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Article

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 (*cpc*BA-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 *cpc*BA genes. There was no correlation between toxic characters based on ELISA, HPLC and mcyA gene and the phylogeny based on partial *cpc*BA 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 *cpc*BA 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 (*cpc*B and *cpc*A, 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 *cpc*BA 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 *cpc*B-IGS-*cpc*A 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 *cpc*BA 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 ml 2X CTAB buffer and 5 µl β-mercapto-ethanol and then incubated at 65°C for 1 h. DNA was extracted twice with chloroform:isopenthylethanol (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 37ºC overnight.

PCR reactions

The *cpc*BA coding regions were amplified using primers PCβF (5´-GGCTGCTTGTTTACGCGACA-3´) and PCαR (5´-CCAGTACCACCAGCAACTAA-3´) (Neilan et al. 1995). PCR conditions were preheating for 3 min at 94ºC, followed by 35 cycles of 1 min at 94ºC, 1 min at 52ºC and 2 min at 72ºC, the final elongation cycle lasted 6 min at 72ºC. 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 55ºC. Amplification of the mcyA coding regions were performed using primers mcyA-Cd 1R (5´-AAAAGTGTTTTATTAGCGGCTCAT-3´) and mcyA-Cd 1F (5´-AAAATTAAAAGCCGTATCAAA-3´) (Hisbergues et al. 2003). PCR conditions were 35 cycles of 94ºC for 1 min., 52ºC for 1 min., and 72ºC for 2 min.

Amplifications were performed in a PTC-100 Programmable Thermal Controler (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 *Hapolosiphon* cf. *delicatulus* (strain HCyG).

Alignment and phylogenetic analyses

The *cpc*BA 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 *cpc*B gene, the *cpc*B-*cpc*A spacer and the first 272 base pairs of *cpc*A gene). The length of the *cpc*B-*cpc*A spacer varied

between 66 and103 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 = $7.6*10^9$. 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 cells (µm)	Form of colonies	
			ELISA	HPLC	mcyA		young stage	mature stage
Chroococcales	*M. aeruginosa* (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
	M. botrys (Figs 9–10)	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
	M. flos-aquae (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)		
	M. panniformis (Figs 11–21)	H19		+	+	2.0–4.3	single cells, zigzag clusters of cells, or a layer of cells	cells regularly densely and evenly aggromerated 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)						
			ELISA	HPLC	mcyA	Filaments	Dimension of cells (µm)(LxW)	Shape of cells	Sheath	Heterocyte	Akinete	Branching
Oscillatoriales	*Arthrospira massartii*	H308	–	–	+	regularly spirally coiled	2.5–3.7 X 5–6.3	cylindrical, shorter than wide	0	0	0	0
		H134	–	–	–		1.7–3 X 5.0–5.5		0	0	0	0
	Planktothrix zahidii	HOs1	–	–	–	straight	1.3–2.5 X 3.3–5	cylindrical, shorter than wide	0	0	0	0
	Phormidium uncinatum	HOs8	–	–	–	straight	1.6 - 5 X 5.5–6.9	cylindrical	1	0	0	0
	Lyngbya sp.	HOs120	–	–	–	straight	1.5–3.5 X 4–7	cylindrical	1	0	0	0
Nostocales	*Anabaena laxa*	HA30	–	–	–	straight	6–7.3	spherical	0	spherical, 6.7–7.5	cylindrical, 16–26 x 5.5–9.1	0
	Anabaena 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
	Sphaerospermopsis aphanizomenoides	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
	Cylindrospermopsis raciborskii	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
	Dolichospermum viguieri	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
Stigonematales	*Hapalosiphon* cf. *delicatulus*	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 (*cpc*BA)

The phylogeny based on partial *cpc*BA 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 cpc*BA 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 *cpc*BA (phycocyanin) genes. Strain numbers and Genbank accession numbers are also provided.

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

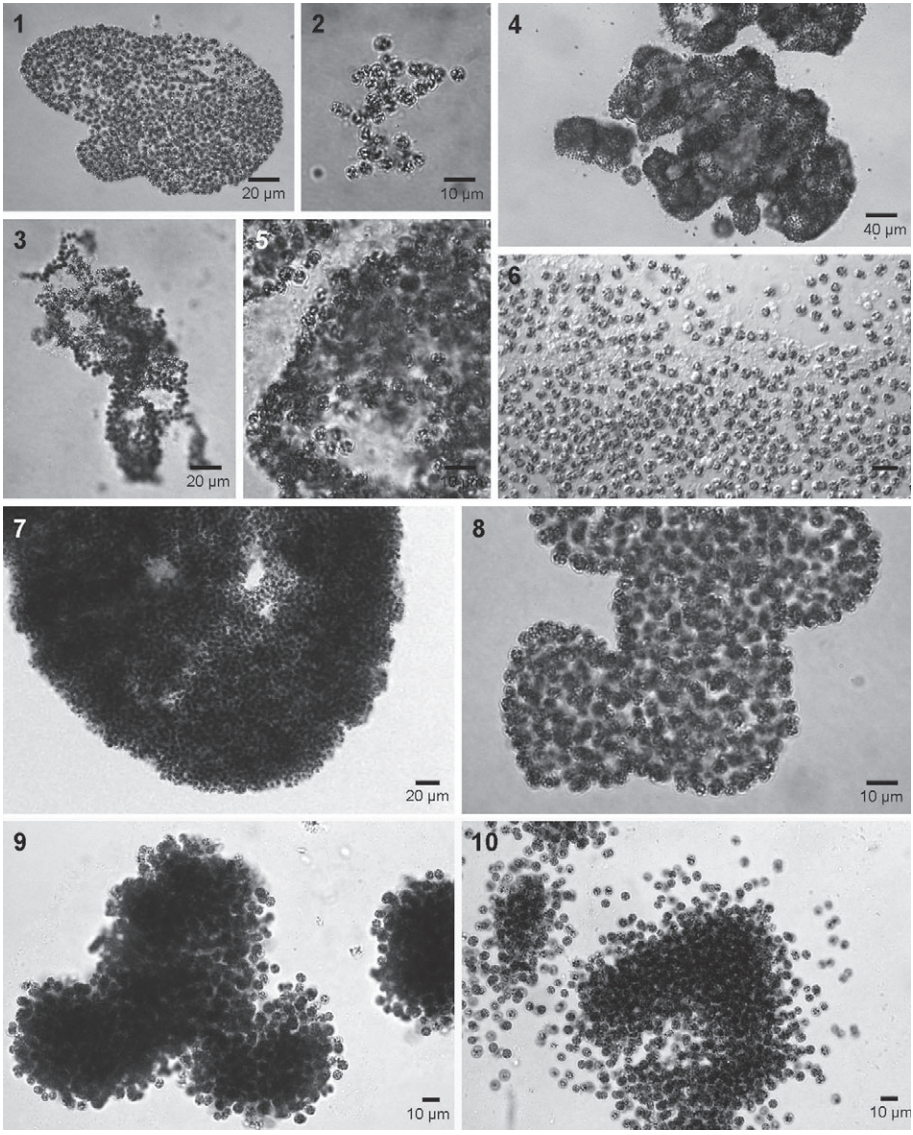
Table 3. cont.

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

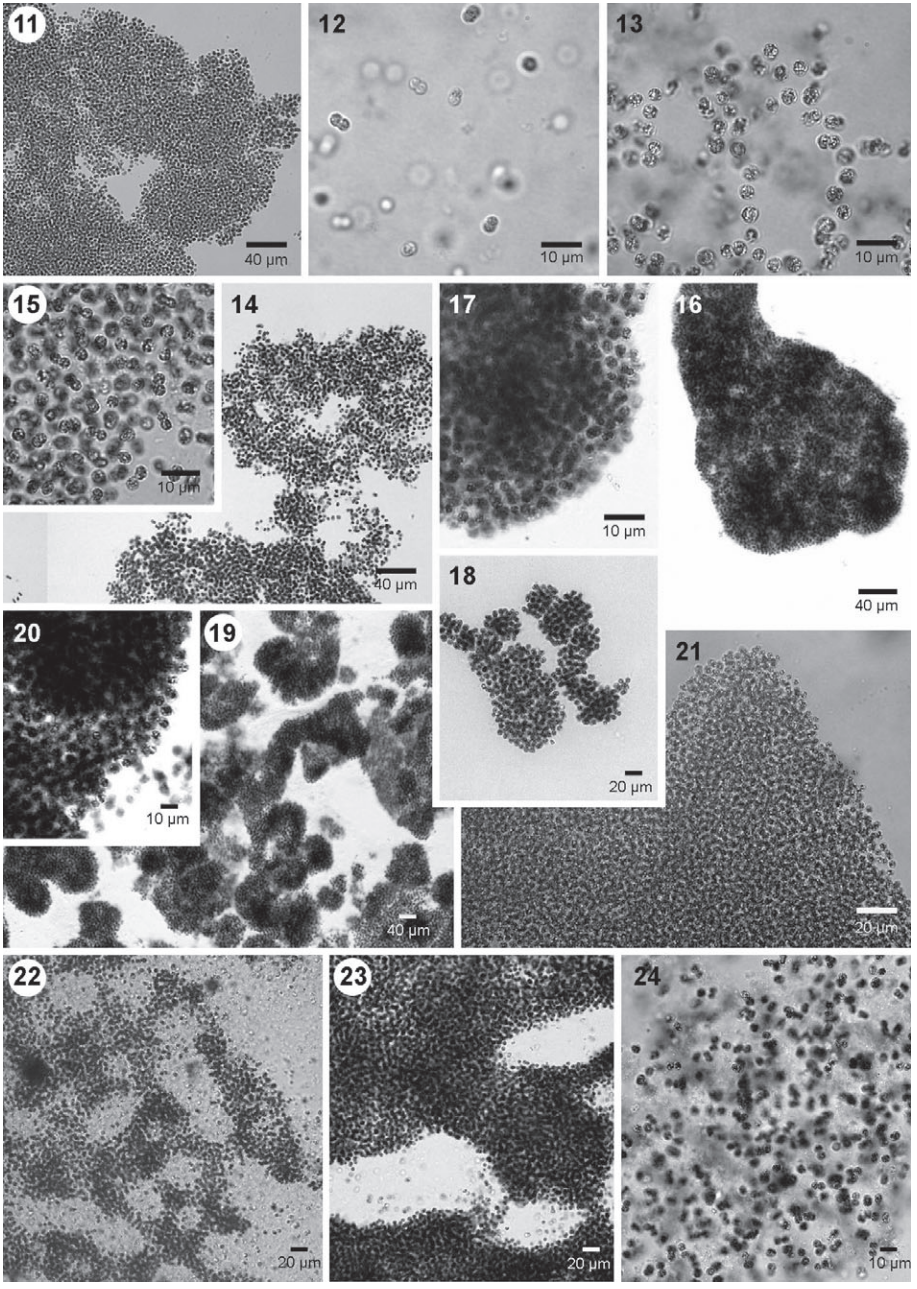
chospermum spp., *Oscillatoria* sp., *Nostoc* sp., *Nodularia* sp., *Cylindrospermopsis raciborskii*, *Aphnizomenon* spp., *Sphaerospermopsis aphanizomenoides*, *Hapalosiphon* cf. *delicatulus* and *Anabaena* spp.

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 *Plantothrix* 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.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22
20 µm
23
20 µm
24
10 µm

(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 *cpc*BA 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).

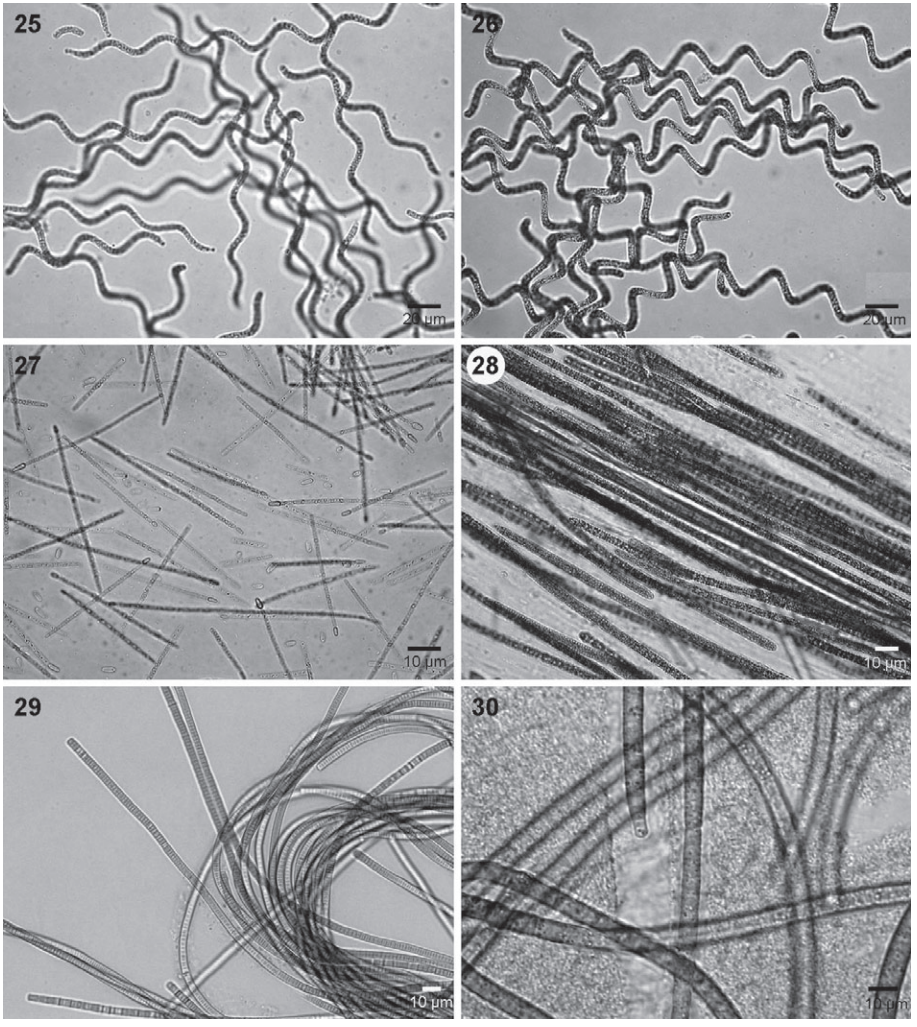
Discussion

Phylogeny of partial *cpc*BA 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 *cpc*BA 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 *cpc*BA 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.

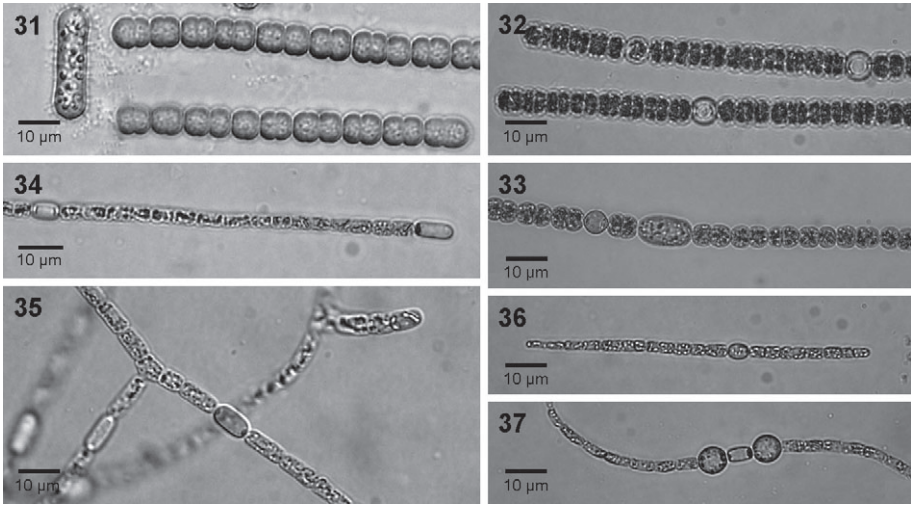


Figs 25–30. Figs 25–26. *Planktothrix spiroides*. Fig. 25. Strain HA134. Fig. 26. Strain HA308. Fig. 27. *Cylindrospermopsis raciborskii* strain HCy90. Fig. 28. *Planktothrix zahidii* strain HOs1. Fig. 29. *Lyngbya* sp. strain HOs120. Fig. 30. *Phormidium uncinatum* strain HOs8.

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 (*cpc*BA-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 *cpc*BA-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 *cpc*BA 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 *Arthospira 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 HOs1, 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 HOs8 was identified as *Phormidium uncinatum*, while HOs120 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 *cpc*BA 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.

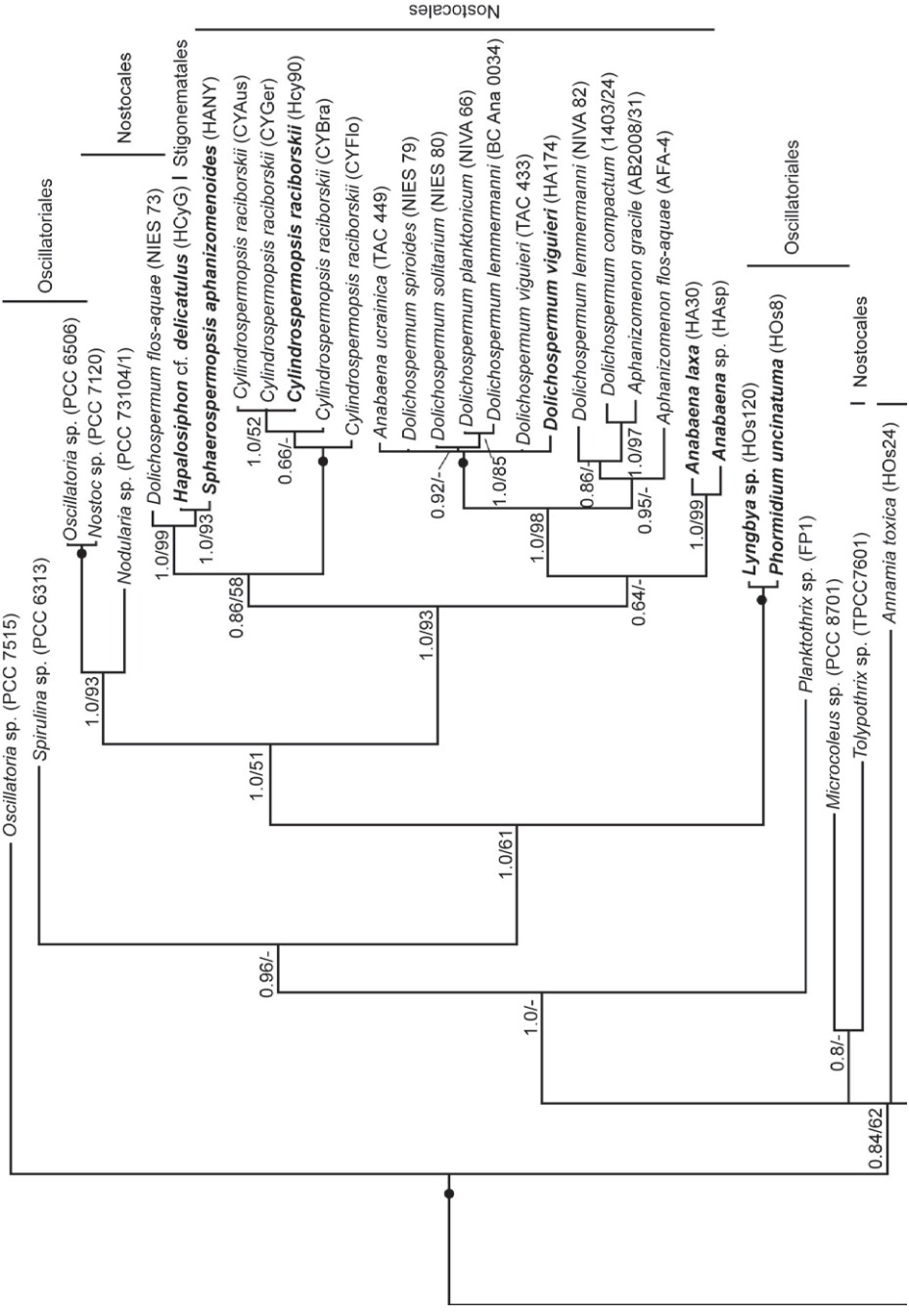
The *Aphanizomenon* strain (HANY) was closely related to *D. flos-aquae* (strain NIES 73) from GenBank. Previous molecular taxonomic studies have also indicated a relationship between strains of *Dolichospermum* and *Aphanizomenon* (see Neilan

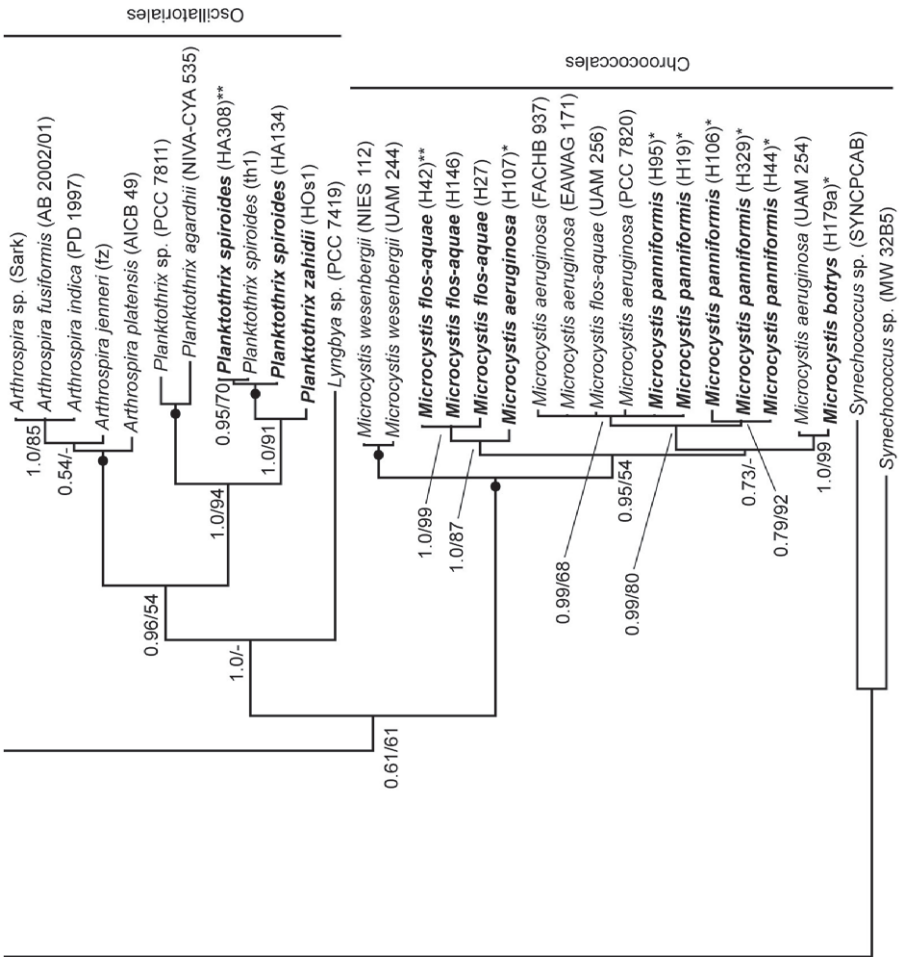
et al. 1995, Lyra et al. 1997, Rudi et al. 1997, Rudi & Jacobsen 1999 for a detailed discussion).

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 *cpc*BA 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 *cpc*BA sequences revealed them almost identical (divergence = 0.6 %). Lateral gene transfer of the *cpc*BA 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 *cpc*B 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 *cpc*BA 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 *mcy*A gene; ** = potential toxic species detected only by the presence of *mcy*A gene. See Wacklin et al. (2009) for taxonomy of *Dolichospermum*.


Nostocales
Oscillatoriales
Nostocales
Stigonematales
Oscillatoriales
Nostocales
Oscillatoriales
Oscillatoria sp. (PCC 7515)
Spirulina sp. (PCC 6313)
Oscillatoria sp. (PCC 6506)
Nostoc sp. (PCC 7120)
Nodularia sp. (PCC 73104/1)
Dolichospermum flos-aquae (NIES 73)
Hapalosiphon cf. delicatulus (HCyG)
Sphaerospermopsis aphanizomenoides (HANY)
Cylindrospermopsis raciborskii (CYAus)
Cylindrospermopsis raciborskii (CYGer)
Cylindrospermopsis raciborskii (Hcy90)
Cylindrospermopsis raciborskii (CYBra)
Cylindrospermopsis raciborskii (CYFlo)
Anabaena ucrainica (TAC 449)
Dolichospermum spiroides (NIES 79)
Dolichospermum solitarium (NIES 80)
Dolichospermum planktonicum (NIVA 66)
Dolichospermum lemmermanni (BC Ana 0034)
Dolichospermum viguieri (TAC 433)
Dolichospermum viguieri (HA174)
Dolichospermum lemmermanni (NIVA 82)
Dolichospermum compactum (1403/24)
Aphanizomenon gracile (AB2008/31)
Aphanizomenon flos-aquae (AFA-4)
Anabaena laxa (HA30)
Anabaena sp. (HAsp)
Lyngbya sp. (HOs120)
Phormidium uncinatuma (HOs8)
Planktothrix sp. (FP1)
Microcoleus sp. (PCC 8701)
Tolypothrix sp. (TPCC7601)
Annamia toxica (HOs24)
1.0/99
1.0/93
0.86/58
1.0/52
0.66/-
1.0/93
0.92/-
1.0/85
1.0/98
0.86/-
1.0/97
0.95/-
1.0/99
0.64/-
1.0/93
1.0/51
1.0/61
0.96/-
1.0/-
0.8/-
0.84/62


Oscillatoriales
Chroococcales
Arthrospira sp. (Sark)
Arthrospira fusiformis (AB 2002/01)
Arthrospira indica (PD 1997)
Arthrospira jenneri (fz)
Arthrospira platensis (AICB 49)
Planktothrix sp. (PCC 7811)
Planktothrix agardhii (NIVA-CYA 535)
Planktothrix spiroides (HA308)**
Planktothrix spiroides (th1)
Planktothrix spiroides (HA134)
Planktothrix zahidii (HOs1)
Lyngbya sp. (PCC 7419)
Microcystis wesenbergii (NIES 112)
Microcystis wesenbergii (UAM 244)
Microcystis flos-aquae (H42)**
Microcystis flos-aquae (H146)
Microcystis flos-aquae (H27)
Microcystis aeruginosa (H107)*
Microcystis aeruginosa (FACHB 937)
Microcystis aeruginosa (EAWAG 171)
Microcystis flos-aquae (UAM 256)
Microcystis aeruginosa (PCC 7820)
Microcystis panniformis (H95)*
Microcystis panniformis (H19)*
Microcystis panniformis (H106)*
Microcystis panniformis (H329)*
Microcystis panniformis (H44)*
Microcystis aeruginosa (UAM 254)
Microcystis botrys (H179a)*
Synechococcus sp. (SYNCPCAB)
Synechococcus sp. (MW 32B5)
1.0/85
0.54/-
0.96/54
1.0/94
0.95/70
1.0/91
1.0/-
0.61/61
1.0/99
1.0/87
0.99/68
0.99/80
0.95/54
0.73/-
0.79/92
1.0/99
0.09

Toxicity based on mcyA gene

All microcystin-producing strains from Hue possessed the mcy A gene. The microcystin-producing morphospecies were *Microcystis aeruginosa*, *M. botrys*, and *M. paniformis*, 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 *cpc*BA 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 *cpc*BA 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 *cpc*BA 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 (*cpc*BA) 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 *cpc*BA gene sequences. Although the resulting tree topo-logy assisted in resolving the phylogeny of *Anabaena*, *Dolichospermum* and *Planktothrix*, taxonomical problems still exist at the species level for *Microcysti*s and *Lyngbya*.

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