



Planktic cyanobacteria from freshwater localities in Thuathien-Hue province, Vietnam.

III. Phylogenetic inference based on partial phycocyanin sequences, morphological and toxicological characters

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With 38 figures and 3 tables

Abstract: The morphology, toxicity and partial sequences of beta and alpha subunits including the intergenic spacer (*cpcBA*-IGS) of the phycocyanin operon were studied in 21 strains of non-axenic cyanobacterial cultures. The cultures were established from water samples collected in the Thuathien Hue province in Vietnam, 2004. Based on testing for the presence of the *mcyA* gene, microcystin-production of *Microcystis aeruginosa*, *M. botrys* and *M. panniformis* was confirmed. The presence of the *mcyA* gene in a strain of *Microcystis flos-aquae* and a strain of *Planktothrix spiroides* (HA308) revealed their toxic potential. The phylogeny of the Vietnamese cyanobacteria was inferred using sequences encoding the *cpcBA* genes. There was no correlation between toxic characters based on ELISA, HPLC and *mcyA* gene and the phylogeny based on partial *cpcBA* sequences. Although the resulting tree topology assisted in resolving the phylogeny of some strains of *Anabaena*, *Dolichospermum* and *Planktothrix*, taxonomical problems still exist at the species level for *Lyngbya* and *Microcystis*. Future morphological and molecular studies will have to solve these issues.

Keywords: morphology, phycocyanin gene sequences, phylogeny, planktic cyanobacteria, Thuathien-Hue, toxicity, Vietnam

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Introduction

Identifications of cyanobacteria based on morphological features are limited due to their relative simple morphology. Furthermore the morphology of species and strains varies considerably under different environmental conditions making species delimitations a challenging task (Barker et al. 1999, Otsuka et al. 1999). However, the application of electron microscopical studies in addition to chemical and molecular methods has helped to solve some of the taxonomical problems (Castenholz & Waterbury 1989). For more than two decades, molecular techniques have been applied intensively to study the diversity and taxonomy of cyanobacteria. Data sets obtained from molecular techniques have proved to be very useful for testing hypotheses generated by other data such as morphology or physiology (e.g. Li et al. 2001, Wilmotte & Golubic 1991, Wilmotte et al. 1993). Hence, a combination of morphological, ultrastructural, as well as chemical and molecular criteria, the so-called polyphasic approach, has recently been employed in taxonomic studies of cyanobacteria (e.g. Abed et al. 2003, Bolch et al. 1999, Hoffmann et al. 2005, Komárek & Kaštovský 2003, Liu et al. 2013, Neilan 1995, Neilan et al. 1997, Nguyen et al. 2013, Rajaniemi et al. 2005a, b, Sanchis et al. 2005, Suda et al. 2002).

Within the domain bacteria only the cyanobacteria possess phycocyanin (*cpcBA*) genes, which encode the main light-harvesting accessory proteins. Therefore, when determining *cpcBA* sequences, clonal cultures of cyanobacteria do not have to be axenic, as PCR co-amplification in contaminating non-photosynthetic bacteria is not possible (Bolch et al. 1996). Recently, the genes encoding the β - and α -phycocyanin subunits of phycobilisomes (*cpcB* and *cpcA*, respectively) have been used in molecular systematics and population genetic studies (e.g. Ballot et al. 2004, Barker et al. 1999, Bittencourt-Oliveira et al. 2001, Bolch et al. 1996, 1999, Manen & Falquet 2002, Nguyen et al. 2013, Teneva et al. 2005). Phylogenetic analyses based on *cpcBA* sequences often corroborate with the results obtained from 16S rDNA sequence analyses (e.g. Crosbie et al. 2003, Robertson et al. 2001). However, there are also some conflicts in the phylogenetic analyses deduced from these genes. For example, a high variation in the *cpcB*-IGS-*cpcA* sequences was found in *Microcystis aeruginosa* strains from Brazil (Bittencourt-Oliveira et al. 2001) while species of *Microcystis* were very similar in 16S rDNA sequences (Otsuka et al. 1998, Lyra et al. 2001). Manen & Falquet (2002), when studying the phylogenetic relationship between *Arthrospira* strains and other cyanobacteria, found a good correlation between 16S rDNA and *cpcBA* data. However, they also discovered intragenic recombination within *Arthrospira* strains that made them separate into three smaller clusters. Future determinations of additional gene sequences will have to examine these differences in greater detail.

Microcystins are hepatotoxic cyanobacterial metabolites, which are produced by some cyanobacterial species such as *Microcystis* (Carmichael et al. 1994, Henriksen 1996), *Phormidium* (Skulberg 1985, Sivonen et al. 1989) and *Planktothrix* (Sivonen et al. 1989, Carmichael 1994). In natural populations of cyanobacteria, both microcystin-producing and non-microcystin-producing individuals may coexist

(Kurmayer et al. 2004). They cannot be distinguished by morphological characters nor by DNA sequence analyses of 16S rDNA (Gugger et al. 2002, Lyra et al. 2001, Otsuka et al. 1998, Tillet et al. 2001). The clusters of genes encoding the microcystin synthetase complex (the *mcy* clusters) have been identified and sequenced in *Microcystis* PCC7806 (Tillett et al. 2001), *Planktothrix agardhii* CYA126 (Christiansen et al. 2003), and *Anabaena* (Rouhiainen et al. 2004). This made it possible to design probes and primers to amplify these genes and allow for discriminating between the toxic and non-toxic genotypes (Hisbergues et al. 2003, Neilan et al. 1999, Nishizawa et al. 2000, Tillet et al. 2001).

In the present study, we constructed the first comprehensive phylogenetic tree of cyanobacterial strains from Hue (Vietnam) based on partial phycocyanin genes and compared these with a selected set (42 taxa) of related cyanobacteria available in Genbank. Additionally we compared the tree topology with morphological characters and microcystin production of toxic species. The cyanobacterial strains were also tested for the presence of the *mcyA* gene to confirm microcystin-producing ability.

Materials and methods

Clonal cultures were established and maintained as described in Nguyen et al. (2007b). For the present study a total of 21 cyanobacterial strains from the Hue area was included. However, the recently described *Annamia toxica* from Hue was also included (Nguyen et al. 2013) for reasons of comparison. The Vietnamese strains covered a rather diverse assemblage of cyanobacteria with a total of 10 either colony or filamentous forming genera belonging to four different orders.

Light microscopy

Live cells forming colonies or filaments were examined using an Olympus BX60 microscope (Olympus, Tokyo, Japan). The forms of colonies and trichomes were observed during different ages of the cultures. At least 50 morphometric measurements were made (Tables 1, 2).

DNA extraction

Exponentially growing cultures (10 ml of each) were centrifuged at 1500 rpm for 15 min at room temperature. The pellets were transferred to 1.5 ml Eppendorf tubes and frozen at -18°C until DNA extraction. Extraction of total genomic DNA was carried out according to the CTAB protocol of Doyle & Doyle (1987). The cell pellets were grinded in preheated (65°C) 500 μl 2X CTAB buffer and 5 μl β -mercapto-ethanol and then incubated at 65°C for 1 h. DNA was extracted twice with chloroform:isopentylethanol (24:1) solution and the precipitation process was initiated by transferring the material to 1 ml 95 % ethanol and 50 μl sodium acetate (3M,

pH 5.5). Precipitated DNA was collected by centrifugation for 10 min at 20 000 rpm, dried in 65°C and re-suspended in approx 30 µl double-distilled water at 37°C overnight.

PCR reactions

The *cpcBA* coding regions were amplified using primers PCβF (5'-GGCTGCTT-GTTTACGCGACA-3') and PCαR (5'-CCAGTACCACCAGCAACTAA-3') (Neilan et al. 1995). PCR conditions were preheating for 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C and 2 min at 72°C, the final elongation cycle lasted 6 min at 72°C. This program was used for *Microcystis* spp., *Planktothrix spiroides*, *Planktothrix zahidii*, *Dolichospermum* spp. *Anabaena* spp., *Aphanizomenon aphanizomenoides* and *Cylindrospermopsis raciborskii*. With *Hapalosiphon* cf. *delicatulus*, *Phormidium uncinatum* and *Lyngbya* sp., the annealing temperature was increased to 55°C. Amplification of the *mcyA* coding regions were performed using primers *mcyA*-Cd 1R (5'-AAAAGTGTTTTATTAGCGGCTCAT-3') and *mcyA*-Cd 1F (5'-AAAATTAAGCCGTATCAAA-3') (Hisbergues et al. 2003). PCR conditions were 35 cycles of 94°C for 1 min., 52°C for 1 min., and 72°C for 2 min.

Amplifications were performed in a PTC-100 Programmable Thermal Controller (MJ Research, Waltham, Mass., USA) or a PTC-200 Peltier Thermal cycler (MJ Research, Waltham, Mass., USA). PCR amplified fragments were electrophoresed in a 2 % Nusieve agarose gel with EtBr and checked under UV light. PCR amplified fragments of correct length were purified using the QIA quick PCR purification kit (Qiagen, Germany) following the commendations of the manufacturer. Purified PCR products (approx. 20 ng µL⁻¹) were sequenced using the same primer PCβF and PCαR (Neilan et al. 1995).

Sequencing reactions were run on an ABI Prism 377 DNA sequencer (Perkin-Elmer, California) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, California, USA). The lengths of the PCR products has a maximum length of 620 base pairs and a negative control (i.e. PCR reagents but no cyanobacteria template) was included in each series of PCR reactions to make sure that contamination products did not affect the results. DNA extractions and PCR amplifications were repeated several times especially for *Hapalosiphon* cf. *delicatulus* (strain HCYG).

Alignment and phylogenetic analyses

The *cpcBA* sequences of the Vietnamese strains were aligned with 42 morphologically related cyanobacterial sequences retrieved from GenBank (see list of taxa in Table 3). The GenBank sequences were selected among the four orders and represented species considered related to the strains from Vietnam. The data matrix comprised a total of 520 base pairs (the last 245 base pairs of *cpcB* gene, the *cpcB-cpcA* spacer and the first 272 base pairs of *cpcA* gene). The length of the *cpcB-cpcA* spacer varied

between 66 and 103 base pairs and could not be aligned unambiguously. Hence, it was excluded prior to phylogenetic analyses.

Bayesian analysis was done with MrBayes (ver. 3.2.2, Ronquist & Huelsenbeck 2003). A total of 10 million generations were run and after 501,000 generations (conservative number), the “burn-in” was reached resulting in a $- \ln = 7.6 \times 10^9$. This resulted in 9,500 trees, which were all used to compute a 50 % majority rule consensus tree. From this we obtained posterior probabilities (≤ 1). Prior to Neighbor-Joining analyses using PAUP *, we used jModeltest (ver. 2.1.3, Darriba et al. 2012) to search for the best model for the PC sequences by hierarchical likelihood ratio tests. The best fit model was GTR+G+I. The settings from this model were applied in Neighbor-joining (NJ) to compute dissimilarity values and these were then used as input to build a NJ tree. NJ bootstrap analyses with the jModeltest maximum likelihood settings were performed with 1,000 replications.

Results

Morphology

The main taxonomic characters of the studied strains, such as the diameter and shape of cells, form of colonies, filaments, presence or absence of sheaths, heterocytes or akinetes, are summarized in Tables 1 and 2. For identification purposes all Vietnamese strains are illustrated in this study (Figs 1–37). However, for more detailed descriptions, the reader is referred to Nguyen et al. (2007a) (except for strain HAsp). Based on the classification system proposed by Anagnostidis & Komárek (Anagnostidis & Komárek 1988, 1990, Komárek & Anagnostidis 1986, 1989), the 21 established strains were assigned to 14 morphological species and 9 genera (excluding *Annamia*). They represented four orders (viz. Chroococcales, Oscillatoriales, Nostocales and Stigonematales).

The strains of *Microcystis* were classified into two groups according to the diameter of the cells, with a minor overlap. Group 1 ranged from 4 to 6.5 μm in diameter and group 2 from 2.5–4.5(5) μm in diameter. Based on observations of colony form and different ages of cultures (young versus mature), the two strains in group 1 were identified as *M. aeruginosa* and *M. botrys*, respectively (Table 1). The strains of group 2 were identified as *M. flos-aquae* and *M. panniformis*. However, this division of the morphospecies in group 2 is weak because of an overlap in cell diameter, the variable shape of the colonies or, in dense cultures, the occurrence of single cells.

Based on the absence or presence of a sheath, the oscillatorialean strains were divided into two groups (one group comprised H308, H134, and HOs1, and the other HOs8 and HOs120). Although the dimensions of filaments are more or less overlapping, the strains H308 and H134 were identified as *Planktothrix spiroides* (Liu et al. 2013) and distinguished from HOs1, which was identified as *Planktothrix zahidii* because of its spiral-shaped filaments. Strain HOs120 differed from HOs8 by the smaller cell length and the absence of the apical cells in HOs120.

Table 1. Toxicity and selected morphological characters of *Microcystis* isolated from Hue (Vietnam); (-) or (+) indicates negative or positive results in the toxic tests, respectively.

Morphospecies	Strains	Toxicity		Diameter of		Form of colonies		mature stage
		ELISA	HPLC	mcyA	cells (µm)	young stage		
<i>M. aeruginosa</i> (Figs 22–24)	H107	+	+	+	4.0–6.3	single cells or small clusters of cells arranged loosely	colonies irregular, lobate, often composed from subcolonies with cells distributed densely but always with gaps between them; later with distinct holes; diffused slime, slightly overlapping the cells; become single cells in old cultures	
	H179a	+	+	+	5.2–6.5 (8)	small spherical colonies with cells densely agglomerated at the center of colonies; mucilage diffluent but distinct, often with a radial structure	colonies irregular or spheroidal, without holes, wide slime margin, with very dense cells which are radially arranged	
<i>M. flos-aquae</i> (Figs 1–8)	H27	-	-	-	3.0–4.0	small spherical or zigzag, irregular clusters of loosely agglomerated cells	spherical or irregular colonies with sparsely to very densely agglomerated cells; not lobate but hollow within the colonies; without a slimy margin; become single cells in old cultures	
	H42	-	-	+	2.5–3.5 (4.5)			
	H146	-	-	-	3.5–4.5 (5)			
<i>M. panniformis</i> (Figs 11–21)	H19	+	+	+	2.0–4.3	single cells, zigzag clusters of cells, or a layer of cells	cells regularly densely and evenly agglomerated in one or more layers, sometimes in distinct rows, cloudy shape, with small holes in old colonies; become single cells in old cultures	
	H95	+	+	+	3.0–5.0			
	H44	+	+	+	3.0–4.0 (5.0)			
	H106	+	+	+	3.0–5.0 (5.2)			
	H329	+	+	+	(2.5) 3.0–5.0			

Table 2. Toxicity and selected morphological characters of filamentous cyanobacteria from Hue (Vietnam). (a): (-) or (+) indicates negative or positive results in the toxicity tests, respectively; (b): (0) or (1) indicates absence or presence of the character, respectively.

Morpho-species	Strains	Toxicity (a)		Morphology (b)					Branching	
		ELISA	HPLC	Filaments	Dimension of cells (μm)(LxW)	Shape of cells	Sheath	Heterocyte		Akinete
<i>Arthrospira massartii</i>	H308	-	-	regularly	2.5-3.7 X 5-6.3	cylindrical,	0	0	0	0
	H134	-	-	spirally coiled	1.7-3 X 5.0-5.5	shorter than wide	0	0	0	0
<i>Planktothrix zabihidi</i>	HOs1	-	-	straight	1.3-2.5 X 3.3-5	cylindrical, shorter than wide	0	0	0	0
<i>Phormidium uncinatum</i>	HOs8	-	-	straight	1.6-5 X 5.5-6.9	cylindrical	1	0	0	0
<i>Lyngbya</i> sp.	HOs120	-	-	straight	1.5-3.5 X 4-7	cylindrical	1	0	0	0
<i>Anabaena laxa</i>	HA30	-	-	straight	6-7.3	spherical	0	spherical, 6.7-7.5	cylindrical, 16-26 x 5.5-9.1	0
<i>Anabaena</i> sp.	HAsp	-	-	straight	3.5-5 X 7-9	spherical, or barrel shaped, slightly shorter than wide	0	spherical, 6.5-9	ellipsoidal, 9-17 x 8-13	0
<i>Sphaerospermopsis aphanizomenoides</i>	HAnNY	-	-	straight	3-10 x 2-5	barrel-shaped to cylindrical	0	spherical to slightly oval, 5-8 x 3.5-5	spherical, 7.5-12.5	0
<i>Cylindrospermopsis raciborskii</i>	HCy90	-	-	straight	2.5-18 x 2.5-3.8	cylindrical	0	arrow shaped, 6-7(8) x 2-3.8	long oval, 10-18 x 3.8-5	0
<i>Dolichospermum viguieri</i>	HA174	-	-	straight	3.4-8 X 5-8(9)	spherical or barrel-shaped	0	spherical, 6-7(9)	ellipsoidal, 7.5-19 x 9-12	0
<i>Hapalosiphon</i> cf. <i>delicatulus</i>	HCyG	-	-	straight	3.7-8 x 2.8-3.8	cylindrical	0	cylindrical, 5.6-8.3 x 3.3-4.2	0	1

The heterocytous strains were identified as the morphospecies *Anabaena viguieri*, *A. laxa*, *Anabaena* sp., *Cylindrospermopsis raciborskii*, *Sphaerospermopsis aphanizomenoides* and *Hapalosiphon* cf. *delicatulus*. Strain HAsp differs from HA30 by the variable cell dimensions and akinetes. Strain HCyG was distinguished by the presence of branches in old cultures. Branches did not form in freshly collected field samples. It was tentatively identified as a species of *Hapalosiphon* within the order Stigonematales.

Toxicity

All studied strains have previously been examined for the production of microcystins using ELISA and HPLC (Nguyen et al. 2007b) and these results are referred to in Tables 1 and 2 of the present paper. The *mcyA* gene was amplified in strains of *Microcystis* (viz. *M. aeruginosa*, *M. botrys* and *M. panniformis*). Additionally, the *mcyA* gene was amplified in strains HA308 and H42 (*Planktothrix spiroides* and *Microcystis flos-aquae*, respectively). However, these strains were found to be non-microcystin-producing based on HPLC and ELISA. All PCR products of *mcyA* gene have the length of 298 base pairs. The *mcyA* PCR products were sequenced and compared to those available in GenBank (data not shown). The *mcyA* sequences were similar to the most closely related ones.

Phylogeny based on partial phycocyanin genes (*cpcBA*)

The phylogeny based on partial *cpcBA* gene sequences including 22 cyanobacteria isolated from Hue and 41 sequences of morphologically related cyanobacteria were retrieved from GenBank was analyzed using Bayesian analyses (BA) and Neighbor-Joining (NJ). A list of strains, their origin and GenBank accession numbers are shown in Table 3. The unrooted tree presented in Figur 38 was deduced from Bayesian analysis (with posterior probabilities and bootstrap values from NJ mapped onto it).

Members of the order Chroococcales, mainly *Microcystis* species, formed one cluster strongly supported by posterior probabilities (pp = 1.0) and bootstrap values (BS = 100 %). Strains H42, H146 and H27 of *M. flos-aquae* formed one clade also strongly supported in both analyses (pp = 1.0 and BS = 99 %). *Microcystis aeruginosa* (strain H107) from Hue formed its own branch and a sister taxon to *M. flos-aquae* (pp = 1.0 and BS = 87 %). *Microcystis botrys* (strain H179a) from Hue and *M. aeruginosa* (strain UAM 254) also formed a highly supported branch (pp = 1 and BS = 99 %). Strains of *M. panniformis* from Hue were divided in two branches: one included H44, H106 and H239 and the other H95, H19 together with three *Microcystis aeruginosa* and one *M. flos-aquae cpcBA* sequences from GenBank (Fig. 38).

The filamentous, non-heterocytous strains were scattered on the tree. The strains of *Lyngbya* sp. (HOs120) and *Phormidium uncinatum* (HOs8) formed a strongly supported clade (pp = 1.0 and BS = 100 %). *Lyngbya* and *Phormidium* formed a sister group to a lineage comprising a diverse assemblage of cyanobacteria including *Doli-*

Table 3. Cyanobacteria included in the phylogenetic analyses of *cpcBA* (phycocyanin) genes. Strain numbers and Genbank accession numbers are also provided.

Species of cyanobacteria	Strain number	Genbank accession numbers
<i>Annamia toxica</i>	HOs24	HQ658459
<i>Anabaena laxa</i>	HA30	KF840312
<i>Anabaena</i> sp.	HAsp	KF840313
<i>Anabaena ucrainica</i>	TAC 449	AY702236
<i>Aphanizomenon flos-aquae</i>	AFA-4	EU822493
<i>Aphanizomenon gracile</i>	AB2008/31	FN552305
<i>Arthrospira fusiformis</i>	AB2002/01	AY575923
<i>Arthrospira indica</i>	PD1997	AY575945
<i>Arthrospira jenneri</i>	fz	HQ828101
<i>Dolichospermum compactum</i>	1403/24	AY702239
<i>Dolichospermum flos-aquae</i>	NIES 73	AY702243
<i>Dolichospermum lemmermannii</i>	BCAna 0034	AY886917
<i>Dolichospermum lemmermannii</i>	NIVA82	AY702242
<i>Dolichospermum planktonicum</i>	NIVA66	AY702220
<i>Dolichospermum spiroides</i>	NIES79	AY702234
<i>Dolichospermum solitarium</i>	NIES80	AY181213
<i>Dolichospermum viguieri</i>	TAC 433	AY702229
<i>Dolichospermum viguieri</i>	HA174	KF840314
<i>Arthrospira platensis</i>	AICB49	AY672714
<i>Arthrospira</i> sp.	Sark31055	AJ310555
<i>Cylindrospermopsis raciborskii</i>	CYAus	AF426804
<i>Cylindrospermopsis raciborskii</i>	CYGer	AF426797
<i>Cylindrospermopsis raciborskii</i>	CYFlo	AF426796
<i>Cylindrospermopsis raciborskii</i>	CYBra	AF426793
<i>Cylindrospermopsis raciborskii</i>	HCy90	KF840317
<i>Hapalosiphon cf. delicatulus</i>	HCyG	KF840318
<i>Lyngbya</i> sp.	PCC7419	AJ401187
<i>Lyngbya</i> sp.	HOs120	HQ658463
<i>Microcoleus</i> sp.	PCC8701	AY768472
<i>Microcystis aeruginosa</i>	UAM254	AY271735
<i>Microcystis aeruginosa</i>	FACHB937	AY568705
<i>Microcystis aeruginosa</i>	EAWAG171	AJ003179
<i>Microcystis aeruginosa</i>	PCC7820	AF195176
<i>Microcystis aeruginosa</i>	H107	KF840319
<i>Microcystis flos-aquae</i>	UAM256	AY271737
<i>Microcystis flos-aquae</i>	H27	KF840320
<i>Microcystis flos-aquae</i>	H42	KF840321
<i>Microcystis flos-aquae</i>	H146	KF840322
<i>Microcystis botrys</i>	H179a	KF840323
<i>Microcystis panniformis</i>	H19	KF840324

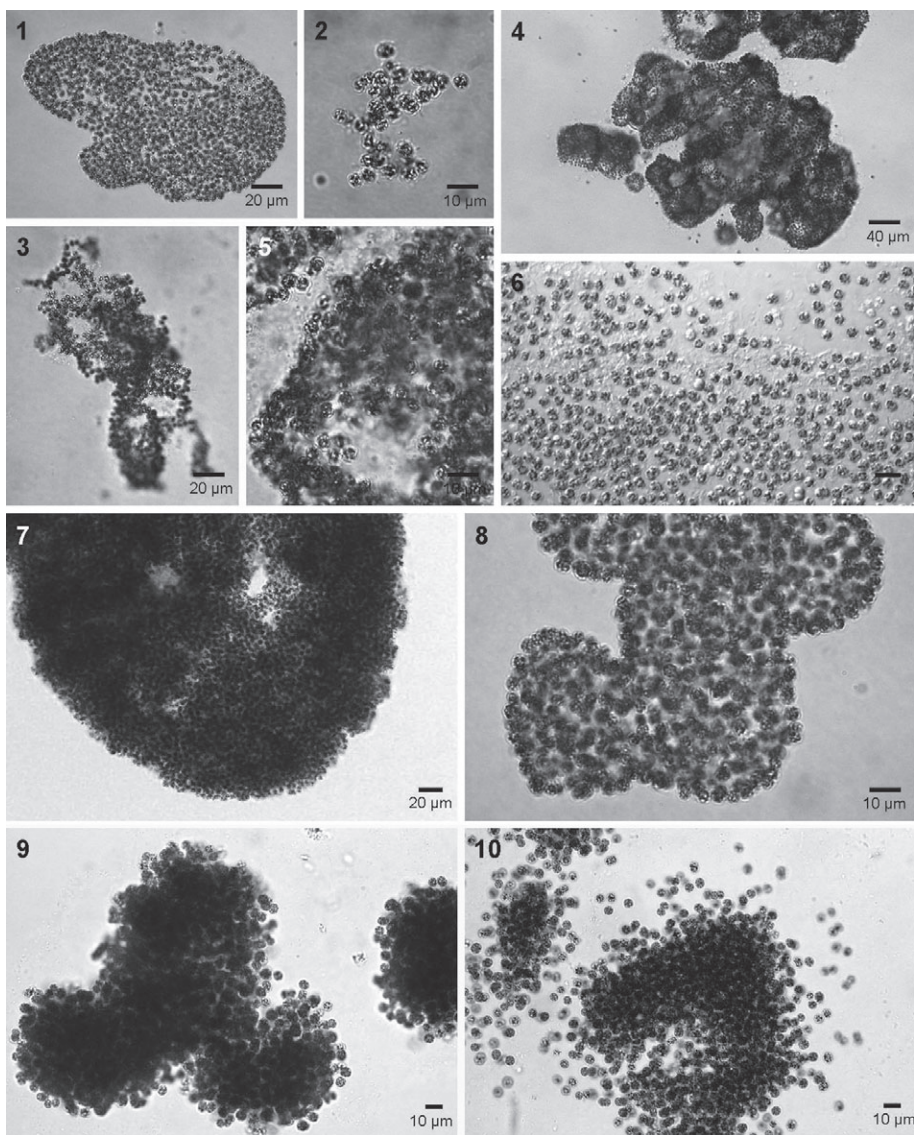
Table 3. cont.

Species of cyanobacteria	Strain number	Genbank accession numbers
<i>Microcystis panniformis</i>	H44	KF840325
<i>Microcystis panniformis</i>	H95	KF840326
<i>Microcystis panniformis</i>	H106	KF840327
<i>Microcystis panniformis</i>	H329	KF840328
<i>Microcystis wesenbergii</i>	NIES112	AF385391
<i>Microcystis wesenbergii</i>	UAM244	AY271728
<i>Nodularia</i> sp.	PCC73104/1	AF367149
<i>Nostoc</i> sp.	PCC7102	AY768464
<i>Oscillatoria</i> sp.	PCC6506	AY768468
<i>Oscillatoria</i> sp.	PCC7515	AJ401185
<i>Phormidium uncinatum</i>	HOs8	HQ658460
<i>Planktothrix agardhii</i>	NIVA-CYA 535	AM490098
<i>Planktothrix spiroides</i>	th1	HQ913018
<i>Planktothrix spiroides</i>	HA134	HQ658462
<i>Planktothrix spiroides</i>	HA308	KF840316
<i>Planktothrix zahidii</i>	HOs1	HQ658461
<i>Planktothrix</i> sp.	FP1	AF212923
<i>Planktothrix</i> sp.	PCC7811	AY768471
<i>Sphaerospermopsis aphanizomenoides</i>	HANY	KF840315
<i>Spirulina</i> sp.	PCC6313	AJ401188
<i>Synechococcus</i> sp.	SYNCP CAB	X59809
<i>Synechococcus</i> sp.	MW32B5	AY151230
<i>Tolypothrix</i> sp.	PCC7601	AY768470

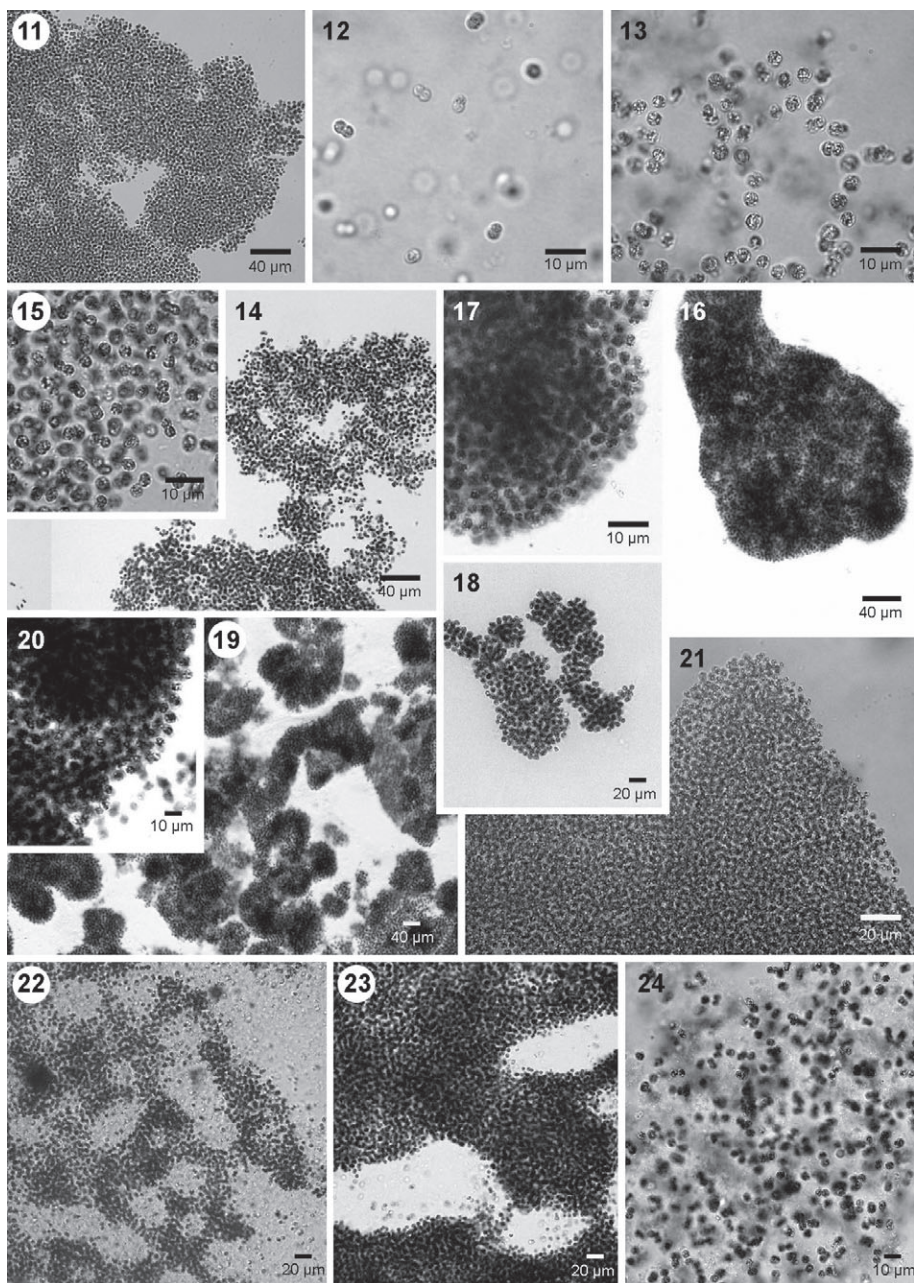
chospermum spp., *Oscillatoria* sp., *Nostoc* sp., *Nodularia* sp., *Cylindrospermopsis raciborskii*, *Aphanizomenon* spp., *Sphaerospermopsis aphanizomenoides*, *Hapalosiphon* cf. *delicatulus* and *Anabaena* spp.

The three strains of *Planktothrix spiroides* (two from Vietnam and one from China) and *Planktothrix zahidii* from Hue formed a distinct clade (pp = 1.0 and BS = 91 %). They had a close relationship with other two species of *Planktothrix* retrieved from GenBank (Fig. 38). The *Planktothrix* species formed a sister group to *Arthrospira* spp. (including the type species, *A. jenneri*). This relationship was highly supported by a posterior probability (pp = 1.0) but weakly supported by NJ bootstrap (BS = 54 %).

The heterocytous cyanobacteria with the exception of *Nostoc* sp. (PCC 7120) and *Nodularia* sp. (PCC 73104/1) formed one well-supported clade (pp = 1.0 and BS = 93 %). *Dolichospermum viguieri* (HA174) from Hue grouped with all other *Dolichospermum* species from GenBank, except *Anabaena ucraina*. *Anabaena laxa* (HA30) and *Anabaena* sp. (HAsp) formed a separate clade with a posterior probability of 1.0 and 99 % bootstrap support in NJ. The strain HCYG of *Cylindrospermopsis raciborskii* from Hue also grouped with *Cylindrospermopsis* from other areas in the world



Figs 1–10. – Figs 1–8. *Microcystis flos-aquae*. Figs 1–2. Strain H27. Fig. 1. Small colony, Fig. 2. Cluster of cells in more than one layer; Figs 3–5. Strain H146. Fig. 3. Zigzag cluster of cells, Fig. 4. Spherical, irregular colonies in which cells agglomerated on the surface of colonies, Fig. 5. Old colonies; Figs 6–8. Strain H42. Fig. 6. Old culture with single cells, Fig. 7. Very dense colony, Fig. 8. Young colony; Figs 9–10. *Microcystis botrys* strain H179a.



(Fig. 38) and was most closely related to German and Australian strains. The two markedly different morphospecies, *Sphaerospermopsis aphanizomenoides* (HANY) and *Hapalosiphon* cf. *delicatulus* (HCyG) had almost identical *cpcBA* sequences (sequence divergence 0.6 %).

The recently described *Annamia toxica* also from the Hue area (Vietnam) formed a distinct lineage but with no clear indication of its closest sister taxon/group. *Annamia* was part of a polytomy comprising most other cyanobacteria included (Fig. 38).

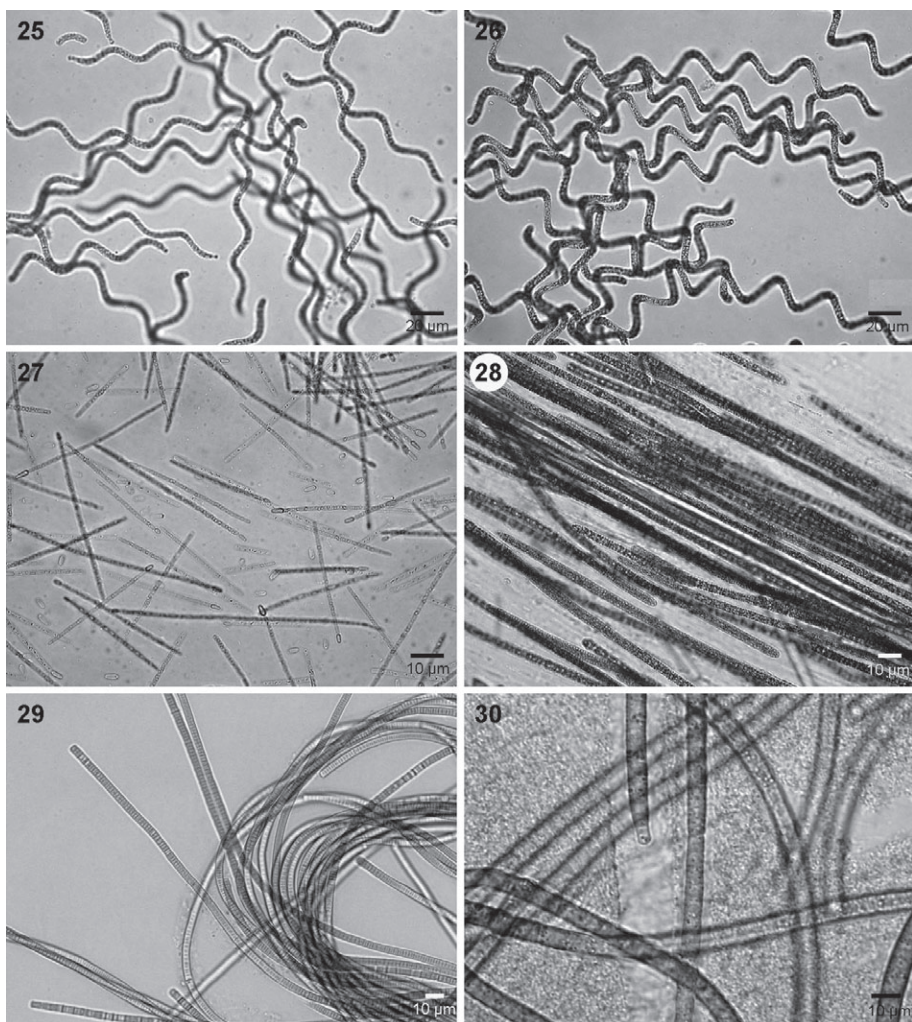
Discussion

Phylogeny of partial *cpcBA* genes and comparison with morphology

Microcystis morphospecies frequently form intensive blooms associated with toxin production all over the world. Species identification is often difficult and there are conflicting reports in the literature on species identity. Yet *Microcystis* species display low phenotypic diversity (Otsuka et al. 1999). Morphological features such as cell diameters may overlap between different species of *Microcystis*, and the morphospecies do not always correlate with genetic data (Bolch et al. 1996, Otsuka et al. 2001). However, several features may be used to differentiate species of *Microcystis* (Komárek & Komárková 2002). Bittencourt-Oliveira et al. (2001) studied the genetic variety of Brazilian strains of *Microcystis aeruginosa* using PC genes and found that some genotypes displayed consistent morphological characteristics. Komárková et al. (2005) showed that cell size may be a main character to differentiate between species, and this work has contributed to the stability of the morphospecies concept within *Microcystis*.

In this study, 4 morphospecies of *Microcystis* isolated from Hue were divided into 2 groups based on cell diameter (4–6.5 µm and 2.5–4.5(5) µm, respectively). A similar division was made for European species of *Microcystis* (Komárek & Komárková 2002). However, the division into size classes is not reflected in the phylogeny based on *cpcBA* sequence data. The two strains of group 1 (H179 identified as *M. botrys* and H107 identified as *M. aeruginosa*) occurred in two clades with the other strains of group 2. Contrary, the morphological identification of *M. flos-aquae* strains agreed with the *cpcBA* analyses. All strains of *M. flos-aquae* from Hue occurred in one cluster and probably constitute the same population. The Vietnamese strains of *M. panniformis* formed two lineages; one lineage comprised H44, H106 and H329 and the

←
Figs 11–24. Figs 11–21. *Microcystis panniformis*. Figs 11–13. Strain H329. Fig. 11. Colony with cells in one layer. Fig. 12. Single cells in old culture. Fig. 13. Zigzag cluster of cells. Figs 14–15. Strain H19, irregular colonies. Figs 16–17. Strain H95, dense colonies. Figs 18–20. Strain H106. Fig. 18. Young colonies with clusters of cells in a single plane. Figs 19–20. Mature colonies in a dense culture. Fig. 21. Strain 44, dense colony with irregularly arranged cells. Figs 22–24. *Microcystis aeruginosa* strain H107. Fig. 22. Old colonies. Fig. 23. Dense colonies in young culture. Fig. 24. Culture with single cells.

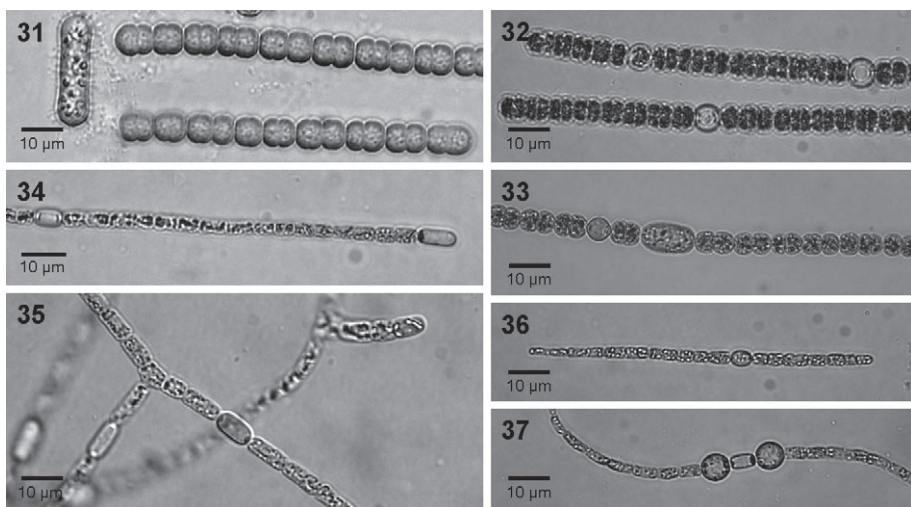


Figs 25–30. Figs 25–26. *Planktothrix spiroides*. Fig. 25. Strain HA134. Fig. 26. Strain HA308. Fig. 27. *Cylandropermopsis raciborskii* strain HCy90. Fig. 28. *Planktothrix zahidii* strain HOs1. Fig. 29. *Lyngbya* sp. strain HOs120. Fig. 30. *Phormidium uncinatum* strain HOs8.

other comprised H95 and H19. The latter strains were more closely related to two other species of *Microcystis* (viz. *M. aeruginosa* and *M. flos-aquae*). Thus, cell diameter at least in the Vietnamese strains seemed to be an unreliable character and size groups were not reflected in the phylogenetic analyses. Although a remarkable variation occurs in cultures, mature colony form in young cultures was stable: irregular, lobate colonies with cells distributed densely in *M. aeruginosa*; irregular or spherical colonies with cells arranged tightly, densely and radially in *M. botrys*, spherical or irregular colonies with cells sparsely agglomerated in *M. flos-aquae*, and cloud-shaped colonies with cells agglomerated regularly in the same layers in *M. panniformis* (Table 2). Colony form was likely a more reliable character for species identification than cell size, at least in the cultures studied here. However, we agree with Bittencourt-Oliveira et al. (2001) in suggesting that a combination of molecular markers and morphological features other than cell dimensions is necessary to discriminate between *Microcystis* at the species level. Although the spiral width of the filaments in strain H308 was slightly larger than that in strain H134 (Figs 25–26), determination of partial *cpcBA* sequences and the intergenic spacer (*cpcBA*-IGS) of phycocyanin operon revealed that they had identical sequences. Wang & Zhao (2005) and Li et al. (2001) observed a similar variation in trichome shape, and the latter authors concluded that coiling was an unreliable character for taxonomy. Therefore, in Nguyen et al. 2007a and 2013, they were identified as *Arthrospira massartii*. Liu et al. (2013) working on Chinese material collected from a pond in the Guangdong Province recently described *Planktothrix spiroides*. The Chinese strains were initially identified as a freshwater *Arthrospira*-like cyanobacterium. However, phylogenetic analyses based on *cpcBA*-IGS and *rpoC1* genes supported the finding that these strains belonged to *Planktothrix* with the new species *P. spiroides*. Furthermore this species had coiled trichomes. A sequence comparison based on *cpcBA* and IGS between Vietnamese strains of *P. spiroides* (HA308 and HA134) and a Chinese strain (th1) of the same species revealed that th1 and HA308 were identical and that these strains only differed by 1 substitution to strain HA134. Hence, we conclude that strains HA134 and HA 308 from Vietnam represent species of *Planktothrix spiroides* and not *Arthrospira massartii* as stated in Nguyen et al. 2007a and 2013.

Species of *Planktothrix* have previously been assigned to the genus *Oscillatoria* within the family Phormidiaceae by Anagnostidis & Komárek (1988). This was mainly due to a number of features such as solitary filaments, which are free-floating, straight, isopolar, and a sheath is usually lacking, cells are cylindrical, shorter than wide and contain aerotopes. It differed from *Arthrospira* in the shape of the filaments. The coiling of the filament was typical for *Arthrospira*. The phylogenetic analyses showed that strain HO1, identified as *Planktothrix zahidii*, was closely related to the two *Arthrospira* strains (posterior probability ≥ 0.99 and bootstrap support values = 100 %). A close relationship between *Planktothrix* and *Arthrospira* was also suggested by Manen & Falquet (2002) based on PC gene sequences.

Based on the presence of apical cells with obtuse end cells, strain HO8 was identified as *Phormidium uncinatum*, while HO120 was identified as *Lyngbya* sp. due to



Figs 31–37. Fig. 31. *Anabaena laxa* strain HA30. Fig. 32. *Anabaena* sp. Strain HA23. Fig. 33. *Dolichospermum viguieri* strain HA174. Figs 34–35. *Hapalosiphon* cf. *delicatulus* strain HCyG. Figs 36–37. *Sphaerospermopsis aphanizomenoides* strain HANY.

the presence of a sheath in our cultures (Nguyen et al. 2007a). The two species also share other characters as shown in Table 3. The highly supported relationship found between the two strains suggests that HOs120 is in fact *Phormidium uncinatum*. Perhaps bringing *Phormidium uncinatum* into culture prevented it from producing the normal apical cell seen in freshly collected material.

Analyses of *cpcBA* sequences showed a monophyletic clustering of heterocytous cyanobacteria of Nostocales except *Nostoc* sp. (strain PCC7120), *Nodularia* sp. (strain PCC 73104/1) and *Tolypothrix* sp. (strain TPCC7601) (Fig. 38). Based on 16S rDNA sequence data, Rajaniemi et al. (2005a) found that heterocytous cyanobacteria formed a monophyletic group.

Our study agrees with the recent taxonomic treatment of Wacklin et al. (2009) separating planktic *Anabaena*-morphotypes into the new genus *Dolichospermum*. In fact *Dolichospermum* spp. did not seem to group with *Anabaena laxa* (HA30) and *Anabaena* sp. (HAsp) as the latter strains was not part of the highly supported clade with mostly *Dolichospermum*. Rather the two *Anabaena* strains formed an unresolved relationship with the largest cluster of Nostocales (Fig. 38) and their sister group relationship to *Dolichospermum* spp. and a few other strains only received low bootstrap support ($pp = 0.64$ and $BS < 50\%$). *Anabaena* sp. may in fact be identified as *A. laxa* with a slightly aberrant morphology (Table 3). *Dolichospermum viguieri* (strain HA174) from Hue shared identical *cpcBA* gene sequences with *Dolichospermum viguieri* (strain TAC 433) from GenBank.

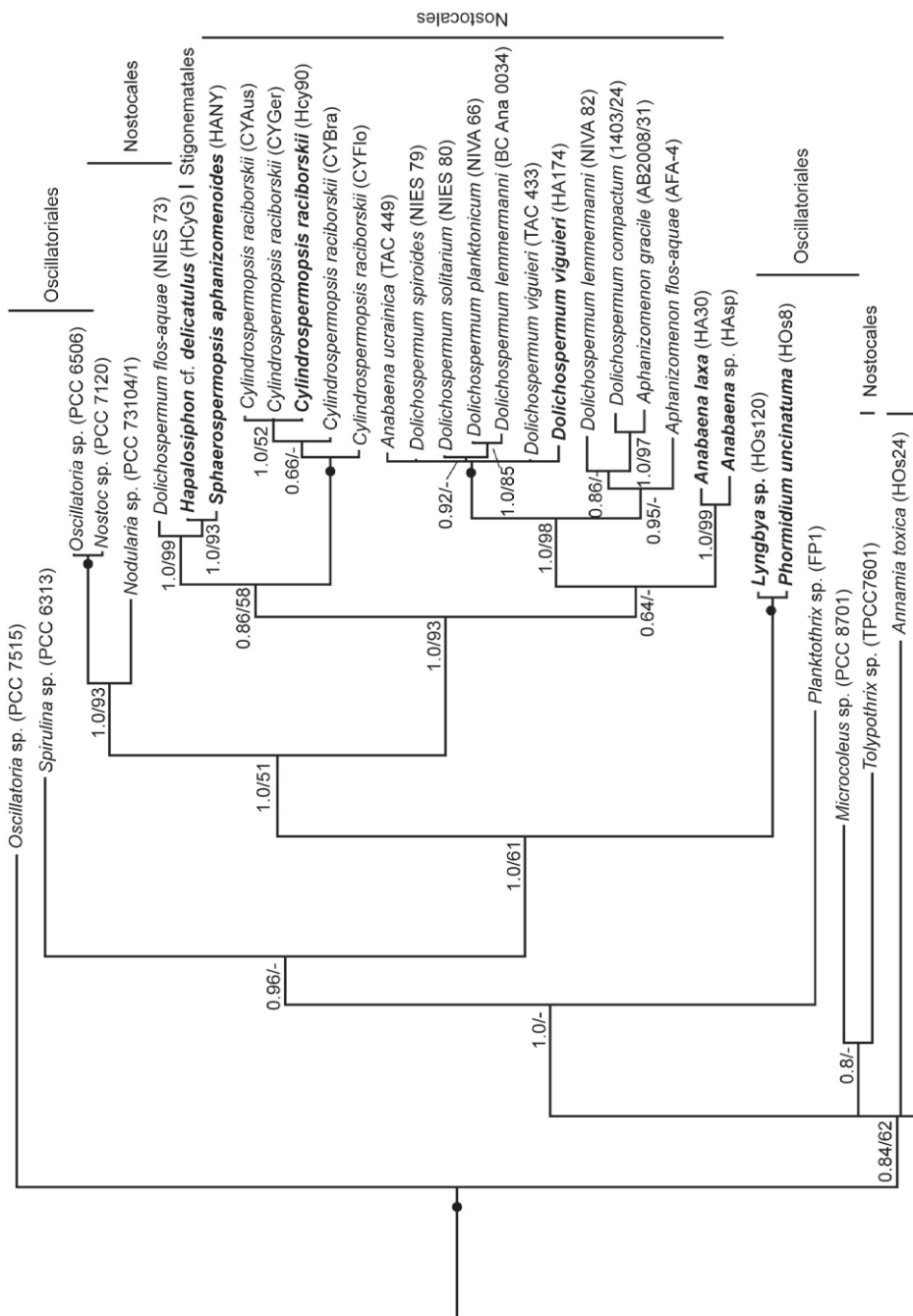
The *Aphanizomenon* strain (HANY) was closely related to *D. flos-aquae* (strain NIES 73) from GenBank. Previous molecular taxonomic studies have also indicated a relationship between strains of *Dolichospermum* and *Aphanizomenon* (see Neilan

et al. 1995, Lyra et al. 1997, Rudi et al. 1997, Rudi & Jacobsen 1999 for a detailed discussion).

The toxic species *Cylindrospermopsis raciborskii* was at first found only in tropical waters but it is now known to be much more widely distributed (Gugger et al. 2005, Neilan et al. 2003). Analyses of *cpcBA* genes revealed a relationship of the Vietnamese *Cylindrospermopsis raciborskii* strain with other *C. raciborskii* strains sampled worldwide. Bayesian analysis even suggest that was most closely related to Australian and German strains (pp = 1.0). However, this relationship received weak support from bootstrap (BS = 52 %) (Fig. 38). Based on 16S rDNA analysis, *C. raciborskii* from Thailand showed a 99 %–100 % sequence similarity to Australian strains (Chonudomkul et al. 2004). Thus, Asian and Australian strains probably form one or few closely related populations.

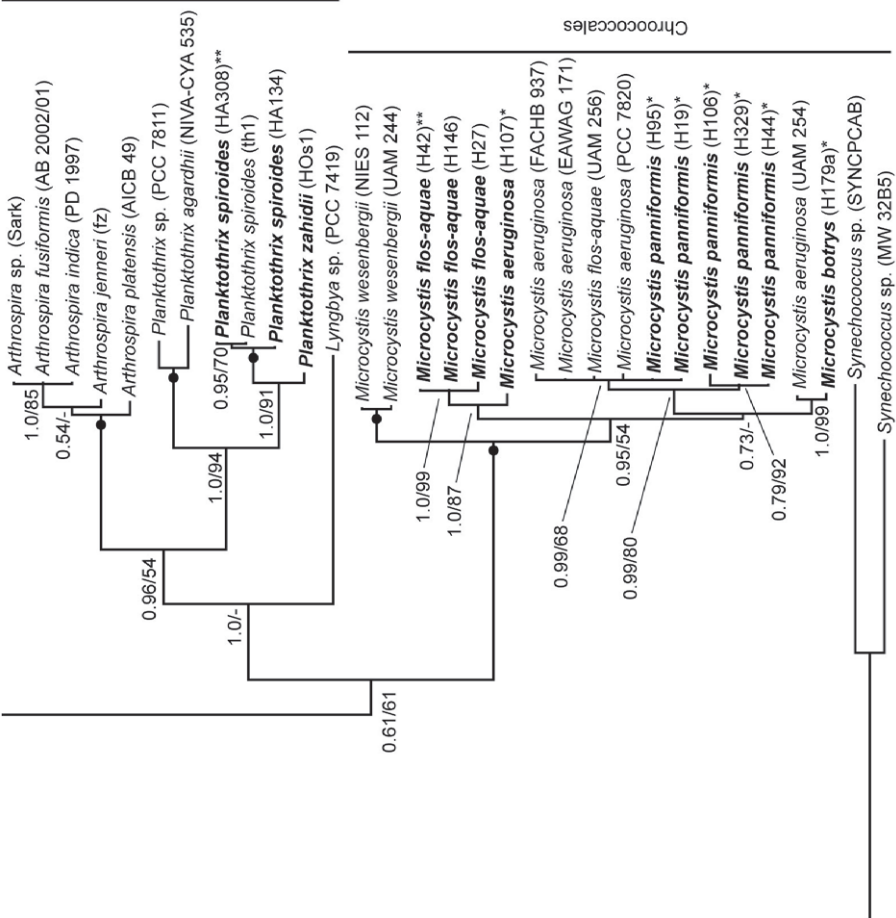
Morphologically, the two strains HANY and HCyG were markedly different. The HANY strain was identified as *Sphaerospermopsis aphanizomenoides* following the main characters of the cells including heterocytes and akinetes (Nguyen et al. 2007a). Strain HCyG on the other hand had straight filaments with cylindrical cells and spherical or cylindrical heterocytes, but it lacked akinetes; occasional branches were found which suggests assignment to Stigonematales. On the basis of accepted morphological criteria (Anagnostidis & Komárek 1990), this material was identified as *Hapalosiphon* cf. *delicatulus* (Nguyen et al. 2007a). However, it should be noted here that the true branching was observed repeatedly in old cultures and not in the freshly collected field samples. The sequence comparison of *cpcBA* sequences revealed them almost identical (divergence = 0.6 %). Lateral gene transfer of the *cpcBA* sequences (including IGS) could potentially explain this observation. The occurrence of lateral gene transfer has been documented a number of times in cyanobacteria (e.g. Barker et al. 2000, Manen & Falquet 2002, Mikalsen et al. 2003, Rudi & Jacobsen 1999, Tanabe et al. 2004). Manen & Falquet (2002) indicated a point of intragenic recombination close to the stop codon of *cpcB* in *Arthrospira* strains and suggested that the capacity to exchange genetic material between strains may explain why morphology and geographical origin do not always correlate with the *cpcBA* operon. Lateral gene transfer, a puzzling factor in phylogenetic studies, is obviously not a rare occurrence in cyanobacteria. Lawrence et al. (2003) even proposed lateral gene transfer to occur more frequently in operational genes than in informational genes (Jain et al. 1999, Woese 1998).

Fig. 38. An unrooted tree deduced from Bayesian analysis and based on partial phycocyanin sequences (520 base pairs, including 3 introduced gaps) for 63 cyanobacteria. Of these 22 taxa were from Vietnam. Numbers at internodes are posterior probabilities (≥ 0.5) and bootstrap values (≥ 50 %) from Bayesian analyses and Neighbor-Joining, respectively. Sequences determined in this study are bold faced. * = toxic species detected by ELISA, HPLC and presence of *mcyA* gene; ** = potential toxic species detected only by the presence of *mcyA* gene. See Wacklin et al. (2009) for taxonomy of *Dolichospermum*.



Oscillatoriales

Chroococcales



0.09

Toxicity based on *mcyA* gene

All microcystin-producing strains from Hue possessed the *mcyA* gene. The microcystin-producing morphospecies were *Microcystis aeruginosa*, *M. botrys*, and *M. panniformis*, while *M. flos-aquae* did not produce microcystins (Nguyen et al. 2007b). This finding is in accordance with previous reports on the toxicity of these species (Bittencourt-Oliveira et al. 2005, Carmichael et al. 1994, Henriksen 1996, Via-Ordorika et al. 2004). However, the non-toxic strain H42 of *Microcystis flos-aquae* and HA308 of *Planktothrix spiroides* were shown here also to possess the *mcyA* gene.

The presence of *mcy* genes in non-toxic strains, called inactive microcystin genotypes, has previously been found in *Microcystis* spp. and *Planktothrix* spp. (Bittencourt-Oliveira et al. 2001, Kurmayer et al. 2004, Via-Ordorika et al. 2004). The non-toxic strains of *Microcystis flos-aquae* and *Planktothrix spiroides* (HA134) from Hue may be considered such inactive microcystin genotypes with the potential to produce microcystin under certain (yet unknown) environmental conditions.

The ability to produce microcystins was not reflected in a phylogeny based on *cpcBA* sequences because strains do not split up in toxic versus non-toxic lineages. Strains of *Microcystis flos-aquae* and *Planktothrix spiroides* with or without this gene still group in one cluster (Fig. 38). Similarly Mikalsen et al. (2003) found no correlation between the phylogenies of *mcyB1*, 16SrDNA and DNA fingerprinting. Toxicity is therefore not a useful chemical marker applicable for taxonomy. The reason why strains possess these *mcy* genes but do not express them is not quite clear. A common ancestor for microcystin synthesis has been suggested, and the absence of the *mcy* gene in some strains has been explained by gene loss (Rantala et al. 2004). Christiansen et al. (2006) demonstrated that different mutations resulting in inactivation of microcystin synthesis occurred frequently in a *Planktothrix* population.

In conclusion, the phylogeny based on *cpcBA* generally supported the morphological classification of cyanobacteria particularly at the genus level. Problems with difficult or dubious identifications based on morphological features were in some cases resolved by support from molecular data as in the case of *Anabaena* sp., *Dolichospermum* sp. and *Lyngbya* sp. However, in some strains such as *Microcystis* and *Oscillatoria* taxonomical problems are still unresolved. A combination of molecular (probably a handful of gene sequences) and morphological data is needed to construct a more stable taxonomy of cyanobacteria.

Phylogenetic status of Chroococcales, Oscillatoriales and Nostocales

Even considering that the phylogenetic tree shown in Figure 38 is unrooted our analyses based on *cpcBA* sequences indicate that Chroococcales, Oscillatoriales and Nostocales are not monophyletic. Future studies will have to examine if this is a result of frequent occurrences of lateral gene transfer in the taxa included.

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Summary

The morphology, toxicity and partial phycocyanin gene sequences (*cpcBA*) were studied in 21 strains of non-axenic cultures of cyanobacteria. These were established from water samples collected in the Thuathien Hue province, Vietnam (2004). There was no correlation between toxic characters based on ELISA, HPLC and *mcyA* gene and a phylogeny based on *cpcBA* gene sequences. Although the resulting tree topology assisted in resolving the phylogeny of *Anabaena*, *Dolichospermum* and *Planktothrix*, taxonomical problems still exist at the species level for *Microcystis* and *Lyngbya*.

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