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Annamia toxica gen. et sp. nov. (Cyanobacteria), a freshwater cyanobacterium from Vietnam that produces microcystins: ultrastructure, toxicity and molecular phylogenetics

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A freshwater cyanobacterium from Hoamy Reservoir, Hue, Vietnam was isolated into clonal culture (April 2004). Based on general morphology (including size and shape) from light microscopy it was identified as *Pseudanabaena* cf. *moniliformis*. However, transmission electron microscopy revealed the thylakoids to be radially arranged, in contrast to the *Pseudanabaena* group *sensu* Komárek & Čáslavská (1991), which was characterized by a more or less concentric arrangement of the thylakoids. Furthermore, the phylogenetic relationship of the Vietnamese culture was investigated by sequencing the phycocyanin gene and the gene encoding 16S rRNA. Using morphology, toxicity and gene sequences, we showed that the Vietnamese culture and a culture of *Pseudanabaena galeata* from the UTEX collection were not related at the generic level. To examine this in greater detail, a search was made for a culture of *P. catenata*, the type species of *Pseudanabaena*. Two cultures with that name were available from the SAG culture collection in Göttingen, Germany. Thin sections revealed that both strains were characterized by concentric thylakoids. Based on our polyphasic approach, the Vietnamese material was therefore described as *Annamia toxica* gen. et sp. nov.

Key Words: *Annamia toxica*, Cyanobacterium, Microcystins, Phylogeny, Ultrastructure, Vietnam

INTRODUCTION

Our understanding of the species diversity of cyanobacteria from tropical waters is generally incomplete. There is a lack of information about their ultrastructure and molecular phylogenetics. Many tropical species have been identified as being similar to species from habitats with a different ecology (e.g. temperate zones). Whether this approach is justified is unknown, and investigations of tropical cyanobacteria are strongly needed (Komárek *et al*. 2005). In our study of planktic cyanobacteria from the Hue area, Vietnam, we tentatively identified a filamentous microcystin-producing species as belonging to the genus *Pseudanabaena*. Its trichomes were short, straight and solitary, lacked a sheath and had deeply constricted cross-walls. Cells were barrel-shaped or long cylindrical, and the end cells were flattened or slightly conical. It was initially named *Pseudanabaena* cf. *moniliformis* Komárek & Kling in Nguyen *et al*. (2007a). Its toxic potential was the incentive for a more detailed study, including determination of its taxonomic placement using ultrastructural and molecular data.

At the generic level, filamentous cyanobacteria without heterocytes and akinetes are traditionally distinguished from one another by the presence or absence of a sheath, the potential for false branching and the morphology of the filament. Important features at the species level are cell size, the presence of constrictions at the cross-walls, cellular inclusions, cell shape and the shape of the terminal cells of the trichomes (Komárek & Anagnostidis 2005). The arrangement and number of thylakoids is also taxonomically important (Whitton 1972; Lang & Whitton 1973) because it is considered to be a stable character (Komárek & Čáslavská 1991). In principle, three basic patterns of thylakoid arrangement have been found. A parietal arrangement of thylakoids is present in all members of Pseudanabaenaceae and Schizotrichaceae, a radial arrangement in members of Borziaceae and Phormidiaceae, and an irregular arrangement in Gomontiellaceae and Oscillatoriaceae (Komárek & Čáslavská 1991; Komárek & Anagnostidis 2005). A good correlation has been found between thylakoid arrangement and phylogenetic analyses based on 16S rRNA sequences (Komárek & Kaštovský 2003; Casamatta *et al*. 2005).

The traditional taxonomic system of cyanobacteria is based on morphology and cytology (Geitler 1932; Desikachary 1959; Bourrelly 1970). A taxonomic scheme was subsequently developed that uses a polyphasic approach based on physiological, biochemical, molecular, ultrastructural and morphological characteristics (Stanier & Cohen-Bazire 1977; Stanier *et al*. 1978; Rippka *et al*. 1979; Anagnostidis & Komárek 1985, 1988; Komárek & Anagnostidis 1986, 1989). Most recently, Hoffmann *et al*. (2005) introduced a system which combines the available genetic, ultrastructural and phenotypic data.

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In this study, the ultrastructure of a Vietnamese strain (HOs24) was examined with emphasis on the arrangement of thylakoids, and the genes encoding phycocyanin (PC) and 16S rRNA were sequenced. The 16S rRNA gene is commonly used to distinguish taxa above species level as well as individual species (Neilan 1995; Nelissen *et al*. 1996). Recently, the 16S rRNA gene was recommended as the main genetic marker for classification into genera (Komárek & Golulic 2006). Additionally, the genes encoding the β- and α-phycocyanin subunits of phycobilisomes (*cpc*B and *cpc*A) have been used in molecular systematics and studies of population genetics (e.g. Bolch *et al*. 1996, 1999; Barker *et al*. 1999; Bittencourt-Oliveira *et al*. 2001; Manen & Falquet 2002; Teneva *et al*. 2005). We compared morphology, toxicity and nucleotide sequence data of the Vietnamese strain with a strain of *Pseudanabaena galeata* Böcher (UTEX SP44). When evidence suggested that the Vietnamese material represented a new genus, two clones identified as *P. catenata* Lauterborn, the type species of *Pseudoanabaena*, were obtained from the SAG culture collection (Göttingen, Germany) and the thylakoid arrangement examined by transmission electron microscopy (TEM). Based on genotypic and phenotypic evidence, the material from Hue (strain HOs24) was identified as a new species of a new genus, *Annamia*.

MATERIAL AND METHODS

Single trichomes of strain HOs24 were isolated in April 2004 from Hoamy Reservoir in the Hue area, central Vietnam (16°00′–16°45′ N; 107°01′–108°13′E). Detailed information about the sampling site was provided in Nguyen *et al*. (2007a). Non-axenic cultures were established by single-cell isolation using capillary glass pipettes. Unfortunately, some years after the present study was completed, all Vietnamese strains were lost. *Pseudanabaena galeata* strain UTEX SP44 was obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX). Two strains (SAG 254.80 and SAG 1464-1) of *Pseudanabaena catenata* Lauterborn were obtained from Sammlung von Algenkulturen at Universität Göttingen, Germany.

Strain HOs24 was maintained in liquid Z8 medium (Kotai 1972), UTEX SP44 in f/2-Si (Guillard 1975) and *Pseudanabaena catenata* in modified Z8 medium (N.H. Larsen, unpublished data). The cultures were illuminated with cool white light at 24°C ± 4 at 20–35 μmol photons m^{-2} s^{-1} under a 12:12 hour light:dark regime. For observations of thylakoid arrangement under low light intensity, a subculture of strain HOs24 was grown at 3–3.5 μmol photons m^{-2} s^{-1} for 20 days before fixing and embedding for TEM.

Live cells of HOs24, *Pseudanabaena galeata* and *P. catenata* were examined using a BX60 microscope (Olympus, Tokyo, Japan) equipped with differential interference contrast. Micrographs were taken with a DP12 digital camera (Olympus, Tokyo, Japan). Two cultures (HOs24, UTEX SP44) were fixed in a mixture of glutaraldehyde (2.5%) and paraformaldehyde (2%) in 0.1 M sodium cacodylate buffer for 2 h at room temperature, and concentrated at 1200 × *g* for 10 min. They were washed several times in the buffer over 1 h (changed every 15 min) and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer overnight at 4°C. After a brief rinse in distilled water, cells were dehydrated in a series of aqueous solutions of acetone (15%, 30%, 50%, 70% and 96%) for 15 min at each concentration, followed by two changes of 99% acetone at room temperature. Cells were finally transferred to 100% acetone for 5 min, and a 1:1 mixture of acetone and Spurr's resin added. Cultures of SAG 254.80 and SAG 1464-1 were fixed for 80 min in 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.7, rinsed three times in buffer over 2 h, post-fixed in 1% osmium tetroxide in phosphate buffer and dehydrated in an ethanol series. The dehydration was completed in propylene oxide (twice, 5 min) and a 1:1 mixture of propylene oxide and resin added. In both cases the mixture of specimen and acetone/resin or ethanol/resin was left 5 h or overnight to allow for evaporation of acetone and propylene oxide. The resin was replaced with fresh 100% resin (two changes, 4 h in each change), and the material polymerized at 70°C for at least 8 h. Thin sections were stained for 30 min in aqueous uranyl acetate, followed by 30 min in lead citrate. Observations were carried out on a JEM-1010 (JEOL Ltd, Tokyo, Japan) transmission electron microscope at the Biological Institute, University of Copenhagen, using Gatan 792 or Gatan SC1000W digital cameras (Pleasanton, CA, USA).

Strains HOs24 and SP44 were tested for microcystin production by high performance liquid chromatography (HPLC) analysis as outlined in Henriksen (1996). Detailed protocols are described in Nguyen *et al*. (2007b).

Exponentially growing clonal cultures (*c*. 10 ml) of HOs24 and five additional filamentous freshwater species of cyanobacteria [viz. *Arthrospira massartii* Kufferath, *Lyngbya* sp., *Phormidium uncinatum* (C. Agardh) Gomont ex Gomont, *Planktothrix zahidii* (Faridi & Khalil) Anagnostidis & Komárek and *Pseudanabaena mucicola* (Naumann & Huber-Pestalozzi) Bourrelly] established from Vietnam were harvested by centrifugation (1200 × *g*) for 15 min. The pellets were kept frozen until DNA extraction. Extraction of total genomic DNA was done according to the CTAB protocol of Doyle & Doyle (1987). Eluded DNA was kept at –18°C until PCR amplification.

Partial sequences of *cpc*B and *cpc*A (including the intergenic spacer) were amplified using primers PCβF and PCαR (Neilan 1995). PCR conditions were as follows: preheating for 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; the final elongation cycle was 6 min at 72°C. PCR amplified fragments were electrophoresed in a 2% Nusieve agarose gel with ethidium bromide and checked under ultraviolet light. Amplified fragments of correct length were purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany) following the recommendations of the manufacturer. Purified products (approx. 20 ng μl^{-1}) were sequenced using the amplification primers. Sequencing reactions were run on an ABI Prism 377 DNA sequencer (Perkin-Elmer, San Jose, California, USA) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA).

The forward primer WAW 8F was used in combination with the reverse primer WAW 1486R (Wilmotte *et al*. 1993) to amplify 1316 base pairs of 16S rRNA. Internal primers CYA781F, CYA781R and CYA359F (Nübel *et al*. 1997)

were used to determine the 16S rRNA sequence. The PCR temperature profile used to obtain nearly complete 16S rRNA sequence was an initial denaturation of 30 s at 94°C, then 35 cycles of amplification of 1 min at 94°C, 30 s at 63°C and 2 min at 72°C, followed by a 7 min extension at 72°C.

PCR products were cloned into competent *Escherichia coli* cells using TOPO TA Cloning Kits® (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA). Positive clones (60 clones of HOs24 and seven clones of UTEX SP44) were PCR amplified, and fragments were visualized on 2% agarose gels to confirm that cloned fragments were of the appropriate length. PCR products were analyzed by restriction fragment length polymorphism analysis, using *Hin*f I and *Hha*I enzymes (New England BioLabs, Ipswich, MA, USA) and grouped according to fragment patterns. From each group two clones were picked at random and sequenced at the sequencing facility provided by Macrogen (Seoul, Korea). The obtained partial sequences were assembled using Sequencher 3.0 and blasted in the NCBI gene database to establish which sequences had cyanobacterial origin.

For molecular phylogeny, PC sequences of strain HOs24 were aligned with sequences of five other filamentous cyanobacteria from Vietnam (Nguyen *et al*. 2007a, b) and 26 cyanobacterial sequences retrieved the GenBank (Table S1). A total of 520 base pairs of partial *cpc*B and *cpc*A DNA sequences excluding the intergenic spacer fragment were analyzed using Maximum Parsimony (MP) and Neighbour-joining (NJ). For these analyses we used PAUP* ver. 4b.10 (Swofford 2003). Phylogenetic analyses were conducted as in Hansen *et al*. (2007). Bayesian analysis (BA) was performed using MrBayes (ver. 3.12; Ronquist & Huelsenbeck 2003). A total of 1×10^6 generations were run, and after 6500 generations the burn-in was reached. This resulted in 19,871 trees, which were all used to compute a 50% majority rule consensus tree.

For 16S rRNA phylogeny, partial sequences of strain HOs24 and *Pseudanabaena galeata* were aligned with 34 cyanobacteria sequences from GenBank (Table S2). The data matrix comprised 1316 base pairs and was analyzed using MP, NJ and BA with the same settings as for the analysis of *cpc*B–*cpc*A.

The new species and genus name was described following the requirements of the International Code of Botanical Nomenclature (McNeill *et al*. 2006).

RESULTS

Live trichomes of strain HOs24 were green, straight and solitary, constricted at the cross walls and lacked a sheath. Trichome length varied from 45 to 230 μm. Cells were cylindrical, about 2–2.5 μm wide and 6–12 μm long. End cells were rounded (Figs 1–4). In thin-sectioned material, the cells mostly contained radially arranged thylakoids, except in the central area, which contained the genetic material (Figs 5, 6 and 9). The cell wall had the usual cyanobacterial structure – a cytoplasmic membrane, a peptidoglycan layer and an outer membrane. It was about 45–50 nm thick (Figs 11–13). The peptidoglycan layer (M2) was 8.5–12.5 nm thick and was separated from the inner membrane (M1) and the outer membrane (M3) by a distance of 25–30 nm. The cross-wall was formed by aggregation of the two peptidoglycan layers (Fig. 13). The outer membrane was a normal three-layered unit membrane (Fig. 12). An indistinct pore system traversing the cell wall was present near the septum (Fig. 13). However, no pores were observed to penetrate the septa. Cyanophycin granules were located between the thylakoids, usually near the cell wall (Figs 7, 11, 13). The peripheral area of the cells also contained scattered polyphosphate granules, polyhedral bodies and glycogen granules (Fig. 9). No aerotopes were found. The thylakoids were arranged more or less radially, approximately at right angles to the cell wall, and ran parallel to the long axis of the trichome. They formed blocks of parallel lamellae. During cell division, thylakoids were cleaved by the developing septa (Fig. 10). To examine whether the arrangement of the thylakoids remained stable at different light intensities, a culture was maintained at low light intensity and examined with TEM. This had no effect on thylakoid arrangement (Fig. 8).

In dense cultures, *Pseudanabaena galeata* (UTEX SP44) formed a green mat of filaments with a fine sheath. Trichomes were straight and constricted at the crosswalls. Individual cells were cylindrical, 1–2 μm wide and 1.5–3.5 μm long. End cells were rounded (Fig. 14). The thylakoids were arranged concentrically in cross section, parallel to the long axis (Figs 15, 16) with four to five thylakoids. Cyanophycin granules were observed in the peripheral area (Figs 15, 16). Aerotopes were not observed.

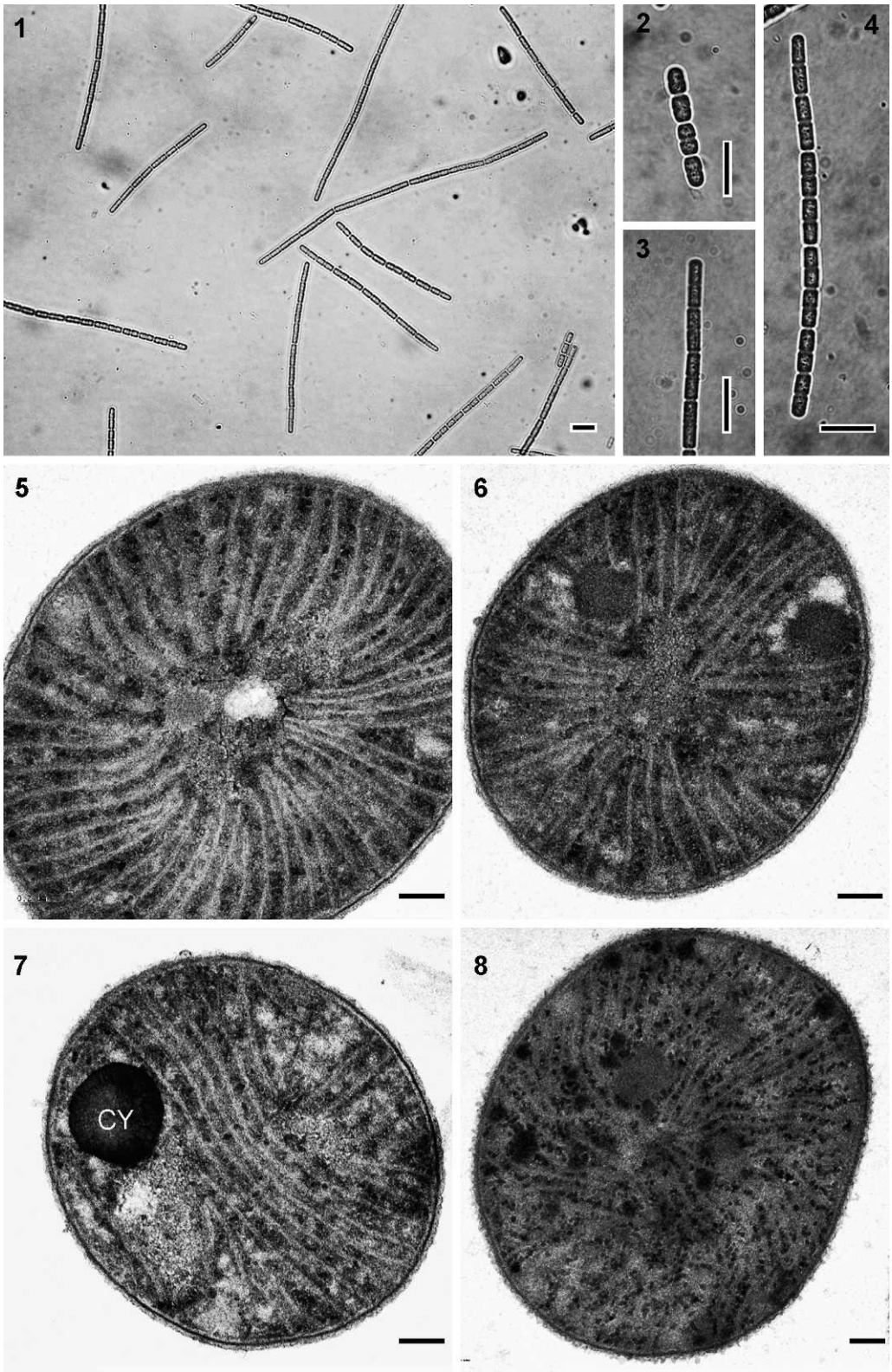
Thin sections of the two strains of *Pseudoanabaena catenata* also showed the presence of concentric thylakoids, similar to *P. galeata* (Figs 17, 18). Strain SAG 254.80 had aerotopes at the cross walls (not illustrated) whereas strain SAG 1464-1 lacked aerotopes (Fig. 18).

When analyzed for microcystin toxicity by HPLC, strain HOs24 produced microcystin while UTEX SP44 did not produce microcystin (compare Fig. 19 and 20).

Phylogenetic analyses based on partial PC sequences and including strain HOs24, five Vietnamese filamentous cyanobacteria (Nguyen *et al*. 2007a, b) and 26 sequences from GenBank are illustrated in Fig. 21. *Pseudanabaena mucicola* (Naumann & Huber-Pestalozzi) Schwabe (strain HP26) from Hue formed a highly supported clade with the two *Pseudanabaena* sequences from GenBank (Fig. 21). Strain HOs24 groups with a cluster comprising *Phormidium molle* (Kützing) Gomont and the three *Pseudanabaena* species. This relationship was highly supported in BA [posterior possibility (pp) = 0.95], but it was not supported by MP and NJ (bootstrap $< 50\%$).

Phylogenetic analyses of partial 16S rRNA sequences of strain HOs24, *Pseudoanabaena galeata* (strain UTEX SP44) and 34 cyanobacterial sequences of 16S rRNA from GenBank are shown in Fig. 22. Support values were generally low, but strain HOs24 was clearly separate from the two species of *Pseudanabaena* included [viz. *P. galeata* (strain SP44) and *P. tremula* Johansen & Casamatta (UTCC471)]. Strain HOs24 formed a sister taxon to *Planktothrix*. This relationship received no support from MP, but it had 89% support from NJ and 0.99 posterior probability from BA.

To further explore the relationship between the Vietnamese strain Hos24 and *Pseudanabaena galeata* (SP44), we estimated the divergence in percentage of the 16S rRNA



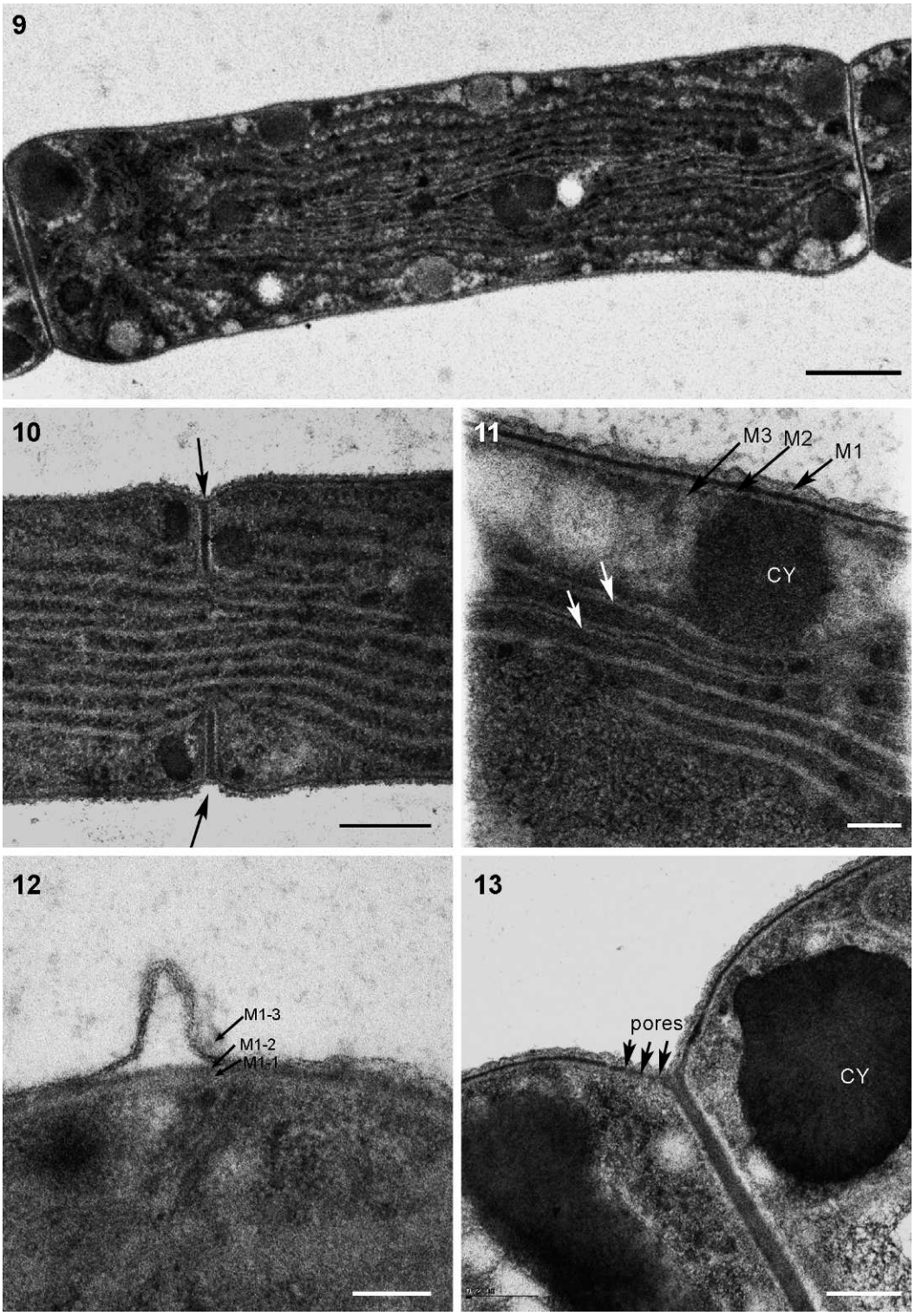
Figs 1–8. *Annamia toxica* gen. et sp. nov. Strain HOs24.

Figs 1–4. Light micrographs of a clonal culture showing solitary, cylindrical, straight trichomes (Fig. 1); trichome deeply constricted at the cross walls (Fig. 2); long trichome with cylindrical cells (Fig. 3); long trichome with flattened end cells (Fig. 4). Scale bars = 10 μm.

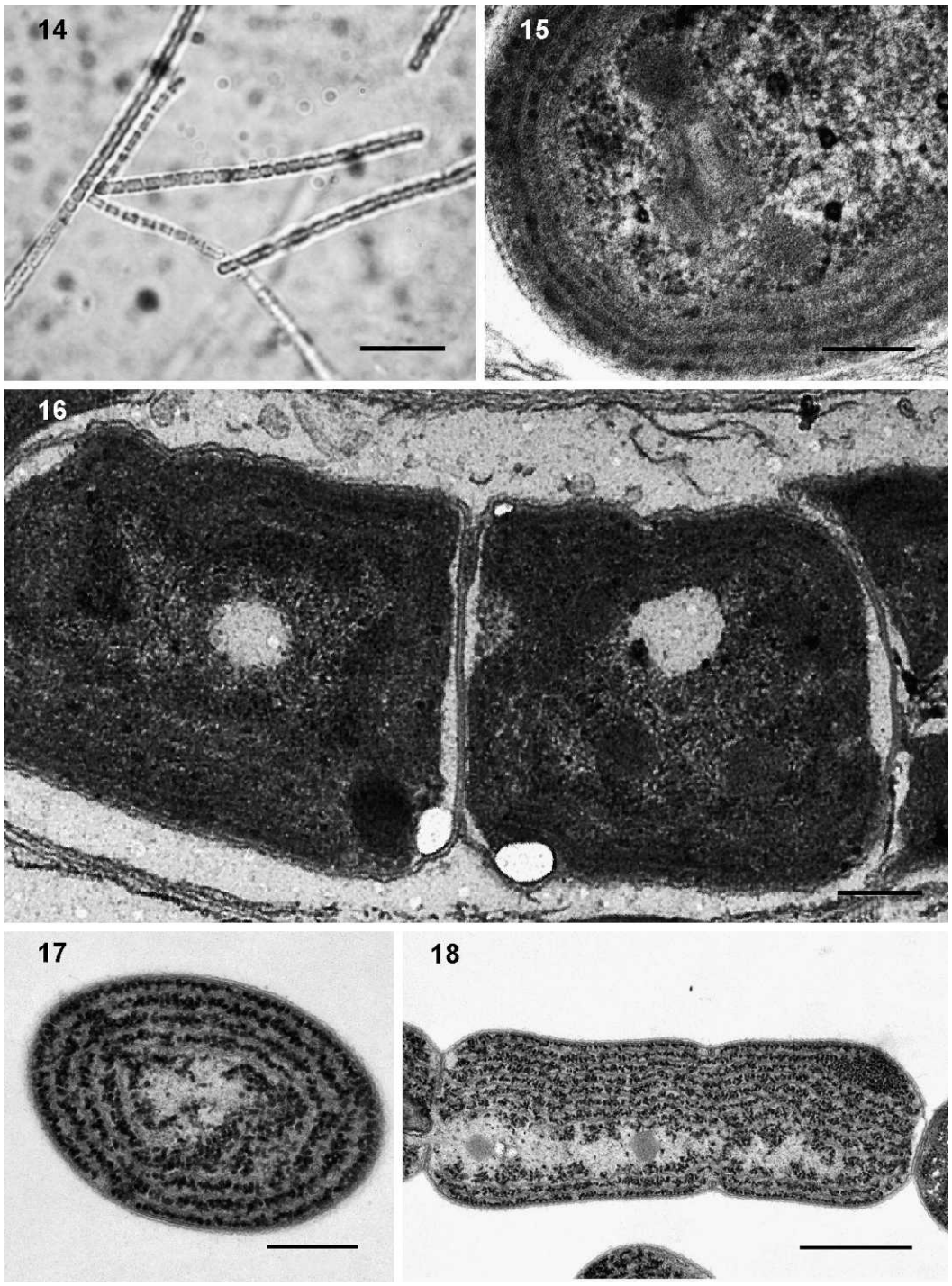
Figs 5–8. TEM, transverse sections showing the arrangement of thylakoids. Scale bars = 0.2 μm.

Figs 5–7. Filaments cultured under normal light intensity. CY = cyanophycin granule.

Fig. 8. Filament grown at low light intensity.



Figs 9–13. *Annamia toxica* gen. et sp. nov. Strain HOs24.
Fig. 9. Longitudinal section of cells showing the constriction at cross walls. Scale bar = 1 μm.
Fig. 10. Longitudinal section through dividing cell showing formation of transverse septum (arrows). Scale bar = 0.5 μm.
Fig. 11. Longitudinal section, cell wall layers (black arrows); M1 = outer membrane; M2 = peptidoglycan layer; M3 = cytoplasmic membrane; CY = cyanophycin granule near the cell wall; additional membrane present within thylakoids (white arrows). Scale bar = 0.1 μm.
Fig. 12. Outer three-layered membrane (arrows). Scale bar = 0.1 μm.
Fig. 13. Longitudinal section through area near transverse septum showing pores (arrows). Scale bar = 0.2 μm.



Figs 14–16. *Pseudanabaena galeata*. Strain UTEX SP44.
Fig. 14. Light micrographs showing single filaments. Scale bar = 10 μm.
Figs 15–16. TEM.
Fig. 15. Transverse section showing parietal arrangement of four to five thylakoids. Scale bar = 0.2 μm.
Fig. 16. Longitudinal section. Note cyanophycin granules and parallel thylakoids. Scale bar = 0.5 μm.
Figs 17–18. *Pseudanabaena catenata*. Strain SAG 1464-1. TEM.
Fig. 17. Transverse section showing parietal arrangement with four thylakoids. Scale bar = 0.5 μm.
Fig. 18. Longitudinal section showing parallel thylakoids and constrictions with cross walls. Scale bar = 1 μm.

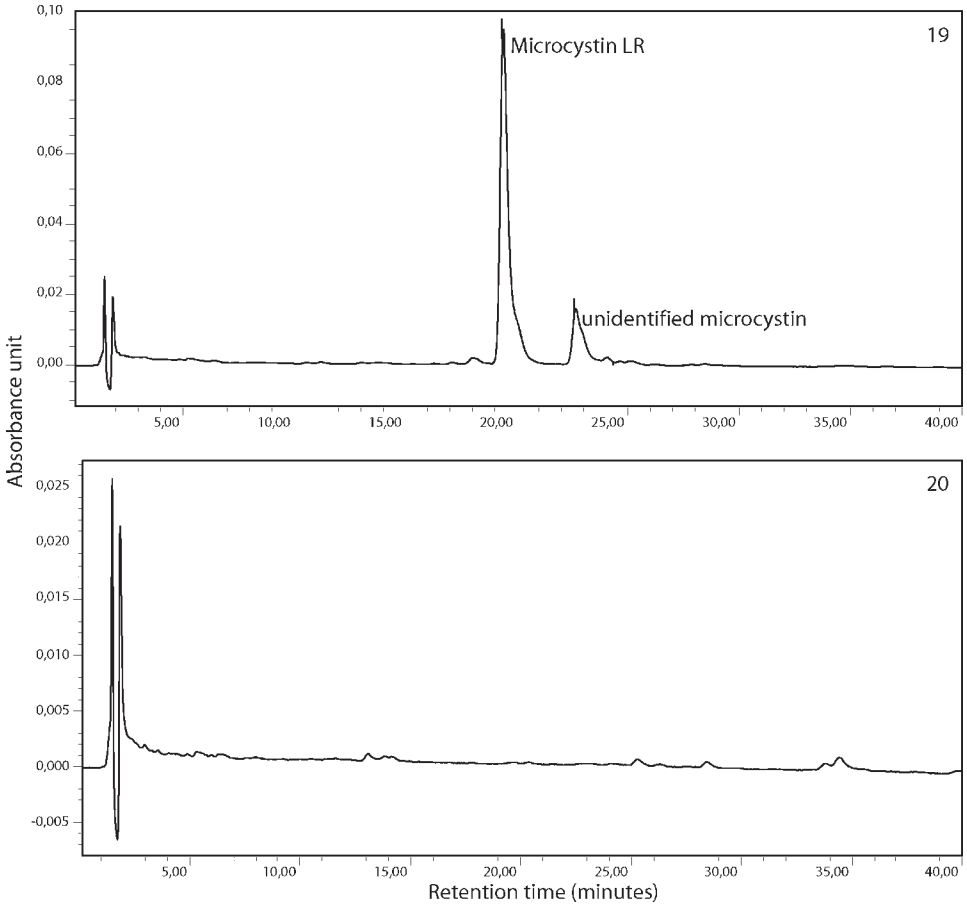


Fig. 19. HPLC chromatograms of extracts of freeze-dried cells of *Annamia toxica* (strain HOs24) and *Pseudoanabaena galeata* (strain SP44).
Fig. 19. High peak of microcystin-LR and one peak of unidentidied microcystin in *A. toxica*.
Fig. 20. Microcystin peaks lacking in *P. galeata*.

sequences using the Kimura-2-parameter model. This revealed a sequence divergence of 15.1% (data not shown). For reasons of comparison at the generic level we also estimated the sequence divergence between the type species of five other cyanobacteria included in this study [i.e. *Limnothrix redekei* (van Goor) M.E. Meffert, *Microcystis aeruginosa* (Kützing) Kützing, *Dolichospermum flos aquae* ([Lyngbye] Bornet & Flahault) Wacklin, Hoffman & Komárek, *Planktothrix agardhii* (Gomont) Anagnostidis & Komárek and *Trichodesmium erythraeum* Ehrenberg]. Here the sequence divergence varied between 11.0% and 12.1%. When including strain HOs24 and *P. galeata* in all pairwise comparisons (i.e. a total of 7 taxa), the sequence divergence varied between 10.3 and 15.1% (data not shown). Hence, the maximum sequence divergence was between strain HOs24 and *Pseudanabaena galeata*, showed that they were only distantly related.

DISCUSSION

Strain HOs24 agrees with typical *Pseudanabaena* in lacking a sheath; having solitary, narrow (1.5–3.5 μm wide), straight trichomes with constrictions at the cross walls; having mostly cylindrical cells; lacking aerotopes; and having non-attenuated trichome ends (Anagnostidis & Komárek 1988). The shape agrees with *Pseudanabaena moniliformis* from Lake Victoria, which has short, deeply constricted trichomes, and up to 18 (–26) cells in the trichome (Komárek & Kling 1991). However, cell dimensions of the Vietnamese cells are larger (2.5–5.6 μm ± 2 μm). The species from Lake Victoria has not been examined by TEM.

In the transmission electron microscope, thin sections of the specimen from Vietnam showed the thylakoids to be arranged radially. This arrangement was independent of the light intensity under which the cultures were grown. *Pseudanabaena* sensu Komárek & Čáslavská (1991), however, is characterized by a more or less concentric arrangement of the thylakoids, and this was confirmed in thin sections of our strain of *P. galeata*. The pattern of thylakoids in HOs24 is similar to the thylakoid arrangement of *Komvophoron bourrellyi* Turon, M. Hernández-Mariné & J. Catalan, an epibiote on ascidians from the Mediterranean Sea (Turon *et al.* 1991). The radial arrangement has also been found in some species of the Phormidiaceae: *Phormidium boryanum* (Bory ex Gomont) Anagnostidis & Komárek (Anagnostidis & Komárek 1988), *Trichodesmium thiebautii* Gomont ex Gomont (Gantt *et al.* 1984) and *Tychonema bourrellyi* (J.W.G. Lund) Anagnostidis & Komárek (Komárek & Albertano 1994). Morphological features considered being diagnostic of Phormidiaceae such as the presence of sheaths

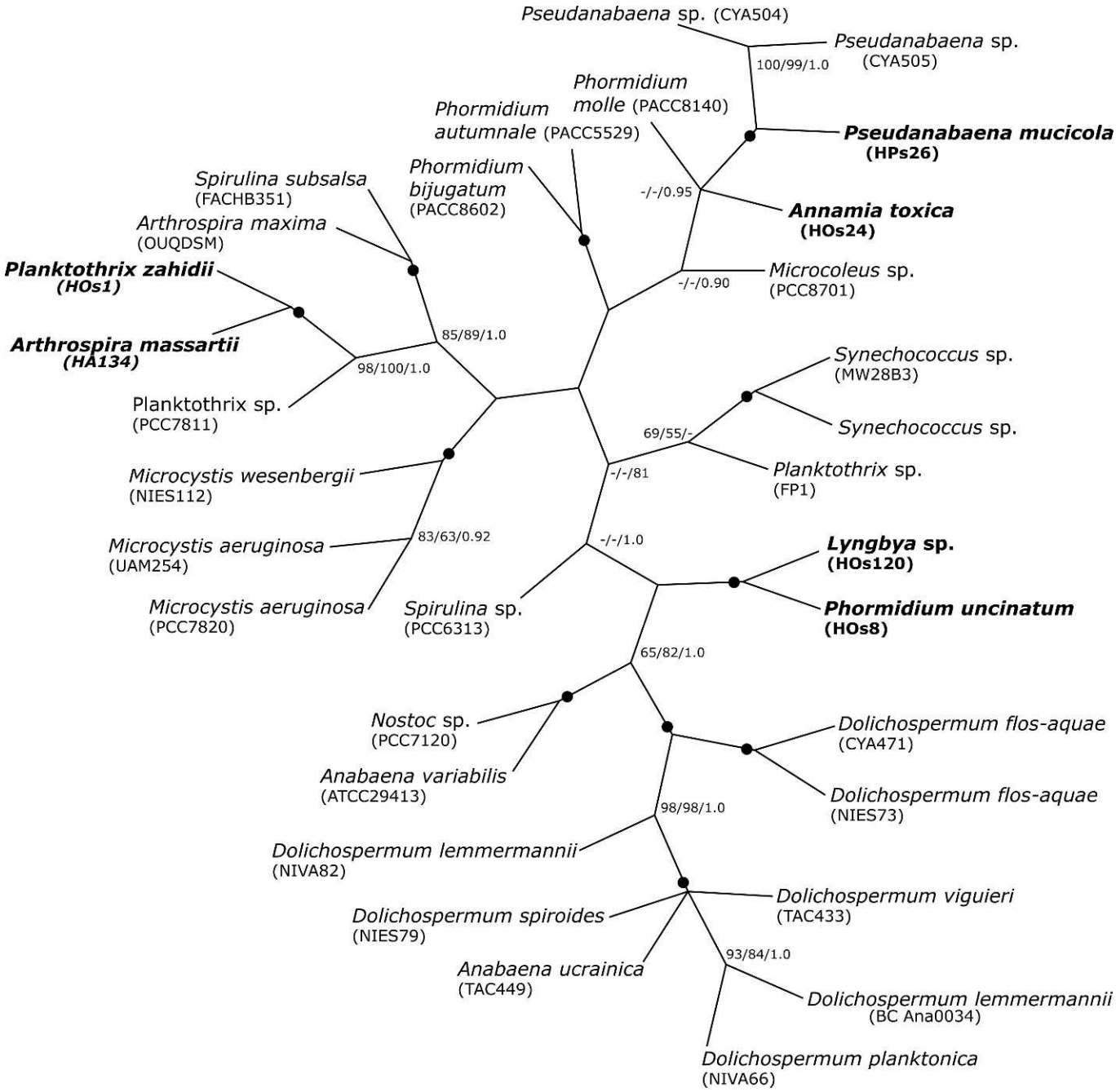


Fig. 21. Unrooted phylogenetic tree of *Annamia toxica* strain HOs24 and 31 other cyanobacteria based on partial phycocyanin sequences (the data matrix comprised 520 base pairs). The tree is a strict consensus of four equally parsimonious trees [each 1427 steps long; CI (consistency index) = 0.420 and RI (retention index) = 0.684]. It was obtained from MP analysis with 1000 random additions. Numbers indicate bootstrap values from MP, followed by bootstrap values from NJ and posterior probabilities from BA. Only bootstrap values $\geq$ 50% or posterior probabilities $\geq$ 0.5 are indicated. Filled black circles indicate the highest possible support values (100% in MP and NJ; posterior probability of 1 in BA). The Vietnamese cyanobacteria determined in this study are bold faced. See Wacklin et al. (2009) for taxonomy of *Dolichospermum*.

and necridic cells were not found in our material, and members of the Phormidiaceae do not have deep constrictions at the cross-walls.

In this study, lines of pores were observed in the area near the cross walls. Pores situated in side walls near the cross walls are characteristic of the family Pseudanabaenaceae (Anagnostidis & Komárek 1988). Pores were also observed near the cross walls in *Komvophoron bourrellyi* (Turon *et al.* 1991). A circular row of pores in the region near the septum has been observed in *Microcoleus chthonoplastes* Thuret ex Gomont (Phormidiaceae) by Hernández-Mariné (1996), who suggested, however, that pore arrangement should be used with care in taxonomy since the same arrangement is present in different families.

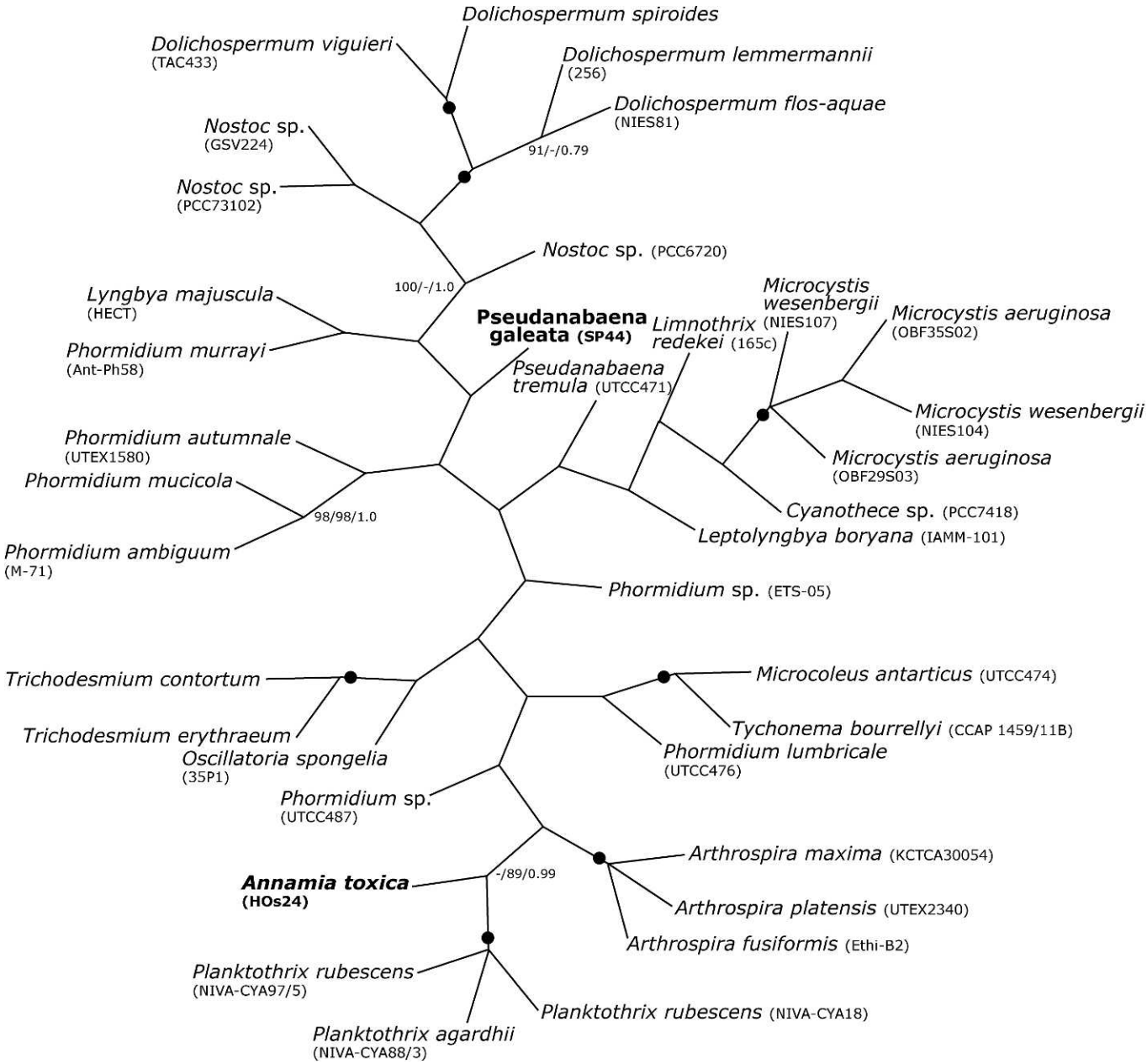


Fig. 22. Unrooted phylogenetic tree of *Annamia toxica* strain HOs24, *Pseudanabaena galeata* strain UTEX SP44 and 34 other cyanobacteria based on partial 16S rRNA sequences (data matrix comprised 1316 base pairs). The tree shown is a strict consensus of six equally parsimonious trees (each 1713 steps long; CI = 0.388 and RI = 0.598). It was obtained from parsimony analysis with 1000 random additions. Numbers indicate bootstrap values from MP, followed by bootstrap values from NJ and posterior probabilities from BA. Only bootstrap values $\geq$ 50% or posterior probabilities $\geq$ 0.5 are indicated. Filled black circles indicate the highest possible support values (100% in MP and NJ; posterior probability of 1 in BA). The cyanobacteria determined in this study are bold faced. See Wacklin et al. (2009) for taxonomy of *Dolichospermum*.

Microcystin-producing ability was first shown in *Microcystis aeruginosa* (Kützing) Kützing (Botes *et al*. 1985; Watanabe *et al*. 1988) and subsequently in other *Microcystis* species (Watanabe *et al*. 1988; Bittencourt-Oliveira *et al*. 2005). Later it was discovered in genera such as *Anabaena*, *Anabaenopsis*, *Nostoc*, *Planktothrix, Hapalosiphon* (as summarized by Codd *et al*. 2005) and *Arthrospira* (Ballot *et al*. 2005). Species of *Pseudanabaena* have also been shown to produce microcystins (Mez *et al*. 1997; Ballot 2003). Oudra *et al*. (2001, 2002) found microcystins in cultured strains of *P. mucicola* and *P. galeata* from Morocco. *Pseudoanabaena galeata* UTEX SP44 was selected for a comparative study; however, this strain proved to be non-toxic. Strains of other species of *Pseudanabaena* (from the NIVA culture collection) have been shown to be toxic (Edvardsen *et al*. 2004). Strain HOs24 was also found to produce microcystins. Its toxicity is further discussed in Nguyen et al. (2007b) as *Pseudanabaena* cf. *moniliformis* (p. 41 and table 2).

In the analyses of phycocyanin and 16S rRNA nucleotide sequences, the heterocytous species were monophyletic, while unicellular and simple filamentous forms were polyphyletic (Figs 21, 22). This pattern has also been found in many other studies as summarized by Hoffmann *et al.* (2005). In our study, phycocyanin and 16S rRNA gene sequence analyses from strain HOs24 separated this species from *Pseudanabaena* (viz. *P. mucicola* strain H126 and *P. galeata* UTEX SP44) and *Pseudanabaena* available in GenBank. This phylogenetic separation was also supported by sequence divergence estimates base on 16S rRNA. The phylogenetic tree based on 16S rRNA suggested that HOs24 is a sister taxon to *Planktothrix*. However, individual species in this cluster differ markedly. The main characters of *Planktothrix* (such as cross walls not constricted, cells shorter than wide, aerotopes present) were not found in strain HOs24.

In the phylogenetic tree based on 16S rRNA (Fig. 22), strain HOs24 clustered with some species having a radial arrangement of thylakoids, e.g. *Tychonema bourrellyi* (Komárek & Albertano 1994) and *Microcoleus antarcticus* Casamatta & Johansen (Casamatta *et al.* 2005). On the other hand, *Pseudanabaena galeata* (SP44) was closer to species with the parietal arrangement of thylakoids, e.g. *Limnothrix redekei* (Van Goor) Meffert (Gkelis *et al.* 2005) and *Pseudanabaena tremula* Johansen & Casamatta (Casamatta *et al.* 2005). The topology based on 16S rRNA and the arrangement of thylakoids therefore appear to support the division of species having a radial and a concentric pattern of thylakoids. Species with a radial arrangement of thylakoids are found in the families Phormidiaceae and Borziaceae of the order Oscillatoriales (Komárek & Kaštovský 2003, Hoffmann *et al.* 2005). The molecular analyses did not reveal a relationship between strain HOs24 and members of the Phormidiaceae. Unfortunately no complete sequences of Borziaceae were available in GenBank (as of July 2012).

Based on the morphological comparison (constriction at the cross-walls, absence of sheaths and necridic cells), strain HOs24 was more closely related to members of the family Borziaceae than to members of the Phormidiaceae. Two of the genera within this family, *Borzia* and *Komvophoron* possess mostly barrel-shaped cells which are shorter than wide. In strain HOs24 cylindrical cells are prevalent. *Borzia* has very short trichomes (a maximum of 16 cells), while those of HOs24 are longer. Two other members of Borziaceae, *Sinaiella* (with attenuated filaments and heteropolar) and *Yonedaella* (with trichomes in spherical or ellipsoid colonies) (Komárek & Anagnostidis 2005) are clearly different from HOs24. Based on sequence data and ultrastructural features, the establishment of a new genus for stain HOs24 from Hue is justified. Furthermore we assign it to the family Borziaceae within the order Oscillatoriales.

Taxonomic descriptions

Annamia* Nguyen *gen. nov.

Trichomata solitaria, cylindrica, ad 3 μm lata, ad septa constricta. Vagina deest. Cellulae cylindricae, longiores quam latae. Cellulae terminales rotundatae. Thylacoides radiales. Akineta heterocytaque non visa.

Trichomes solitary, cylindrical, up to 3.5 μm wide, constricted at the cross walls. Sheaths absent. Cells cylindrical, longer than wide. Terminal cells rounded. Thylakoids arranged radially. Akinetes and heterocytes not observed.

TYPE SPECIES: *Annamia toxica* sp. nov.

Annamia toxica* Nguyen *sp. nov.
Figs 1–13

Trichomata recta, mobilia (tremula); cellulis 4–8 vel numerosis, typice 20–46 μm sed in cultura ad 229 μm longis. Cellulae vulgo glaucae, granulis aliquot dispersis, divisione incipiens vulgo longe cylindricae vel cupiformes, 1.5–3.5 μm latae et 6.3–12.0 μm longae. Cellulae terminales complantae vel subconicae. Cellulae necridiae et akineta et hyterocytes non visa.

Trichomes straight, motile (trembling) with 4–8 or many cells. Trichomes typically 20–46 μm, but up to 229 μm long in culture. Cells mostly blue-green, with scattered granules, usually long cylindrical or barrel-shaped when starting division, 1.5-3.5 μm wide and 6.3-12.0 μm long. Terminal cells flattened or slightly conical. Necridic cells, akinetes, aerotopes and heterocytes not observed.

This species was found in Huong River, Hoamy Reservoir and in some bloom samples from Dapda, Nhuy and Anluu in the Hue City, Vietnam (Nguyen *et al.* 2007a).

HOLOTYPUS: CAT 2458, fixed material embedded in resin and deposited at the Botanical Museum, Copenhagen.

ETYMOLOGY: *Annamia* is derived from the name of the ancient imperial kingdom of Annam, in which Hue was the capital.

TOXICITY: Produces microcystin.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/10-097.1.s1.

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