

Genetic diversity and molecular phylogeny of *Planktothrix* (Oscillatoriales, cyanobacteria) strains from China

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ABSTRACT

The genus *Planktothrix* Anagnostidis et Komárek 1988 represents an important, water-bloom forming cyanobacterial group, and in recent years it has been found to occur in dense water blooms around China. In this study, genetic diversity and phylogeny of *Planktothrix* strains isolated from seventeen Chinese water bodies, based on 16S rRNA, *rbclX* and *rpoC1* genes, were investigated. These *Planktothrix* strains, all phycocyanin-containing, were morphologically classified into two groups as *Planktothrix agardhii* and *Planktothrix mougeotii*. However, phylogenetic analyses, based on the three gene regions, revealed that *Planktothrix* strains analyzed in this study were mainly divided into three clades, corresponding to *P. agardhii*, *Planktothrix pseudagardhii*, and *P. mougeotii* respectively. The strains in the *P. pseudagardhii* clade were shown to be more genetically divergent, reflecting possible existence of cryptic species in the genus *Planktothrix*.

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1. Introduction

The genus *Planktothrix* Anagnostidis et Komárek 1988 represents an important, water-bloom forming cyanobacterial group, and it has been classified within the order Oscillatoriales, family Phormidiaceae, subfamily Phormidioideae under the current botanical taxonomic system (Anagnostidis and Komárek, 1988; Komárek and Anagnostidis, 2005). The species of the genus *Planktothrix* were originally classified into the genus *Oscillatoria* (Geitler, 1932), due to their solitary trichomes without sheaths, heterocysts and akinetes. However, they are planktonic since their trichomes possess gas vesicles with the uniform distribution through the whole cells (Pringsheim, 1965; Anagnostidis and Komárek, 1988). Since it had been encountering long-termed difficulties in taxonomic delimitation of genus *Oscillatoria*, the establishment of genus *Planktothrix* was proven to make a great contribution to the solution of this problem, and separation of *Planktothrix* from *Oscillatoria* was supported by molecular 16S rRNA sequences (Rippka and Herdman, 1992; Wood et al., 2001; Suda et al., 2002). Suda et al. (2002) performed a thorough taxonomic research on water-bloom forming oscillatoriid cyanobacteria, and established a new genus *Planktothricoides* with the type of *Planktothricoides raciborskii* (originally *Planktothrix*

raciborskii) and a new species *Planktothrix pseudagardhii*. Since then, it is generally recognized that both the genera *Planktothrix* and *Planktothricoides* represent genetically two genetically delimited genera (Komárek and Komárková, 2004). They are well separated from other related oscillatoriid planktonic genera (*Tychonema*, *Limnothrix*) based on phylogenetic, phenotypic and cytological characters (Anagnostidis and Komárek, 1988; Wood et al., 2001; Komárek and Komárková, 2004; Suda et al., 2002).

Anagnostidis and Komárek (1988) described fourteen species of the genus *Planktothrix* when they founded the genus, but molecular data from several studies did not support the criteria used in differentiating species within the genus *Planktothrix* (Beard et al., 1999; Humbert and Le Berre, 2001), and these studies suggested the whole genus is monospecific. Suda et al. (2002) reevaluated the taxonomy of water bloom forming oscillatoriid cyanobacteria using a polyphasic approach based on a large number of strains, and provided clearly delimited descriptions for several *Planktothrix* species: *Planktothrix agardhii*, *Planktothrix rubescens*, *Planktothrix mougeotii* and *Planktothrix pseudagardhii*. Komárek and Komárková (2004) reviewed the taxonomy of the genus *Planktothrix*, and posed the revised description of fifteen species in this genus. These latter two studies provided a good basis for further solutions in addressing natural diversity and taxonomic classification within the genus of *Planktothrix*.

Planktothrix has been considered to be an important genus of harmful cyanobacteria since it is one of the most frequent microcystin (MC) producers (Kurmayer et al., 2005). It is well

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known that in European lakes natural *Planktothrix* populations are mainly dominated by either the red-pigmented *P. rubescens* or the green-pigmented *P. agardhii*, being both species producers of MCs with specific ecological niches in lakes of the temperate zone (Scheffer et al., 1997; Muret and Skulberg, 1999). In general, green-colored *P. agardhii* co-occurs with other planktonic cyanobacteria, such as *Microcystis* spp. and *Anabaena* spp., which form dense blooms in shallow and eutrophic lakes (Rücker et al., 1997). By contrast, red-colored *P. rubescens* occurs in deep stratified and less eutrophic lakes, and often dominates phytoplankton communities completely (Anneville et al., 2004).

Molecular diversity of *Planktothrix* has been discussed based on 16S rDNA sequences (Suda et al., 2002), gas vesicle genes (Beard et al., 1999, 2000; Becker et al., 2005), the *rbcLX* gene (Rudi et al., 1998) and the *mcy* gene cluster (Mbedi et al., 2006). Nonetheless, these studies mainly focused on the *Planktothrix* populations and strains from European countries. Towards a refined view of the phylogenetic relationship and delimited species within the genus of *Planktothrix*, strains from other global regions (outside Europe), are needed for further examination. Additionally, it has been pointed out that morphological species delimitation tends to be problematic within the microalgae (cyanobacteria): many cases of cryptic species diversity are being disclosed through application of molecular phylogenetic methods (Kooistra, 2002; Zuccarello and West, 2003; Verbruggen et al., 2007). Therefore, we anticipated the

present study following a molecular genetic approach will verify and elucidate whether cryptic species exist within species of the genus *Planktothrix* by using molecular genetic approaches.

During the last decades, China has been reported high incidences for the occurrence of water blooms, with the worst cyanobacterial bloom occurring in a large shallow lake—Taihu Lake in 2007 (Guo, 2007). *Planktothrix* spp., mostly green-pigmented *P. agardhii* like species, have been found to occur or co-occur with *Microcystis* spp. together as major species of water blooms in shallow lakes, reservoirs and ponds (Lin et al., 2008). In this study, we intended to expand the current phylogenetic framework of the *Planktothrix* genus using more gene sequence data from more strains. To achieve the primary knowledge of water bloom forming *Planktothrix* species in China, the following examinations were performed in the present study: (1) the morphological and molecular diversity of *Planktothrix* strains from Chinese waters; (2) the phylogenetic relationships within these strains.

2. Materials and methods

2.1. Strains and culture conditions

The strains used in this study are listed in Table 1. As shown in Fig. 1, sampling sites included seventeen fresh waterbodies located in eleven provinces of China, covering thirty-three degrees of

Table 1
Planktothrix strains investigated in this study.

| Strains | Origin | Collection date | Accession number | | |
|--------------------|-------------------------------------|-------------------|------------------|--------------|--------------|
| | | | 16S rRNA | <i>rbcLX</i> | <i>rpoC1</i> |
| <i>P. agardhii</i> | | | | | |
| HAB001 | Dianchi Lake, Kunming, Yunnan | 2006 | FJ184432 | FJ184497 | FJ173026 |
| HAB113 | Dianchi Lake, Kunming, Yunnan | May 7, 2006 | FJ184416 | NK | NK |
| HAB201 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184395 | FJ184491 | FJ173037 |
| HAB202 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184424 | FJ184479 | FJ173062 |
| HAB203 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184402 | FJ184487 | FJ173058 |
| HAB204 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184403 | FJ184484 | FJ173028 |
| HAB205 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184406 | FJ184492 | FJ173047 |
| HAB206 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184431 | FJ184504 | FJ173055 |
| HAB207 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184425 | FJ184481 | FJ173038 |
| HAB208 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184404 | FJ184482 | FJ173036 |
| HAB209 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184408 | FJ184490 | FJ173030 |
| HAB210 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184405 | FJ184494 | FJ173046 |
| HAB211 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184427 | FJ184469 | FJ173043 |
| HAB212 | A pond in Kunming City, Yunnan | August 1, 2006 | FJ184394 | FJ184503 | FJ173027 |
| HAB325 | A pond in Kunming City, Yunnan | April 27, 2006 | FJ184418 | FJ184498 | FJ173041 |
| HAB326 | A pond in Kunming City, Yunnan | April 27, 2006 | FJ184436 | FJ184474 | FJ173056 |
| HAB602 | A pond in Kunming City, Yunnan | October 25, 2006 | FJ184393 | FJ184475 | FJ173048 |
| HAB603 | A pond in Kunming City, Yunnan | October 25, 2006 | FJ184413 | FJ184486 | FJ173040 |
| HAB631 | A pond in Kunming City, Yunnan | January 8, 2007 | FJ184417 | FJ184499 | FJ173025 |
| HAB633 | A pond in Kunming City, Yunnan | January 8, 2007 | FJ184423 | FJ184501 | FJ173032 |
| HAB217 | South Lake, Wuhan, Hubei | August 10, 2006 | FJ184415 | FJ184502 | FJ173024 |
| HAB218 | South Lake, Wuhan, Hubei | August 10, 2006 | FJ184428 | FJ184485 | FJ173050 |
| HAB236 | South Lake, Wuhan, Hubei | August 10, 2006 | FJ184397 | FJ184480 | FJ173033 |
| HAB237 | South Lake, Wuhan, Hubei | August 10, 2006 | FJ184433 | FJ184483 | FJ173064 |
| HAB240 | South Lake, Wuhan, Hubei | August 10, 2006 | FJ184419 | NK | NK |
| HAB241 | South Lake, Wuhan, Hubei | August 10, 2006 | FJ184429 | FJ184495 | FJ173042 |
| HAB243 | Hang-Yong Canal, Shaoxing, Zhejiang | August 20, 2006 | FJ184396 | FJ184488 | FJ173049 |
| HAB244 | Hang-Yong Canal, Shaoxing, Zhejiang | August 20, 2006 | FJ184412 | FJ184473 | FJ173054 |
| HAB604 | Yuyuantan Park, Beijing | November 11, 2006 | FJ184399 | NK | NK |
| HAB605 | Yuyuantan Park, Beijing | November 11, 2006 | FJ184430 | FJ184489 | FJ173052 |
| HAB607 | Yuyuantan Park, Beijing | November 11, 2006 | FJ184407 | FJ184493 | FJ173044 |
| HAB612 | Yuyuantan Park, Beijing | November 11, 2006 | FJ184434 | FJ184471 | FJ173034 |
| HAB613 | Yuyuantan Park, Beijing | November 11, 2006 | FJ184398 | FJ184470 | FJ173060 |
| HAB619 | Yuyuantan Park, Beijing | November 11, 2006 | FJ184414 | FJ184467 | FJ173045 |
| HAB622 | Yuyuantan Park, Beijing | November 11, 2006 | FJ184400 | FJ184496 | FJ173053 |
| HAB635 | Yuyuantan Park, Beijing | November 11, 2006 | FJ184411 | FJ184449 | FJ173035 |
| HAB638 | East Lake, Wuhan, Hubei | February 2, 2007 | FJ184401 | FJ184477 | FJ173029 |
| HAB645 | Guangqiao Pond, Wuhan, Hubei | March 5, 2007 | FJ184409 | FJ184478 | FJ173051 |
| HAB655 | Ulungur Lake, Xinjiang | April 26, 2007 | FJ184420 | FJ184466 | FJ173059 |
| HAB1448 | Ulungur Lake, Xinjiang | April 26, 2007 | FJ184420 | FJ184464 | FJ173061 |

Table 1 (Continued)

| Strains | Origin | Collection date | Accession number | | |
|-------------------------|--|--------------------|------------------|--------------|--------------|
| | | | 16S rRNA | <i>rbcLX</i> | <i>rpoC1</i> |
| HAB677 | Nuoshan Reservoir, Qingdao, Shandong | April 1, 2007 | FJ184426 | FJ184500 | NK |
| NIVA-CYA126 | Lake Langsjon, Aland, Finland | 1984 | AB045914 | FJ184465 | FJ173039 |
| <i>P. rubescens</i> | | | | | |
| NIES-928 | Cambria, UK | 1975 | FJ184422 | FJ184476 | FJ173023 |
| NIES-1266 | Neuglobsow, Brandenburg | August 25, 2000 | FJ184421 | FJ184472 | FJ173022 |
| <i>P. pseudagardhii</i> | | | | | |
| HAB414 | The inner river in Jinxian, Jiangxi | September 3, 2006 | FJ184390 | FJ184458 | FJ173011 |
| HAB2310 | The inner river in Jinxian, Jiangxi | September 3, 2006 | FJ184386 | FJ184455 | FJ173010 |
| HAB526 | The inner river in Jinxian, Jiangxi | September 3, 2006 | FJ184388 | FJ184456 | FJ173009 |
| HAB366 | West Lake, Fuzhou, Fujian | September 4, 2006 | FJ184387 | FJ184457 | FJ173012 |
| NIES-914 | Lake Dalai-Mongolia-China | NK | AB045907 | FJ184448 | FJ173017 |
| HAB662 | Taihu Lake, Wuxi, Jiangsu | June 1, 2007 | FJ184438 | FJ184445 | FJ173016 |
| HAB639 | Shiyan Reservoir, Shenzhen, Guangdong | April 1, 2007 | GQ457311 | FJ184444 | FJ173020 |
| HAB1128 | The inner river in Fusha Country, Zhongshan, Guangdong | June 13, 2006 | FJ184439 | FJ184452 | FJ173031 |
| HAB1129 | The inner river in Fusha Country, Zhongshan, Guangdong | June 13, 2006 | GQ457309 | GQ457314 | FJ173021 |
| HAB1130 | The inner river in Fusha Country, Zhongshan, Guangdong | June 13, 2006 | FJ184437 | FJ184446 | FJ173063 |
| HAB1131 | The inner river in Fusha Country, Zhongshan, Guangdong | June 13, 2006 | FJ184440 | FJ184447 | FJ173013 |
| HAB1133 | Fish Pond, Beijing | September 22, 2006 | GQ457310 | FJ184451 | FJ173014 |
| HAB1346 | Cidong Lake, Qichun, Hubei | August 26, 2006 | GQ457312 | FJ184454 | GQ457308 |
| HAB1347 | Cidong Lake, Qichun, Hubei | August 26, 2006 | FJ184442 | FJ184453 | FJ173065 |
| HAB1379 | South Lake, Wuhan, Hubei | August 26, 2006 | FJ184443 | GQ457313 | FJ173019 |
| HAB417 | Cidong Lake, Qichun, Hubei | August 26, 2006 | FJ184441 | FJ184468 | FJ173015 |
| <i>P. mougeotii</i> | | | | | |
| HAB002 | Dianchi Lake, Kunming, Yunnan | 2006 | FJ184392 | FJ184460 | FJ173007 |
| HAB626 | A pond in Kunming City, Yunnan | October 25, 2006 | FJ184391 | FJ184459 | FJ173006 |
| HAB3342 | Manmade Wetland, Guangzhou | April 10, 2007 | FJ434247 | FJ184461 | NK |
| HAB3343 | Manmade Wetland, Guangzhou | April 10, 2007 | FJ434248 | NK | NK |
| HAB3346 | Manmade Wetland, Guangzhou | April 10, 2007 | FJ434249 | FJ184462 | FJ173008 |
| HAB3348 | Manmade Wetland, Guangzhou | April 10, 2007 | FJ434250 | FJ184463 | NK |

Culture collections are abbreviated as HAB (Harmful Algal Biology, from Institute of Hydrobiology, Chinese Academy of Sciences, PR China), NIES (National Institute for Environmental Studies, Japan) and NIVA (Norwegian Institute for Water Research). Scientific names are given according to culture collection catalogues (HAB, NIES and NIVA). NK, not known.

geographical latitudes and twenty-seven degrees of geographical longitudes, and ranging from tropical to temperate zones. All clonal strains were isolated using the Pasteur Micropipette method (Rippka, 1988) and maintained as unialgal strains. These strains were cultured in liquid CT medium (Ichimura, 1979) under constant white light intensity at $25 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, on a 12:12 L:D cycle, and at a temperature of $25 \pm 1^\circ\text{C}$.

2.2. Morphological examination

All strains examined were identified according to Komárek and Anagnostidis (2005), and morphological studies were carried out on strains during the exponential growth phase. At least 50 measurements on fresh materials were conducted on each morphological character by using a light microscope-Olympus BX 51 light microscope with a digital camera (Olympus, Japan). The image was analyzed using the Image-pro Express Iplite Application 5.1 (Media Cybernetics, Inc., USA).

2.3. DNA extractions

Total genomic DNA of *Planktothrix* strains were extracted according to Neilan et al. (1995) with a minor modification. Briefly, lysis buffer (100 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 9.0) and lysozyme (10 mg ml^{-1}) were added to the harvested cell pellets. The samples were incubated at 37°C for 30 min. After 30 min, proteinase K (20 mg ml^{-1}) and 10% SDS were added to the samples, which were incubated in 55°C for 1–2 h. The samples were then extracted using phenol-chloroform-isoamyl (25:24:1), and centrifuged at 12,000 rpm for 5 min. Sodium acetate (10 M) and equal volume of ethanol were added to the DNA-containing supernatants for DNA precipitation.

2.4. PCR and sequencing

Fragments of three genes including 16S rRNA, *rbcLX* and *ropC1*, were chosen as the sequencing targets in the present study. The primer sequences for these three genes are listed in Table 2. The 16S rRNA gene was amplified as two overlapping fragments with two sets of primers. P1F, P2R, PcfF1/2 and PcfR 1/2 were degenerate primers used in this study, and were verified for specificity to *Planktothrix* species by BLAST (Altschul et al., 1997). Most sequences were amplified using a combination of primer sets of F1/P2R and P1F/R4, while part of *P. pseudagardhii* sequences were amplified under combination of primer sets of PcfF1/PcfR1 and PcfF2/PcfR2. The polymerase chain reactions (PCR) were performed in a volume of $50 \mu\text{L}$, containing 5–10 ng total genomic DNA, 1 U Taq DNA polymerase (Takara, Japan), $1 \times$ PCR reaction buffer with 1.5 mM MgCl_2 , 10 pmol of each primer, and $200 \mu\text{M}$ concentrations of each deoxyribonucleoside triphosphate (dNTPs). PCR was performed in a PTC-100 thermal cycler (MJ Research, USA). The amplification program was set at 94°C for 5 min, followed by 40 cycles of 94°C for 40 s; 55°C for 50 s, and 72°C for 2 min, followed by a final extension at 72°C for 5 min. The amplified products were purified using the PCR purification kit (Omega, USA) and sequenced with the ABI 3730 Automated Sequencer (PerkinElmer Biosystems, USA).

2.5. Phylogenetic analysis

DNA sequences, both examined in this study and obtained from GenBank, were aligned using CLUSTAL X, version 2.0 (Larkin and Blackshields, 2007). Genetic distances were calculated using the method of Kimura's two-parameter (K2P). DNA sequences were assessed for the best fit model to explain sequence evolution



Fig. 1. Map showing sampling sites all over China. *Planktothrix* strains were isolated from spots marked in the map.

through the modeltest (Posada and Crandall, 1998). The phylogenetic trees were constructed from the multiple-aligned data using the neighbor-joining (NJ) algorithmic Kimura's two-parameter as implemented within the MEGA4 program package (Tamura and Dudley, 2007). The maximum likelihood (ML) algorithms were constructed in PHYML version 3.5c (Guindon and Gascuel, 2003). 100 bootstrap replicates were performed, and only bootstrap values above 50% are indicated at the nodes of the trees. Clade support was estimated utilizing a Markov chain Monte Carlo

(MCMC) algorithm nchains = 4 (three hot and one cold). The program MrBayes was used to execute the Bayes algorithms. Parameters in MrBayes were set to five million generations and 50,000 trees, sampled every 100th generation, using the general time-reversible (GTR/HKY) model of DNA substitution, Nst = 2/6 (corresponding to the modeltest result), rates = gamma, burnin = 10,000 and the default random tree option was set to begin the analysis. The Genbank accession numbers for the 16S rRNA, *rbclX* and *rpoC1* sequences used in this study are shown in Table 1.

Table 2
Primers used in this study.

| Gene region and primer | Sequence ^a | Product length (bp) | Ref. |
|------------------------|---------------------------|---------------------|----------------------|
| 16S rRNA | | | |
| F1 | TTGATCCTGGCTCAGGATGA | 1388 | Otsuka et al. (2006) |
| R4 | TACGGCTACCTTGTACGAC | | Neilan et al. (1997) |
| P1F | AGCGAGGCTTAACCTCGTAAAGG | | This study |
| P2R | TCCCTTTCAAGGAGATTCCAGAG | | This study |
| PcfF1 | TGCAAGTCGAACGGAATCCTTAGGG | 1330 | This study |
| PcfR1 | TCAAAGGGATTCCCGAAATGTCAAG | | This study |
| PcfF2 | GCGTAAACGATGGAAACTAGGTGT | | This study |
| PcfR2 | TCAAAGGGATTCCCGAAATGTCAAG | | This study |
| <i>rbclX</i> | | 824 | |
| <i>PrbcF</i> | GGACATCCCTGGGTAAT | | This study |
| <i>PrbcR</i> | TTGGACTTGCTGACGAT | | This study |
| <i>rpoC1</i> | | 608 | |
| RPOF | TGGTCAAGTGGTTGGAGA | | This study |
| RPOR | GCCGTAATCGGGAGGAA | | This study |

^a 5'-3' orientation.

2.6. Analysis of genetic diversity

DnaSP (Rozas and Rozas, 1999) was used to analyze nucleotide diversity (π) and haplotype diversity (h).

3. Results

3.1. Morphological characteristics of *Planktothrix* strains in China

A total of sixty-three *Planktothrix* strains were isolated from Chinese waters and examined for their morphological characteristics. All strains were found to be green-colored due to the presence of only phycocyanin (Fig. 2). Following the taxonomic criteria of the genus *Planktothrix* defined by Suda et al. (2002), these Chinese *Planktothrix* strains could be morphologically divided into two species: *P. agardhii* (Gomont) Anagnostidis & Komárek and *P. mougeotii* (Kützing ex Lemmermann) Anagnostidis & Komárek. There were slight differences in some features, such as constriction at the crosswalls, granulation at the crosswalls, trichome colors (ranging from pale blue-green, yellow-green or olive green), cell lengths and widths (Figs. 2 and 3), found in some strains within the same defined *Planktothrix* species. However, these divergences were insufficient for the classification of the divergent strains into different species morphologically.

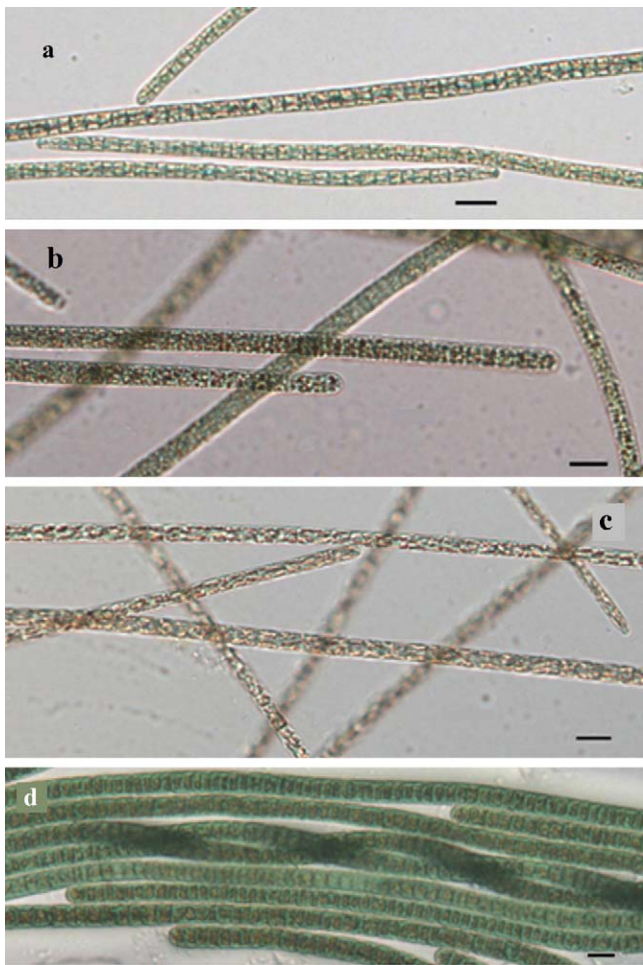


Fig. 2. Micrographs of *Planktothrix* species in the present study. (a) *P. agardhii* HAB 637; (b) *P. pseudagardhii* HAB 1131; (c) *P. rubescens* NIES-1266; (d) *P. mougeotii* HAB3348. Scale bar = 10 μ m. The *P. pseudagardhii* strains were phylogenetically defined and detailed described in phylogenetic analysis.

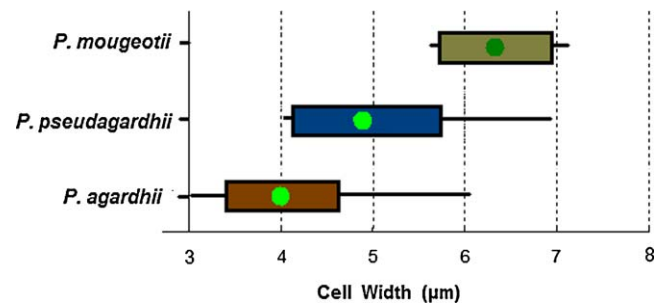


Fig. 3. Cell width distribution of *Planktothrix* strains of different *Planktothrix* species examined in this study. (–) range, (○) mean, (□) standard deviation.

3.2. Phylogenetic analysis of *Planktothrix* strains based on 16S rRNA gene

Phylogenetic trees of the *Planktothrix* strains based on 16S rRNA, *rbcLX* and *rpoC1* sequences, were constructed by NJ, ML and Bayesian methods. As shown in Figs. 4–6, these trees generally gave a highly similar clustering pattern of the *Planktothrix* strains. Partial 16S rRNAs sequenced in this study and twenty-six previously sequenced 16S rRNAs from GenBank of *Planktothrix* strains were used to construct the phylogenetic tree. The tree based on 16S rRNA gene revealed that all *Planktothrix* strains analyzed were grouped into one big cluster significantly separated from other related oscillatorioid cyanobacterial genera such as *Arthropira*, *Lyngbya*, *Trichodemium*, *Tychonema* and *Planktothricoides* (Fig. 7 and Supplementary Fig. 1), confirming *Planktothrix* as a monophyletic genus. However, a high genetic diversity among strains within the genus *Planktothrix* was phylogenetically revealed through phylogeny, and all the *Planktothrix* strains examined in this study, were divided into four distinct clades (Fig. 4). Clade I contained forty-eight strains of *P. agardhii* in which forty from China, and eight strains from other geographical regions. Strain NIVA-CYA126, a representative of microcystin-producing *P. agardhii*, was included in this clade, clustering together with all the non MC-producing strains isolated from China (data not shown). All the *P. rubescens* strains investigated in this study were grouped into clade II, clustering with some non-Chinese *P. agardhii* strains. All the *P. pseudagardhii* strains were included in clade III, which, was furthermore divided into two subgroups: subclade IIIa with three strains isolated from Jinxian, Jiangxi province and one from Fuzhou, Fujian province, and the subclade IIIb including twelve strains isolated in China and four from outside of China. All *P. mougeotii* strains used in this study, including six strains from China and four from Thailand, were clustered into clade IV, in which strains HAB3342, HAB3343, HAB3345 and HAB3346 from the manmade wetland were distinguished from the other strains and forming a subclade. The lowest similarities of 16S rDNA gene sequences in genus *Planktothrix*, clades I, II, III and IV were 98.98%, 99.78%, 97.26% and 97.86% respectively. It is noted that the strains in the *P. pseudagardhii* clade (III) had 16S rDNA sequence similarity as low as 97.2%, indicating the larger genetic divergence within the strains of *P. pseudagardhii*.

Basic 16S rRNA gene structure using MEGA elucidated that 1388 nucleic acid sites in the 16S rRNA gene of Chinese *Planktothrix* strains were useful for the analysis of nucleotide diversity. Among which, 1148 conserved sites, 238 variable sites, 104 parsim informative sites and 134 singleton informative sites were included. Forty-six haplotypes were identified according to these variable sites. The nucleotide frequencies were 0.265 (A), 0.189 (T), 0.225 (C), and 0.322 (G). The transition/transversion rate ratios were $k_1 = 2.460$ (purines) and $k_2 = 2.757$ (pyrimidines). The overall transition/transversion bias was $R = 1.31$, where $R = [A \times G \times k_1 + T \times C \times k_2] / [(A + G) \times (T + C)]$.

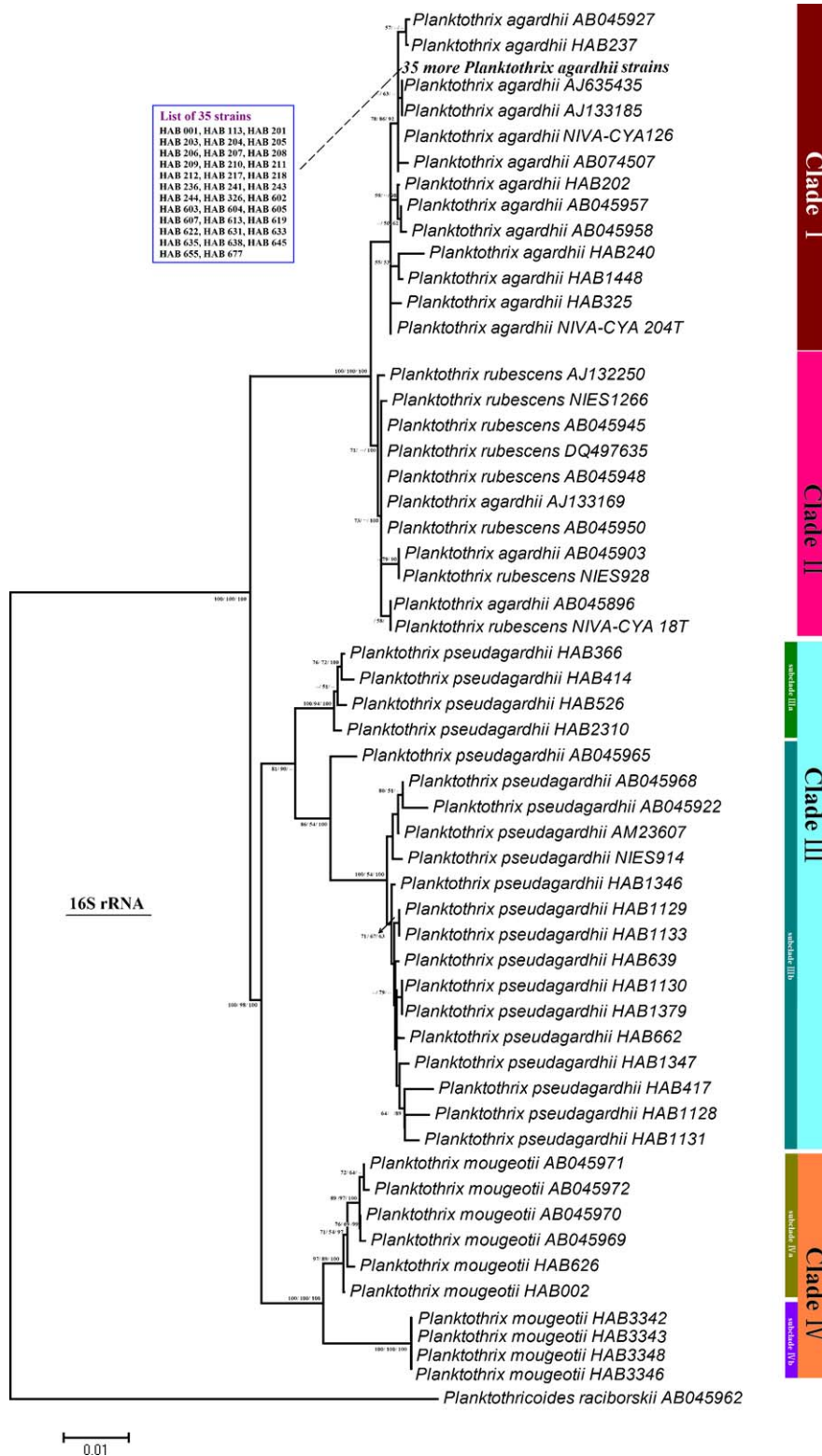


Fig. 4. Phylogenetic tree based on 16S rDNA region sequences (1387 bp) of eighty-nine *Planktothrix* strains in this study. Bootstrap values greater than 50% with ML/Bayers/NJ methods are indicated on the tree. Strains isolated from China are shown with HAB Code. *Planktothricoides raciborskii* (AB045962) was used as the outgroup. Roman numerals denote *Planktothrix* clades I–V.

3.3. Phylogenetic analysis of *Planktothrix* strains based on *rbclX*

The *rbclX* sequences of sixty Chinese *Planktothrix* strains analyzed in this study and fourteen non-Chinese strains obtained from GenBank were used to construct the phylogenetic tree. The

region covered the end of the *rbcl* gene, an intergenic spacer (IGS1), the complete *rbclX* gene and a second intergenic spacer (IGS2) (Rudi et al., 1998; Gugger et al., 2002). Different from the clustering based on 16S rDNA sequences, *rbclX* sequence based phylogenetic tree revealed that these seventy-four *Planktothrix* strains could be

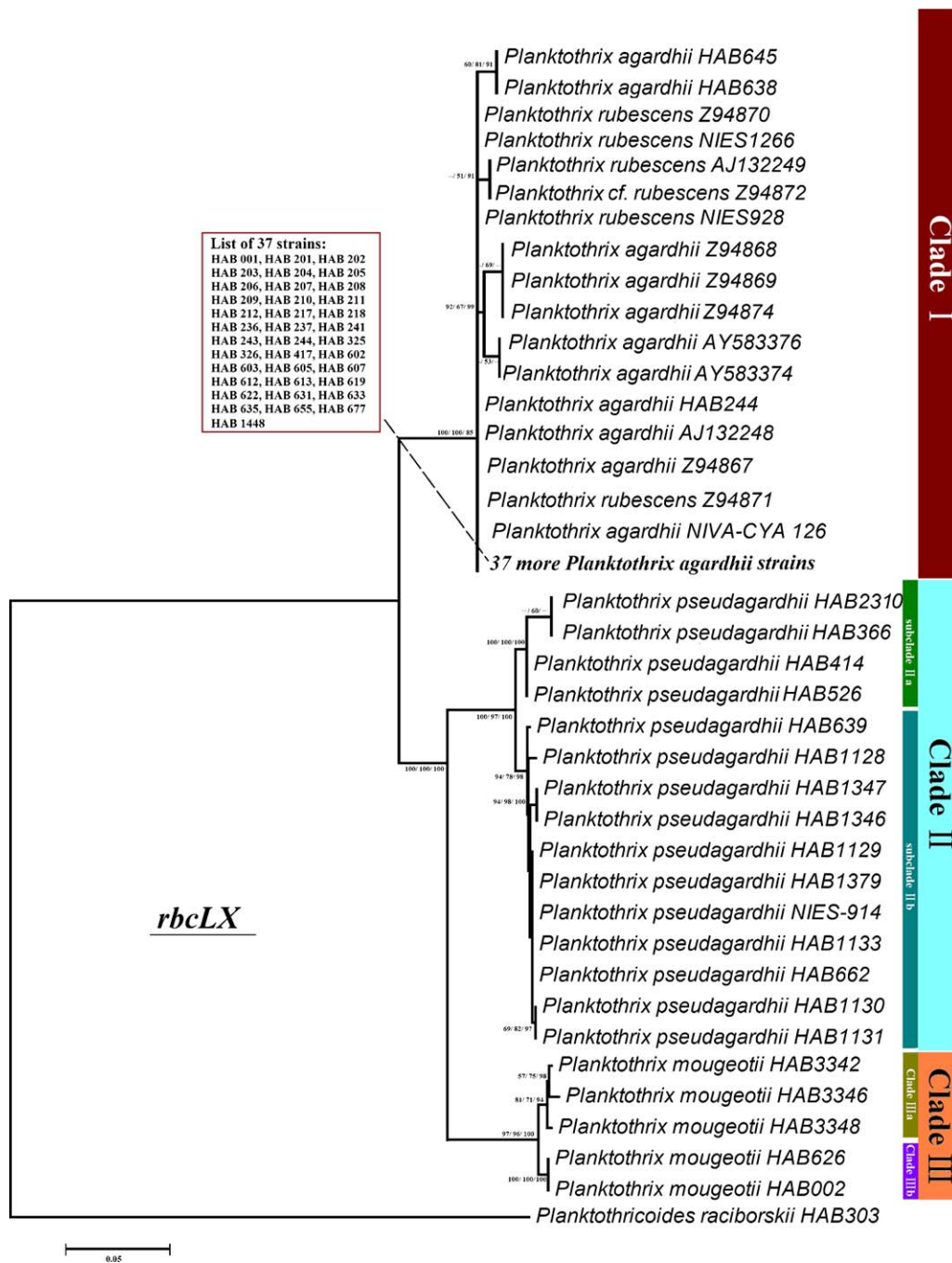


Fig. 5. The Neighbor-Joining tree based on *rbcLX* region sequences (824 bp) of seventy-one *Planktothrix* strains in this study. Bootstrap values greater than 50% with ML/Bayers/NJ methods are indicated on the tree. Sequences from Genbank are indicated with accession. Roman numerals denote *Planktothrix* clades I–IV.

clustered into three clades (Fig. 5). Clade I was shown as a large group, including all the *P. agardhii* strains from China, two strains of *P. rubescens* sequenced in this study, eight strains of *P. agardhii* strains and five strains of *P. rubescens* previously sequenced from GenBank. Clade II contained all *P. pseudagardhii* strains isolated from China, and this clade could also be divided into two subclades corresponding to those in the clade III in the 16S rDNA phylogeny. Clade III included all five strains of the *P. mougeotii* strains isolated from China, and could be further divided into two subclades. Three strains in one subclade were all isolated from Dianchi Lake in Yunnan province, and two strains in the other one were obtained from Guangzhou, Guangdong province. The lowest similarities of *rbcLX* sequences in the clades I, II, III within the *Planktothrix* genus,

were 98.87%, 97.69% and 98.48% respectively. As shown in Fig. 8, three types differing in sequence and length of IGS1 region in *rbcLX* were found among *Planktothrix* strains. *P. pseudagardhii* and *P. mougeotii* strains all belonged to the type I with the shortest IGS1 sequences, and *P. agardhii* and *P. rubescens* strains were divided into type II and III, and type II had one more GATTGA box than type I, while type III had two more GATTGA boxes than type I.

3.4. Phylogenetic analysis of *Planktothrix* strains based on *rpoC1*

The DNA dependent RNA polymerase (*rpoC1*) gene has been suggested to be more discriminatory than 16S rRNA gene analysis at the species level (Palenik and Haselkon, 1992). The cyanobacterial

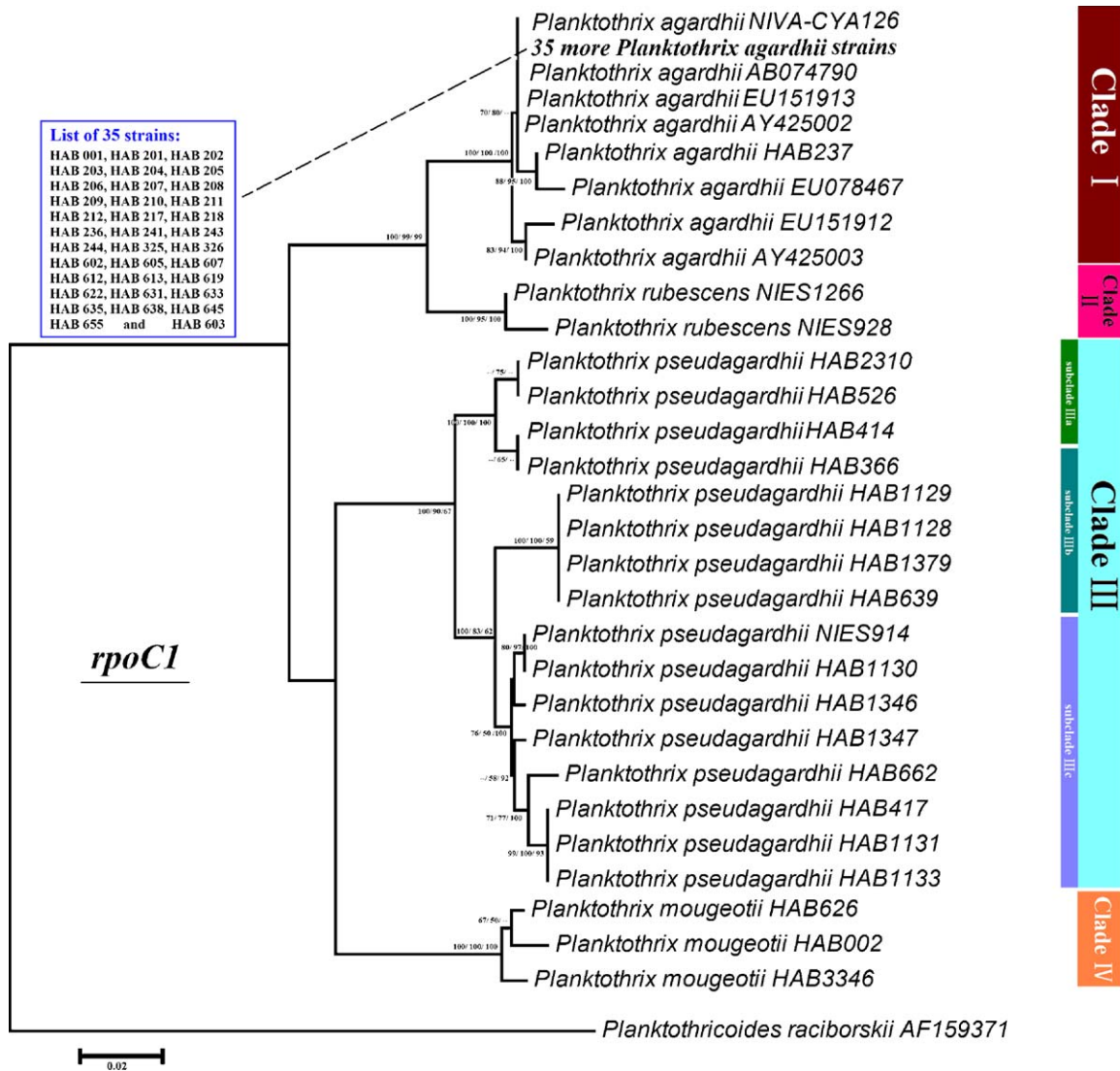


Fig. 6. The Neighbor-Joining tree based on *rpoC1* region sequences (608 bp) of sixty-four *Planktothrix* strains in this study. Bootstrap values greater than 50% with ML/Bayers/NJ methods are indicated on the tree. Strains originated from China are shown with HAB Code. Roman numerals denote *Planktothrix* clades I–V. *Cylindrospermopsis raciborskii* (AF159371) was used as the outgroup.

rpoC1 gene encodes the gamma subunit of RNA polymerase and exists as a single copy in the genome (Palenik and Swift, 1996). However, until now very scarce information about *rpoC1* gene sequences of cyanobacteria especially of oscillatorioid cyanobacteria was available prior to this study. We employed *rpoC1* gene sequences of fifty-five Chinese *Planktothrix* strains and nine strains isolated from outside of China to construct the phylogenetic tree. The resultant tree revealed that four clades were identified by the clustering based on *rpoC1* gene sequences (Fig. 6). In detail, clade I includes all the *P. agardhii* strains, and clade II contained two *P. rubescens* strains sequenced in this study. All the *P. pseudagardhii* strains were included in clade III in which three subclades could be identified. Subclades IIIa contained the four strains isolated from Jinxian, Jiangxi province, while the other two subclades corresponded to the subclade IIIb of the 16S rRNA phylogeny. All *P. mougeotii* strains belonged to clade V. The lowest similarities of *rpoC1* in genus *Planktothrix*, clades I, II, III and IV were 97.65%, 99.01%, 94.29% and 98.16% respectively.

The result of DNA polymorphism analyses revealed that 16S rRNA, *rbclX* and *rpoC1* genes in *Planktothrix* strains demonstrated different diversities of haplotype and nucleotide. It is unexpected to find that *rbclX* and *rpoC1* sequences of *Planktothrix* strains had

Table 3
Nucleotide diversity test for *Planktothrix* species.

| Gene | Haplotype number | Haplotype diversity, <i>h</i> | Nucleotide diversity, π |
|--------------|------------------|-------------------------------|-----------------------------|
| 16S rRNA | 46 | 0.946 ± 0.014 | 0.0225 |
| <i>rbclX</i> | 21 | 0.710 ± 0.058 | 0.0432 |
| <i>rpoC1</i> | 18 | 0.636 ± 0.069 | 0.0542 |

lower haplotype but higher nucleotide diversities than 16S rRNA gene (Table 3).

4. Discussion

Molecular characterization of the *Planktothrix* species in China using a number of strains was for the first time conducted to determine their genetic diversity. Sixty-three *Planktothrix* strains were isolated from seventeen water bodies, and they were all found to be phycocyanin-containing species. Morphological observations of these strains showed that they were categorized into two morphological species, as *P. agardhii* and *P. mougeotii*. In contrast to morphological classification, analyses of the 16S rDNA,

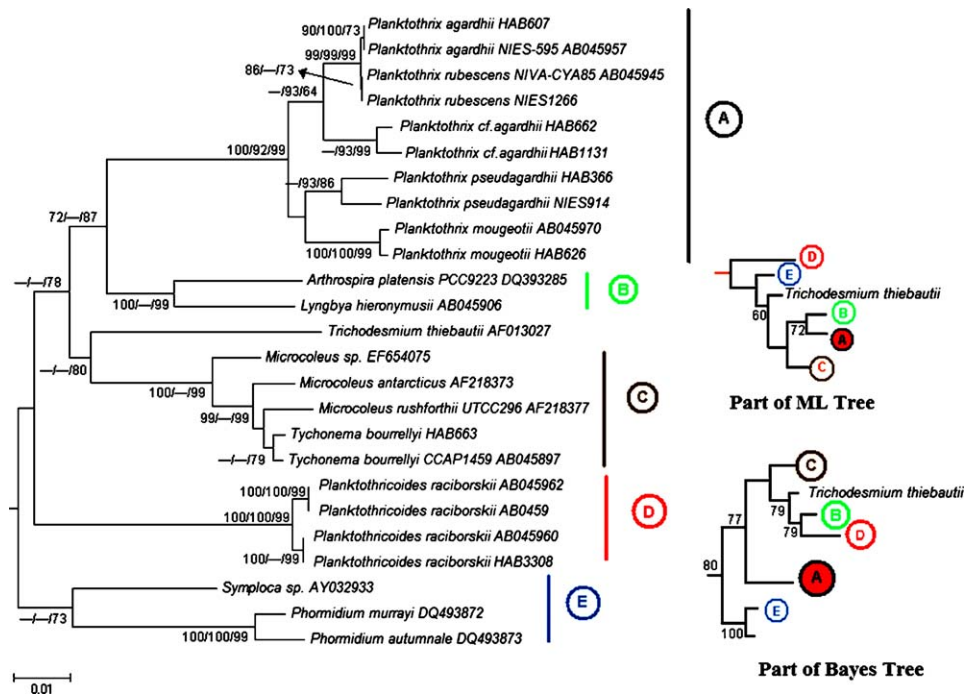


Fig. 7. A part of the phylogenetic tree based on 16S rDNA region sequences (1360 bp) of genus *Planktothrix* and sixty-one other cyanobacteria species displaying the positions of genus *Planktothrix*. Bootstrap values greater than 50% with ML/Bayers/NJ methods are indicated on the tree. Strains originating from China are shown with HAB Code. *Gloeobacter* sp. (EF032784) was used as the outgroup.

rbcLX and *rpoC1* sequences of *Planktothrix* species showed a genetic variation among the strains of each *Planktothrix* species especially for the *P. agardhii* morphotype. A previous study conducted by Suda et al. (2002) already demonstrated that *P. agardhii* strains displayed a high genetic divergence, and therefore a new species—*P. pseudagardhii* was established primarily based on 16S rRNA gene sequences. As shown in the 16S rDNA phylogenetic tree (Fig. 4), the clade I and clade II formed by both strains of *P. agardhii* and *P. rubescens* were significantly distinct from the morphologically *P. agardhii* alike species *P. pseudagardhii* clade. A closer phylogenetic relationship was found between the *P. pseudagardhii* clade and the *P. mougeotii* clade compared to that between the *P. agardhii* clade and the *P. mougeotii*.

The phylogenetic trees based on 16S rRNA, *rbcLX* and *rpoC1* genes exhibited highly consistence. It seemed that the clustering pattern, such as the clades of *P. pseudagardhii* strains and *P. mougeotii* strains, was similar among the phylogenetic trees considering all the three gene regions. Clade I or II always contained *P. agardhii* strains mixing with *P. rubescens* strains.

Rudi et al. (1998) indicated that phylogeny based on *rbcLX* was incongruent with 16S rDNA phylogeny within the cyanobacteria. However, Gugger et al. (2002) performed phylogenetic comparative studies between cyanobacterial genera *Anabaena* and *Aphanizomenon* based on the 16S rDNA and *rbcLX* sequence, and their analysis revealed that clusters in 16S rDNA tree were grouped similarly in the *rbcLX* tree. To date, the only recourse of *rbcLX* sequences of *Planktothrix* strains recorded in the GenBank were contributed by Rudi et al. (1998). Our study provided more information on the phylogeny of genus *Planktothrix* based on *rbcLX* region sequences. In the *rbcLX* tree of *Planktothrix* strains in this study, clades II and III corresponded to *P. pseudagardhii* and *P. mougeotii* respectively. Some difference was found in the clade I, which was corresponding to the combination of clade I and II in the 16S rDNA phylogeny representing the mixture of strains of *P. agardhii* and *P. rubescens*. This difference was actually in agreement with previous results by Suda et al. (2002), who described no difference in 16S rDNA sequences between strains of *P. agardhii* and *P. rubescens*.

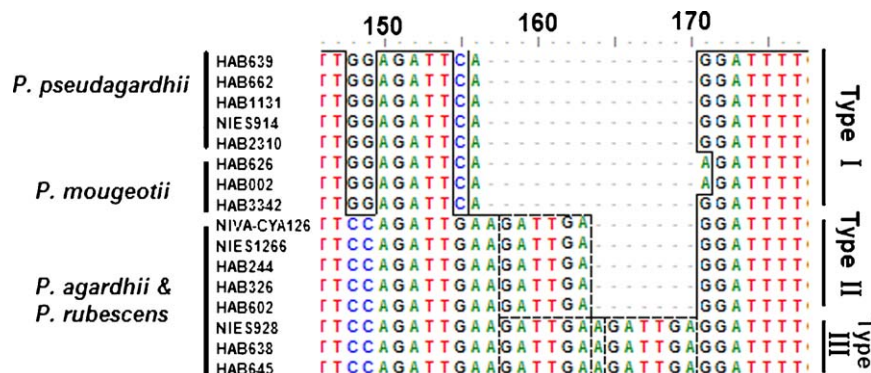


Fig. 8. Three different insertion type clusters found in IGS1 between *rbcL* and *rbcX* genes for *Planktothrix* species.

rpoC1, as one subunit encoding the core of RNA polymerase in cyanobacteria, is always present as a single copy in the cyanobacterial genome (Bergslund and Haselkorn, 1991). *rpoC1* has been shown to have greater divergence between two related strains and thus able to resolve genus and species issues (Toledo and Palenik, 1997). The analysis of *rpoC1* gene has proven to complement the phylogeny based on 16S rRNA gene in various cyanobacterial groups (Seo and Yokota, 2003). Analysis of *rpoC1* has been repeatedly performed on strains of heterocystous *Cylindrospermopsis raciborskii* which has been regarded as an invasive cyanobacterium from the tropical regions of the African continent (Wilson et al., 2000; Gugger et al., 2005; Haande et al., 2008). On the other hand, very limited *rpoC1* sequences of *Planktothrix* strains were available from GenBank. In this study, phylogeny based on *rpoC1* sequences of sixty-four *Planktothrix* strains, including fifty-five new isolates from China and nine strains from outside of China, revealed the similar clustering to the 16S rDNA tree. It is worth noting that in the *rpoC1* based tree two strains of *P. rubescens* formed a single cluster supported by a high bootstrap value to differ from strains of *P. agardhii*. This result is of particular value since the genetic divergence between *P. agardhii* and *P. rubescens* has not yet been found as yet, and *rpoC1* was expected to be a potentially genetic tool to distinguish these two related species when more *P. rubescens* strains would have been examined.

It is still uncertain for the modern species concept of cyanobacteria. However, several studies, using the bacteriological standard for the cut-off points of 97.5% and 95.0% 16S rRNA gene sequence similarity for species and genus definition respectively, have been performed to analyze the genetic diversity in closed related cyanobacterial strains (Halinen et al., 2008; Rajaniemi et al., 2005). Suda et al. (2002) established the new species—*P. pseudagardhii* primarily based on 16S rRNA gene sequences by the phylogenetic analysis. The *P. pseudagardhii* strains investigated in this study demonstrated a greater diversity since the similarity of 16S rDNA sequences between the subclades was as low as 97.26%, and even 94.29% for the similarity of *rpoC1* gene sequences. By the criteria of cut-off points of 97.5%, the present the putative cases of cryptic species may exist in the species of *P. pseudagardhii*. The high nucleotide diversity, especial the species *P. pseudagardhii*, reflected the rapid evolution and frequent gene mutation in genus *Planktothrix*.

rbclX and *rpoC1* genes still presented more rapid evolutionary rate and higher divergence than 16S rRNA gene. However, it is interesting to find that *rbclX* and *rpoC1* genes had lower haplotype diversity than 16S rRNA gene. Since no integrated information of haplotype and nucleotide diversity in prokaryotes was available, it remains difficult to explain the inconsistency between haplotype and nucleotide diversities of the genes examined in this study.

The phylogenetic analyses of the *Planktothrix* genus in the present study provide more information on the diversity of water bloom forming cyanobacterial species. The genetic divergence in *P. pseudagardhii* clade shown in this study, revealing the possible existence of cryptic cyanobacterial species, whose classification is mainly based on morphological properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2009.08.004.

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