The Journal of Microbiology, December 2002, p.335-339 Vol. 40, No. 4

NOTE

Inferring the Molecular Phylogeny of Chroococcalian Strains (Blue-green algae/Cyanophyta) from the Geumgang River, Based on Partial Sequences of 16S rRNA Gene

Wook Jae Lee and Kyung Sook Bae*

Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Yusong, Daejeon 305-600, Korea

(Received November 4, 2002 / Accepted November 28, 2002)

Partial sequences of 16S rRNA gene of five chroococcalian blue-green algal strains, *Aphanothece nidulans* KCTC AG10041, *Aphanothece naegelii* KCTC AG10042, *Microcystis aeruginosa* KCTC AG10159, *Microcystis ichthyoblabe* KCTC AG10160, and *Microcystis viridis* KCTC AG10198, which were isolated from water from the Geumgang River, were determined and were inferred their phylogenetic and taxonomic positions among taxa of order Chroococcales. Most taxa of Chroococcales whose partial 16S rRNA gene sequences were aligned in this study, are clustered with other related taxa. *Aphanothece nidulans* KCTC AG10041 and *Aphanothece naegelii* KCTC AG10042 made a cluster with other European species of these genera, which supported 100% of the bootstrap trees with a very high sequence similarity (97.4-99.4%) in this study. Three strains, *Microcystis aeruginosa* KCTC AG10159, *M. ichthyoblabe* KCTC AG10160, and *M. viridis* KCTC AG10198, formed a cluster with other *Microcystis* spp. supported 100 % of the bootstrap trees with a similarity of 97.0-99.9% except for two strains. However, this phylogentic tree made no resolution among the species of *Microcystis* spp. The topology of the tree reconfirmed the taxonomic status of three species of *Microcystis*, identified in this study based on the morphology, as three colonial types of *Microcystis aeruginosa* com. nov. Otsuka *et al.* (1999c). The genera of chroococcalian cyanophytes are heterogeneously clustered in these sequence analyses. We suggest that more molecular studies on the genera of Chroococcales with reference strains, widely collected from restricted geographic or environmental ranges, get accurate taxonomic or phylogenetic determinations.

Key words: 16S rRNA, phylogeny, blue-green algae, Cyanophyta, Cyanobacteria, Chroococcales

Although the photosynthetic blue-green algae (cyanobacteria) occur in a wide range of habitats and their service to the global environment has been widely understood (Tyagi *et al.*, 1999; Rai *et al.*, 2000), reports on molecular data have suggested that there are lots of taxonomic problems in their traditional classification system based on the morphology (Komárek and Anagnostidis, 1986; Kürger *et al.* 1995; Lee and Bae, 2001). Cyanobacteria have usually been treated as a cluster of the plant kingdom, called as cyanophytes, in common blue green algae (Rai *et al.*, 2000) and have been classified into five groups including Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, and Stigonematales (Castenholz and Waterbury, 1989). Woese (1987) suggested that cyanophytes should be classified within the eubacteria of prokaryotes based on the small subunit of ribosomal RNA gene sequences.

Chroococcales have a unicellular habit with or without mucilage cluster. Other characteristics, which have been used as criteria for genera in the Chroococcales, depend on the shape of its cell and mucilage sheathes known to be variable according to physical conditions in the culture. These morphological characteristics have made classification of these taxa very difficult (Komarek and Anagnostidis, 1986; Otuska *et al.*, 1999b). Some nucleotide sequence analyses showed that cyanophycean species are genetically heterogeneous and they do not agree with the traditional classification system based on morphology (Wilmontte *et al.*, 1992; Nelissen *et al.*, 1994; Wilmontte *et al.*, 1994; Nelissen *et al.*, 1996). Recent studies on the 16S rRNA gene sequences have extended the knowledge

* To whom correspondence should be addressed.
(Tel) 82-42-860-4610; (Fax) 82-42-860-4677
(E-mail) ksbae@mail.kribb.re.kr

of the phylogenetic relationship among some prokaryotic strains and are usually used in identification of other cyanophytes (Nelissen *et al.*, 1994; Nelissen *et al.*, 1996; Ishida *et al.*, 1997; Otuska *et al.*, 1999a; Lee and Bae, 2001). Nübel *et al.* (1997) developed and tested a set of oligonucleotide primers for very specific amplification of partial 16S rRNA gene segments from photosynthetic cyanophytes and planktonic algae.

Several unicellular cyanophytes have been known to form blooms annually in the Geumgang River in Korea (Lee, 1999). However, there is little molecular taxonomic information on these species and a few morphological descriptions of these species.

In this study we isolated five unicellular cyanophytes (cyanobacteria) from the several habitats along the Geumgang River and determined the partial 16S rRNA gene sequences of these strains. We also inferred their phylogenetic and taxonomic position among taxa of the order Chroococcales based on these sequences.

The nonaxenic unicellular cyanophycean strains used in this study are *Aphanothece naegelii* KCTC AG10042, *A. nidulans* KCTC AG10041, *Microcystis aeruginosa* KCTC AG10159, *M. ichthyoblabe* KCTC AG10160, and *M. viridis* KCTC AG10198. After unialgal isolation with the spreading method, all of the strains were cultured in ALLEN medium by incubating in capped tubes without aeration at 23°C with fluorescent lamp illumination of 1500 Lux.

The cells were harvested by a process of centrifugation in a 1.5 ml micro tube and washed three times with 1 ml of distilled water and dried briefly in a vacuum before the DNA extraction. The genomic DNA extraction was made with a Dneasy® Plant Mini Kit (Qiagen Co., Gernmy) following the manufacturer's instruction.

The 16S rRNA gene segments were amplified by Polymerase Chain Reaction (PCR) in a fragment by using the primer sets CYA106-CYA781 (Nübel *et al.*, 1997). A negative control without the template was included in every set of PCR. The PCR reaction mixtures were prepared by following the manufacturers instruction (Taq DNA Polymerase™, Bioneer Co., Korea). PCR was performed in an automated thermal cycler (PE Applied Biosystems, Foster City, CA, U.S.A.). The initial cycle was carried out at 95°C for 2 min, followed by 20 cycles of 30 s at 94°C, 30 s at 60°C, and 2 min at 72°C. The final extension cycle was at 72°C for 10 min to achieve a complete primer extension. The amplified DNA was further purified using a High Pure PCR Product Purification Kit™ (Borhringer Mannheim Co., Germany).

The purified DNA was sequenced directly by using a BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, U.S.A.) following the manufacturer's recommendations. The sequence data were collected with an ABI Model PRISM 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA, U.S.A.). The sequences were aligned with other known 16S rRNA gene sequences of chroococcalian cyanophytes (Neilan *et al.*, 1997; Otuska *et al.*, 1999a) by using the PHYDIT program (Chun, 1995). The tree topology that was based on these aligned 16S rRNA gene sequences was reconstructed with Kimura's 2-parameter distance model and neighbor-joining methods by using the PHYLIP 3.5 package (Felsenstein, 1993; Kimura, 1980). The statistical significance of tree branches was assessed by incorporating the bootstrap resampling method with 1,000 replications (Felsenstein, 1985). The parsimony tree reconstructed by the heuristic search option of the PAUP version 4.0 beta (Swofford, 1998) was compared with the distance tree. The resultant phylogenetic tree was visualized by using the TreeView Program (Page, 1996).

All PCR amplifications of nonaxenic chrooroccalian cyanophytes from the Geumgang River were successful by use of a probe showing high specificity for cyanophytes (Nübel *et al.*, 1997) without cloning. The sequencing strategy generated a partial sequence ranging from position 65 to 710 of *Arthrospira* sp.'s sequences (accession number X75044). The total number of nucleotides compared was 660 base pairs including gaps or sites that were not determined. The sequences were submitted to the GenBank database under accession numbers from AY121353 to AY121357.

The Phylogenetic tree based on the aligned partial 16S rRNA gene sequences was constructed by using the neighbor-joining method and the most parsimony method. The topology of the tree was consistent between these trees (the most parsimony trees are not shown). A phylogenetic tree constructed by the neighbor-joining method is shown in Fig. 1.

Most taxa of Chroococcales whose partial 16S rRNA gene sequences were aligned in this study, are heterogenously clustered except for species of *Microcystis* and *Aphanothece*. Three strains isolated and identified in this study, *Microcystis aeruginosa* KCTC AG10159, *M. ichthyoblae* KCTC AG10160, and *M. viridis* KCTC AG10041, formed a cluster with other *Microcystis* strains supported 100% of the bootstrap trees with high similarity (97.0-99.9%). However, there is no phylogenetic resolution among species of *Microcystis* within a clade. Although it could not determine exactly their phylogentic relationship among chroococcalian taxa, the other two strains *Aphanothece nidulans* and *A. naegelii* formed a clade closely related with European strains, which supported 100% of the bootstrap trees with a very high sequence similarity (97.4-99.4%) in this study.

Aphanothece nidulans KCTC AG10041 is characterized by a spherical or ovoid colony with a gelatinous sheath and a cylindrical cell with 1.0-1.5 μm broad and up to ca. 3.0 μm long. The cells with straight or curved shape and gray to light blue green color were found in this strain. However, these characteristics were not confirmed

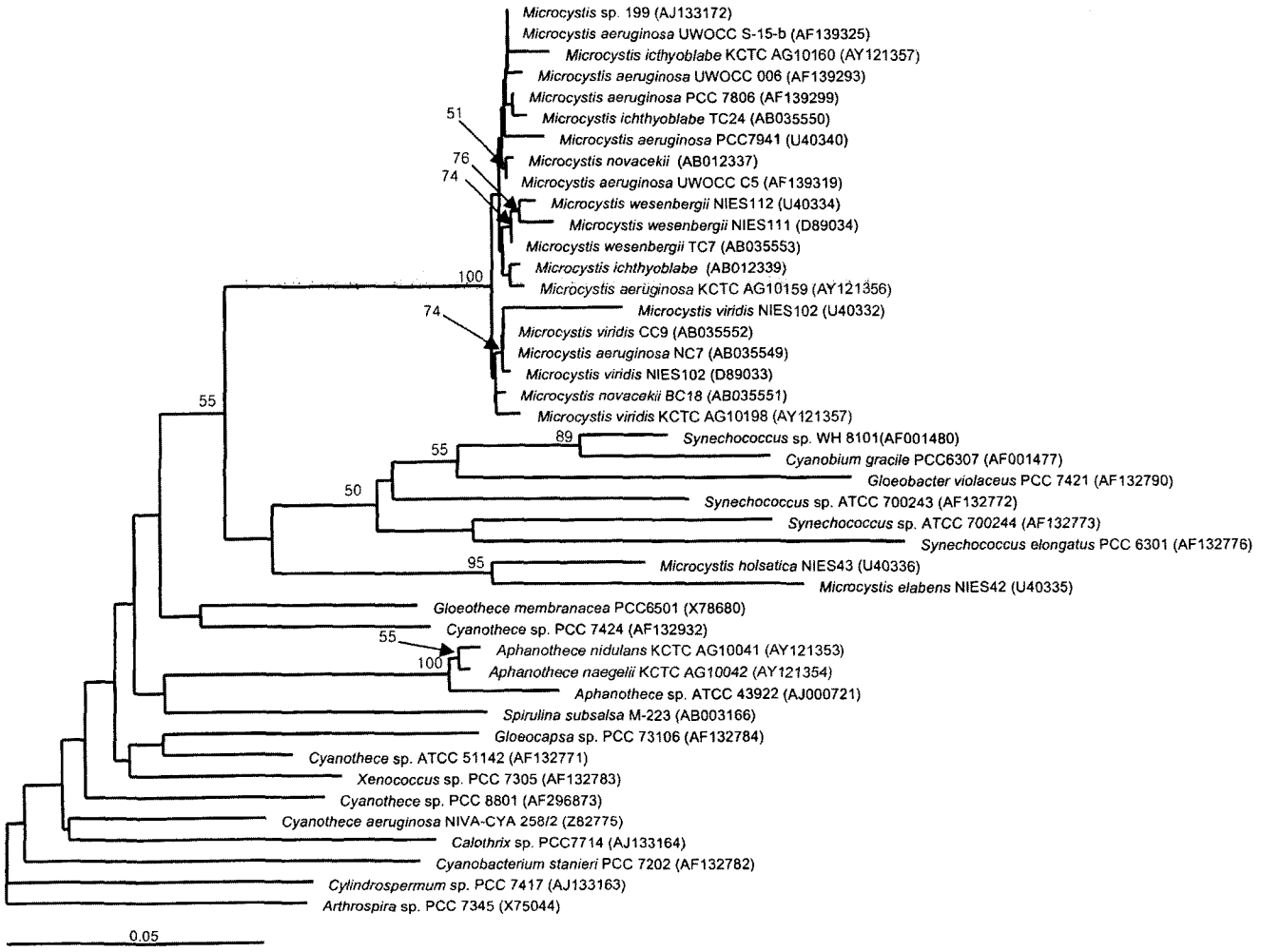

Fig. 1. Distance tree based on an alignment of 43 partial 16S rRNA gene sequences (660 positions) from chroococcalian cyanophytes, constructed by means of the neighbour-joining method. Bootstrap percentages higher than 50% are placed alongside the node considered. The scale bar indicates 0.05 substitutions per nucleotide.

in the cultured strains.

Aphanothece naegelii KCTC AG10042 is distinguished from strains of *A. nidulans* by an elongated ovoid cell 2.5-4.0 μm broad and up to ca. 6.0 μm long. The cells irregularly aggregated in the gelatinous sheath were collected from a small pit covered water plant on the side of the streams. These characteristics were less confirmed in the cultured strains.

As there have been few molecular and demographic reports on the species of *Aphanothece* except halotolerant strains from America (Gracia-Pichel *et al.*, 1998), it is difficult to determine the taxonomic and phylogenetic position among the species based on the 16S rRNA gene sequences. But two strains from the Geumgang River are clustered in a clade and formed a monophyletic relationship with strains of *Aphanothece* sp. ATCC43922 in this study.

The species *Microcystis* is characterized by a colony of spherical cells. There has been lots of discussion on the delimitation or the validity of the genus, however well conserved, following the suggestion of Kormárek (1957), with *M. aeruginosa* as type species. Nine species of *Microcystis* have been identified in Korean water (Lee *et al.*, 1997). *M. aeruginosa* with several other species, M. *ichthyoblae*, *M. viridis*, and *M. wessenbergii*, are the dominant species forming blooms in reservoirs and rivers(Lee *et al.*, 1997; Lee, 1999). A few reports (Lee, 1999) on chrooroccalian cyanophytes in the Geumgang River were done in ecological research but without any description of the morphological characteristics, in addition to their morphological plasticity, which has made a great confusion on identification of the species collected in this area.

M. aeruginosa KCTC AG10159 collected in this study has morphological characteristics such as densely arranged cells 3.5-6.7 μm in diameter, irregular colony shapes and conspicuous gas vacuoles. This species is the most common in the phytoplankton community and is a major species of water blooms in Korea, but there must be misleading

identification in listing because of the large morphological variation especially in colony shape and cell size.

M. ichthyoblabe KCTC AG10160 is characterized by spherical cell colonies with 2.5-4.5 μm in diameter and a thin colonial sheath. The thin sheath and soft aggregation of small cells are characteristic in identification. Young plants are very similar to *M. novacekii* in that they have a spherical shape and the same range in cell size but *M. novacekii* has a very thick and visible sheath (Lee *et al.*, 1997).

M. viridis KCTC AG10198 can be distinguished from two other strains by the refractive colony and perpendicular arrangements of large cells 5.0-8.5 μm in diameter. They have a gelatinous and visible colonial sheath with an undulate margin of sheath.

However, these characteristics of *Microcystis* are quite variable in the culture. Especially, the shapes of the colony and the gas vacuole could not be easily determined. Although there is a report on the transitional change in morphotypes between *M. aeuginosa* and *M. ichthyoblabe* (Kormárek, 1991), strains of each species used in this study are distinguished from each other based on the partial 16S rDNA sequences with very low similarity (Fig. 1). *M. viridis* KCTC AG10198 is closely related to other European strains and forms a clade including a strain of *M. aeuginosa*.

The phylogenetic relationships among the species of *Microcystis* based on rDNA sequence in this analysis did not agree with the morphological classification system. The *M. aeruginosa* strain collected in this study formed a clade with European *M. aeruginosa, M. novacekii*, and *M. viridis*. All of the species, *M. aeruginosa, M. ichthyoblabe, M. novacekii, M. viridis,* and *M. wesenbergii*, arranged in a clade, which normally has internal clades supported by very low bootstrap value (Fig. 1). The results were generally supported by recent phylogenetic studies based on DNA sequences that also noted a high level of genetic polymorphism among strains classified as species of *Microcystis* (Otuska *et al.*, 1998; Otuska *et al.*, 1999a; Bittencourt-Oliveira *et al.*, 2001). Otsuka *et al.* (1999a; 1999b) divided five species of *Microcystis* into three broad clusters based on the 16S rRNA gene and ITS sequences, in which two clusters contained primarily *M. viridis* and *M. wesenbergii*, but also *M. aeruginosa* strains. The others were genetically highly variable and contained a mix of strains classified as *M. aeruginosa*, *M. novacekii*, and *M. ichthyoblabe*. They also said this poor overall correlation of genotypes and phenotypes results from induced variation from culture, environmental effects on morphology and overlapping colonial morphology (Kato *et al.*, 1991; Kormárek, 1991, Kürger, 1995). The obscure taxonomic placement of strains into particular morphological species may result from all these factors. Otsuka *et al.* (1999c) classified six species of genus *Microcystis* into seven colonial types of *Microcystis aeruginosa* and made taxonomic determination under the International Code of Nomenclature of Bacteria (ICNB) based on the DNA sequences and other biochemical reports. Although three strains were identified as different species of *Microcystis* from the Geumgang River by cell size and colonial shape, they are also closely clustered with those strains of *Microcystis aerugionsa* com. nov. Otsuka *et al.*(1999c) based on these sequences. However, it is difficult to discuss the overall relationship of genus *Microcystis* with other genera, because other species, *M. holsatica* NIES43 and *M. elabens* NIES42, are more closely related to other genera of Chroococcales in the phylogenetic tree. We say that molecular studies on the genera of Chroococcales with the reference strains, widely collected from restricted geographic or environmental ranges, could have accurate taxonomic or phylogenetic determinations.

References

Bittencourt-Olveira, M.C., M.C. de Oliveira, and C.J.S. Bolch. 2001. Genetic variability of Brazilian strains of the *Microcystis aeruginosa* complex (Cyanobacteria/ Cyanophyceae) using the phycocyanin intergenic spacer and flanking regions (cpcBA). *J. Phycol.* **37**, 810-818.

Castenholz, R.E. and J.B. Waterbury. 1989. Taxa of the Cyanobacteria. *In* Bergeys manual of Systematic Bacteriology. Vol. 3, ed. by Staley, J.T., M.P. Bryant, N. Pfeng, and J.G. Holt, Williams and Wilkins, Baltimore, pp. 1727-1728.

Chun, J. 1995. Computer-assisted classification and Identification of actinomycetes. Ph. D. Thesis. University of Newcastle, UK.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783-791.

Felsenstein, J. 1993. PHYLIP (Phylogenetic inference package) version 3.5c. University of Washington, USA.

Gracia-Pichel, F., U. Nübel, and G. Muyzer. 1998. Ulrich, The phylogeny of unicellular, extremely halotolerant cyanobatceria. *Arch. Microbiol.* **169**, 469-482.

Ishida, T., A. Yokota, and J. Sugiyama. 1997. Phylogenetic relationships of filamentous cyanobacterial taxa inferred from 16S rRNA sequence divergence. *J. Gen. Appl. Microbiolol.* **43**, 237-241.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111-120.

Kato, T., M.F. Watanabe, and M. Watanabe. 1991. Allozyme divergence in *Microcystis* (Cyanophyceae) and its taxonomic inference. *Arch. Hydrobiol. Suppl. Algol. Stud.* **64**, 129-140.

Kormárek, J. 1957. Das "*Microcystis*"-problem. *Taxon* **6**, 145-149.

Kormárek, J. 1991. A review of water-bloom forming *Microcystis* species, with regard to populations from Japan. *Arch. Hydrobiol. Suppl. Algol. Stud.* **64**, 115-127.

Komárek, J. and K. Anagnostidis. 1986. Mordern approach to the classification system of Cyanophytes, 2-Chroococcales. *Arch. Hydrobiol. Suppl. Algol. Stud* **43**, 157-226.

Kürger, G.H., H. De Wet, J.L.F. Kock, and A.J.H. Pieterse. 1995. Fatty acid composition as a taxonomic characteristic for *Microcystis* and other coccoid cyanobacteria (blue-green alga) isolates. *Hydrobiologia* **308**, 145-151.

Lee, J.A., A.R. Choi, and M. Watanabe. 1997. Taxonomic implications of the genus *Microcystis* (cyanophyta) from the Naktong River. *Angae* **12**, 167-176.

Lee, J.H. 1999. Dynamics of phytoplankton community in Lake Daechung. *Korean J. Limnol.* **32**, 358-366.

Lee, W.J. and K.S. Bae. 2001. The phylogenetic relationships of several oscillatorian cyanobacteria, forming blooms at Daecheong Reservoirs, based on partial 16S rRNA gene sequences. *J. Microbiol. Biotechnol.* **11**, 504-507.

Neilan, B.A., D.Jacobs, T. De Dot, L.L. Blackall, P.R. Hawkins,P.T. Cox, and A.E. Goodman. 1997. rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. *Int. J. Syst. Bacteriol.* **47**, 639-697.

Nelissen, B., A. Willmontte, J. -M. Neefs, and R. De Wachter. 1994. Phylogenetic relationships among filamentous helical cyanobacteria investigated on the basis of 16S ribosomal RNA gene sequence analysis. *Syst. Appl. Microbiol.* **17**, 206-210.

Nelissen, B., R. De Baere, A. Willmontte, and R. De Wachter. 1996. Phylogenetic relationships of nonaxenic filamentous cyanobacterial strains based on 16S rRNA sequence analysis. *J. Mol. Evol.* **42**, 194-200.

Nûbel, U., F. Garcia-Pichel, and G. Muyzer. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl. Environ. Microbiol.* **63**, 3327-3332.

Otuska, S., S. Suda, R. Li, M. Watanabe, H. Oyaizu, S. Masumoto, and M.M. Watanabe. 1998. 16S rDNA sequences and phylogenetic analyses of *Microcystis* strains with and without phycoerythrin. *FEMS Microbiol. Lett.* **164**, 119-124.

Otuska, S., S. Suda, R. Li, M. Watanabe, H. Oyaizu, S. Masumoto, and M.M. Watanabe. 1999a. Phylogenetic relationships between toxic and nontoxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence. *FEMS Microbiol. Lett.* **172**, 15-21.

Otuska, S., S. Suda, R. Li, M. Watanabe, H. Oyaizu, S. Masumoto, and M.M. Watanabe. 1999b. Characterization of morpho-species and strains of the genus *Microcystis* (cyanobacteria) for reconsideration of species classification. *Phycol. Res.* **47**, 189-197.

Otuska, S., S. Suda, R. Li, M. Watanabe, H. Oyaizu, S. Masumoto, and M.M. Watanabe. 1999c. A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kützing ex Lemmermann 1907 under the rules of the bacterial code. *Int. J. of Syst. and Microbiol.* **51**, 873-879.

Page, R.D.M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *ACBIOS* **12**, 357-358.

Rai, L.C., H.D. Kumar, F.H. Mohn, and C.J. Soeder. 2000. Services of algae to the environment. *J. Microbiol. Biotechnol.* **10**, 119-136.

Swofford, D.L. 1998. PAUP. Phylogenetic analysis using parsimony. Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tyagi, M.B., J.K. Thakur, D.P. Singh, A. Kumar, E.G. Prasuna, and A. Kumar. 1999. Cyanobacterial toxins: The current status. *J. Microbiol. Biotechnol.* **9**, 9-21.

Wilmontte, A., S. Turner, Y. Van de Peer, and N. R. Pace. 1992. Taxonomic study of marine oscillatoriacean strains (cyanobacteria) with narrow trichomes. II. Nucleotide sequence analysis of the 16S ribosomal RNA. *J. Phycol.* **28**, 828-838.

Wilmontte, A., J.-M. Neefs, and R. De Wachter. 1994. Evolutionary affiliation of the marine nitrogen fixing cyanobacterium *Trichosedmium* sp. strain NIBB 1067, derived by 16S ribosomal RNA sequence analysis. *Microbiology* **140**, 2159-2164.

Woese, C.R. 1987. Bacterial evolution. *Microbiol. Rev.* **51**, 221-271.