.

Schopf (1994) proposed, based on similarities between fossils and extant groups of cyanobacteria, that they are evolving at exceptionally slow rates (hypobradyletic). In fact it seems that cyanobacteria have not shown any apparent morphological changes over hundreds, or even thousands of millions of years. The hypobradyletic mode of evolution may have been characteristic of the Precambrian history of life. Our study is the first attempt to make a time estimate for genera and strains within cyanobacteria. The lapses of time presented here agree with the hypobradyletic hypothesis, indicating extremely large time points for the evolution and separation of genera ( $\sim 1400$  MYA) and strains ( $\sim 300$  MYA). Schopf (1994) suggests that this slow mode of evolution is in accordance with what Simpson defined in his study 'Tempo and Mode in Evolution' (1944). Hypobradyletely would apply to species with a large population size, ecologic versatility and a large degree of adaptation to an ecological position and continuously available environment. Cyanobacteria fit this definition, being a remarkable lineage considering their longevity, ease of dispersal (resulting in a wide cosmopolitan distribution), as seen in low-temperature ecotypes (Jungblut *et al.*, 2010), and their ability to survive wide abiotic ranges, including intense desiccation and radiation. Also, analysis of cyanobacterial populations from hot springs and geothermal environments following a molecular ecology approach has shown that geographic isolation can play an important role in shaping phylogenies and distribution patterns in certain environments (Papke *et al.*, 2003). The need to generate additional information aimed at unraveling the evolutionary relationships within Cyanobacteria is evident. To date, approximately 50 sequenced cyanobacterial genomes (complete or in progress) are available. However, 41 represent members of the unicellular subsection/group I, with the vast majority being representatives of only two genera: *Prochlorococcus* and *Synechococcus*. Only eight genomes of the genus-rich group IV heterocystous cyanobacteria have been sequenced despite their obvious evolutionary and ecological importance, and deeper phylogenetic inferences are needed to clear relationships within this group.

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## References

- Bergman B, Gallon JR, Rai AN & Stal LJ (1997) Nitrogen fixation by non-heterocystous cyanobacteria. *FEMS Microbiol Rev* **19**: 139–185.
- Berman-Frank I, Lundgren P, Chen Y-B, Küpper H, Kolber Z, Bergman B & Falkowski P (2001) Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. *Science* **294**: 1435–1537.
- Berrendero E, Perona E & Mateo P (2008) Genetic and morphological characterization of *Rivularia* and *Calothrix* (Nostocales, Cyanobacteria) from running water. *IJSEM* **58**: 447–460.
- Capone DG, Zehr J, Paerl H, Bergman B & Carpenter EJ (1997) *Trichodesmium*: a globally significant marine cyanobacterium. *Science* **276**: 1221–1229.
- Castenholz RW (2001) Cyanobacteria *et al.* *Bergey's Manual of Systematic Bacteriology. The Archaea and the Deeply Branching Phototrophic Bacteria, Vol. 1*, 2nd edn (Boone DR, Castenholz RW & Garrity GM, eds), pp. 473–597. Springer-Verlag, New York.
- Falcón LI, Magallón S & Castillo A (2010) Dating the cyanobacterial ancestor of the chloroplast. *ISME J* **4**: 777–783.
- Foster RA, Goebel N & Zehr JP (2010) Isolation of *Calothrix rhizosoleniae* (Cyanobacteria) strain SC01 from Chaetoceros (Bacillariophyta) spp. diatoms of the subtropical North Pacific ocean. *J Phycol* **46**: 1028–1037.
- Gkelis S, Rajaniemi P, Vardaka E, Moustaka-Gouni M, Lanaras T & Sivonen K (2005) *Limnothrix redekei* (Van Goor) Meffert (Cyanobacteria) strains from Lake Kastoria, Greece form a separate phylogenetic group. *Microb Ecol* **49**: 176–182.
- Gupta RS & Mathews DW (2010) Signature proteins of the major clades of cyanobacteria. *BMC Evol Biol* **10**: 24.
- Haselkorn R (2007) Heterocyst differentiation and nitrogen fixation in cyanobacteria. *Associative and Endophytic Nitrogen-Fixing Bacteria and Cyanobacterial Associations* (Elmerich C & Newton WE, eds), pp. 233–255. Springer, The Netherlands.
- Henson BJ, Hesselbrock SM, Watson LE & Barnum SR (2004) Molecular phylogeny of the heterocystous cyanobacteria (subsections IV and V) based on *nifD*. *IJSEM* **54**: 493–497.
- Holland HD (2002) Volcanic gases, black smokers, and the great oxidation event. *Geochim Cosmochim Acta* **66**: 3811–3826.
- Honda D, Yokota A & Sugiyama J (1998) Detection of seven major evolutionary lineages in cyanobacteria based on the 16S rRNA gene sequence analysis with new sequence of five marine *Synechococcus* strains. *J Mol Evol* **48**: 723–739.
- Hongmei J, Aitchison JC, Lacap DC, Peerapornpisal Y, Sompong U & Pointing SB (2005) Community phylogenetic analysis of



- moderately thermophilic cyanobacterial mats from China, the Philippines and Thailand. *Extremophiles* **9**: 325–332.
- Huelsenbeck JP & Ronquist FR (2001) MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.
- Ichimura T & Itoh T (1977) The complete 16S rDNA sequence similarities among *Microcystis* species including *M. aeruginosa*, *M. viridis*. *Preservation Methods of Microorganisms* (Nei T, ed), pp. 355–373. University of Tokyo Press, Tokyo.
- Jungblut AD, Lovejoy C & Vincent WF (2010) Global distribution of cyanobacterial ecotypes in the cold biosphere. *ISME J* **4**: 191–202.
- Komárek J & Anagnostidis K (1989) Modern approach to the classification system of Cyanophytes 4-Nostocales. *Arch Hydrobiol Suppl* **82**: 247–345.
- Lane DJ (1991) 16S/23S rDNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrand E & Goodfellow M, eds), pp. 115–175. John Wiley & Sons Ltd, London.
- Lehtimäki J, Lyra C, Suomalainen S, Sundman P, Rouhiainen L, Paulin L, Salkinoja-Salonen M & Sivonen K (2000) Characterization of nodularia strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. *IJSEM* **50**: 1043–1053.
- Lukesová A, Johansen JR, Martin MP & Casamata DA (2009) *Aulosira bohemensis* sp. nov.: further phylogenetic uncertainty at the base of the Nostocales (Cyanobacteria). *Phycology* **48**: 119–129.
- Lyra C, Laamanen M, Lehtimäki JM, Surakka A & Sivonen K (2002) Benthic cyanobacteria of the genus *Nodularia* are non-toxic, without gas vacuoles, able to glide and genetically more diverse than planktonic *Nodularia*. *IJSEM* **55**: 555–568.
- Marquardt J & Palinska KA (2006) Genotypic and phenotypic diversity of cyanobacteria assigned to the genus *Phormidium* (Oscillatoriales) from different habitats and geographical sites. *Arch Microbiol* **187**: 397–413.
- Miura S & Yokota A (2006) Isolation and characterization of cyanobacteria from lichen. *J Gen Appl Microbiol* **52**: 365–374.
- Narayan KP, Tiwari S, Pabbi S & Wattal-Dhar D (2006) Biodiversity analysis of selected cyanobacteria. *Curr Sci* **91**: 947–951.
- Papke RT, Ramsing NB, Bateson MM & Ward DM (2003) Geographical isolation in hot spring cyanobacteria. *Environ Microbiol* **5**: 650–659.
- Pentecost A & Whitton B (2000) Cyanobacteria and limestone. *The Ecology of Cyanobacteria. Their Diversity in Time and Space* (Whitton BA & Potts M, eds), pp. 257–279. Kluwer Academic Press, Amsterdam.
- Posada D & Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.
- Rajaniemi P, Hrouzek P, Kačotvská K, Willame R, Rantala A, Hoffman L, Komárek J & Sivonen K (2005) Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *IJSEM* **55**: 11–26.
- Raymond J, Siefert JL, Staples CR & Blankenship RE (2004) The natural history of nitrogen fixation. *Mol Biol Evol* **21**: 541–554.
- Rippka R & Herdman H (1992) *Pasteur Culture Collection of Cyanobacteria Catalogue & Taxonomic Handbook*. 1. Catalogue of Strains. Institut Pasteur, Paris.
- Rippka R, Deruelles J, Waterbury JB, Herdman M & Stanier RY (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microb* **111**: 1–61.
- Rippka R, Waterbury JB, Herdman M & Castenholz RW (2001) The Cyanobacteria: Subsection 2 (Formerly Pleurocapsales Geitler 1925, emend. Waterbury and Stanier 1978). *Bergey's Manual of Systematic Bacteriology* (Boone DR & Castenholz RW, eds), pp. 514–539. Springer, New York.
- Sanderson MJ (2006) r8s (ver. 1.70.) University of California Davis, Davis, CA.
- Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Mol Biol Evol* **19**: 101–109.
- Sandh G, El-Shehawry R, Díez B & Bergman B (2009) Temporal separation of cell division and diazotrophy in the marine diazotrophic cyanobacterium *Trichodesmium erythraeum* IMS101. *FEMS Microbiol Lett* **295**: 281–288.
- Schopf JW (1994) Disparate rates, differing rates: tempo and mode of evolution changed from the Precambrian to the Phanerozoic. *P Natl Acad Sci USA* **91**: 6735–6742.
- Seo P-S & Yokota A (2003) The phylogenetic relationships of cyanobacteria inferred from 16S rRNA, *gyrB*, *rpoC1* and *rpoD1* gene sequences. *J Gen Appl Microbiol* **49**: 191–203.
- Shi T & Falkowski PG (2008) Genome evolution in cyanobacteria: the stable core and the variable shell. *P Natl Acad Sci USA* **105**: 2510–2515.
- Sihvonen LM, Lyra C, Fewer P, Rajaniemi-Wacklin P, Lehtimäki JM, Wahlsten M & Sivonen K (2007) Strains of the cyanobacterial genera *Calothrix* and *Rivularia* isolated from Baltic Sea display cryptic diversity and are distantly related to *Gloeotrichia* and *Tolypothrix*. *FEMS Microb Ecol* **61**: 74–84.
- Simpson GG (1944) *Tempo and Mode in Evolution*. Columbia University Press, New York.
- Stucken K, John U, Cembella A, Murillo AA, Soto-Liebe K, Fuentes-Valdés JJ, Friedel M, Plominsky AM, Vásquez M & Glöckner A (2010) The smallest known genomes of multicellular and toxic cyanobacteria: comparison, minimal gene sets for linked traits and the evolutionary implications. *PLoS One* **5**: e9235. DOI: 10.1371/journal.pone.0009235.
- Swingley WD, Blankenship RE & Raymon J (2008) Integrating Markov clustering and molecular phylogenetics to reconstruct the cyanobacterial species tree from conserved protein families. *Mol Biol Evol* **25**: 643–654.
- Swofford DL (2002) *PAUP\*: Phylogenetic Analysis Using Parsimony (\* and other methods) Version 4.0b 10 for Macintosh, and 4.0b 10 for UNIX*. Sinauer Associates, Sunderland, MA.
- Taton A, Grubisic S, Ertz D et al. (2006) Polyphasic study of antarctic cyanobacteria strains. *J Phycol* **42**: 1257–1270.
- Thomazeau S, Houdan-Fourmont A, Couté A & Duval C (2010) The contribution of Sub-Saharan African strains to the

- phylogeny of cyanobacteria: focusing on the Nostocaceae (Nostocales, Cyanobacteria). *J Phycol* **46**: 564–579.
- Tomitani A, Knoll AH, Cavanaugh CM & Ohno T (2006) The evolutionary diversification of cyanobacteria: molecular-phylogenetic and paleontological perspectives. *P Natl Acad Sci USA* **103**: 5442–5447.
- Waterbury JB (1991) The cyanobacteria: isolation, purification and identification. *The Prokaryotes*. 2nd edn (Balows A, Dworkin M, Schlegel HG & Truper H, eds), pp. 2058–2078. Springer-Verlag, Berlin.
- West GS (1970) Report of the freshwater algae including phytoplankton of the Third Tanganyika Expedition. *J Linn Soc Bot* **38**: 81–197.
- Whitton BA & Potts M (2000) Introduction to the cyanobacteria. *The Ecology of Cyanobacteria. Their Diversity in Time and Space* (Whitton BA & Potts M, eds), pp. 1–11. Kluwer Academic Press, Dordrecht.