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PHYTOPLANKTON

Taxonomic and phylogenetic evaluation of *Limnothrix* strains (Oscillatoriales, Cyanobacteria) by adding *Limnothrix planktonica* strains isolated from central China

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Abstract Six *Limnothrix* strains, isolated for the first time from a shallow eutrophic lake in central China, were taxonomically and phylogenetically evaluated by investigating their polyphasic characteristics, including morphological features, cellular ultrastructures, and 16S rRNA gene sequences. All the six strains were morphologically similar, and their trichomes were in average 1.7 μm wide and cells 4.0 μm long, and having small gas vesicles within cells, and therefore identified as *Limnothrix planctonica* (Woloszynska) Meffert. Cellular ultrastructures of them showed that peripheral thylakoids with 3–5 parallel layers were parietally distributed in the cells. The phylogenetic results based on the 16S rRNA gene sequences showed that all the *Limnothrix* strains, including the six in this study and those from the Genbank, formed two distinct clusters. The similarity in 16S rDNA sequences between these two clusters was lower than 90%, indicating that these *Limnothrix* strains belong to different genera. This is the first report on the morphology and phylogeny of *L. planctonica* strains, providing the new information on taxonomy of the genus *Limnothrix*.

Keywords Cyanobacteria · *Limnothrix* · Morphology · Phylogeny · Taxonomy

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Introduction

The cyanobacterial genus *Limnothrix* Meffert has been classified within the order Oscillatoriales, family Pseudanabaenaceae, subfamily Pseudanabaenoideae, under the current botanical taxonomic system (Anagnostidis & Komárek, 1988; Komárek, 2003; Komárek & Anagnostidis, 2005), and *L. redekei* (Van Goor) Meffert was established as the type species (Meffert, 1987). The *Limnothrix* species are characterized by solitary, unsheathed, and mostly unconstricted trichomes, mainly consisting of narrow cylindrical cells with polar and/or central aerotopes. Some species contain both phycocyanin (PC) and phycoerythrin (PE) as well as display complementary chromatic adaptation (CCA) (Kohl & Nicklisch, 1981). Such an adaptation allows these species to maximize the absorption of available light by regulating the ratio of PC to PE (Stomp et al., 2004, 2008).

With the difficulties in taxonomic delimitation, the genus *Limnothrix* has become a large group with 15 species and described forms, in which *L. redekei*, *L. planctonica*, and *L. rosea* are occasionally dominant in freshwater phytoplanktons (Meffert, 1987; Komárek & Anagnostidis, 2005). *Limnothrix* populations are also known to be present in marine environments in South Africa (Silva & Pienaar, 2000). *L. redekei* populations in Europe have been frequently reported to be massively present in shallow eutrophic lakes for decades (Bailey-Watts, 1972; Rojo & Cobelas, 1994; Vardaka et al., 2000; Moustaka-Gouni et al., 2007). Along with the development of molecular phylogenetics in Cyanobacteria, a few strains of *L. redekei* were shown to be polyphyletic. One mixed cluster is usually composed of *L. redekei* and *Pseudanabaena* strains (Gkelis et al., 2005; Willame et al., 2006; Acinas et al., 2009; Nishizawa et al., 2010). Therefore, the term *Pseudanabaena/Limnothrix* group, instead of two different genera, has been used in current researches (Zwart et al., 2005; Willame et al., 2006; Nishizawa et al., 2010). Another *Limnothrix* cluster typically represented by three Greek strains of *L. redekei* has been reported (Gkelis et al., 2005), and this cluster was shown to be complicated by adding strains morphologically identified as genus *Geitlerinema* (Perkerson et al., 2010; Bernard et al., 2011). Another difficulty related to the taxonomic assignment at the species level is that the conducted studies on the genus *Limnothrix* rarely documented other species than *L. redekei*. Hence, further evaluation of the phylogenetic position of the genus *Limnothrix* and further exploration of the defined taxonomy within this genus by applying more strains of *Limnothrix* and related genera, preferably from other geographic regions outside Europe, is necessary.

The occurrence of *Limnothrix* has never been reported in China since the establishment of this genus. In this study, six *Limnothrix* strains were isolated for the first time from a shallow eutrophic lake in central China. We aimed to perform the polyphasic examination of these six Chinese strains, to add further knowledge on the taxonomy and phylogeny of the *Limnothrix* genus.

Materials and methods

Isolation and cultivation of *Limnothrix* strains

The *Limnothrix* strains (Table 1) were isolated from the Donghu Lake in Wuhan City, Hubei Province in 2009. The micropipette method (Rippka, 1988) was used to isolate the cultivated strains. Six uni-algal strains initially identified at the *Limnothrix* genus level were obtained. All the six strains were cultured in liquid CT medium (Ichimura, 1979) under a constant white light intensity of 25 μmol photons m^{-2} s^{-1} on a 12:12 L:D cycle at 25 ± 1°C.

Morphological examination

The examined strains were morphologically identified according to the description of Meffert (1987) as well as that of Komárek & Anagnostidis (2005). Morphological and morphometrical studies were performed on specimens collected from cultures on the exponential growth phase. Trichome size was measured from ≥50 individuals per strain using a Nikon eclipse 80i light microscope with DS-Ri1 digital camera (Nikon, Japan). The image was analyzed using the NIS-Elements D 3.2.

Ultrastructure observation

The ultrastructural features of the isolated strains were studied using transmission electron microscopy

Table 1 Morphological characteristics of the *Limnothrix planktonica* isolates studied

Strain	Width (μm)	Length (μm)	L:W ratio	PE	Motility	Calyptra
CHAB709	1.3–1.7–2.0	2.3–4.1–6.2	1.9–2.4–3.1	+	+	−
CHAB751	1.2–1.7–2.0	2.0–3.8–6.0	1.7–2.4–3.1	+	+	−
CHAB753	1.3–1.8–2.0	1.8–4.1–6.7	1.8–2.4–3.2	+	+	−
CHAB756	1.3–1.9–2.2	2.2–3.9–5.7	1.6–2.2–3.2	+	+	−
CHAB759	0.8–1.6–2.0	2.0–4.2–6.0	2.0–2.7–3.1	+	+	−
CHAB763	0.9–1.6–2.2	2.0–4.0–6.8	2.0–2.5–3.2	+	+	−

(TEM). The strains during the exponential growth phase were fixed following a procedure similar to that described by Gkelis et al. (2005) and then observed under a TEM (FEI TECNAI G^2 20 TWIN, USA) at an accelerating voltage of 200 kV using the iTEM FEI software.

DNA extraction and PCR amplification

Total genomic DNA was extracted according to the method of Neilan et al. (1995) and the xanthogenate-SDS (XS) DNA extraction protocol (Tillett & Neilan, 2000). A 1 mL portion of the cultivated solution at the logarithmic growth phase was used, and 50 μL DNA solutions were finally obtained with 5–10 ng/μL DNA concentration.

The 16S rRNA gene sequences were amplified from the genomic DNA using the PCR primers 27F1 (5′-AGAGTTTTGATCCTGGCTCAG-3′) (Neilan et al., 1997) and B23S: 5′-CTTCGCCTCTGTGTGCC TAGGT-3′ (Taton et al., 2003). The polymerase chain reactions (PCR) were performed in a 50 μL reaction according to Lin et al. (2010). All PCR products were examined on 1% (w/v) agarose gels dyed with ethidium bromide and purified by the PCR purification kit (Omega, USA), and the purified PCR products were directly sequenced by Invitrogen Biotechnology Co. Ltd. (Shanghai, China).

Phylogenetic analysis

The 16S rDNA sequences, consisting of those examined in this study and those obtained from GenBank, were aligned using CLUSTALW integrated into the BioEdit package (Hall, 1999). Phylogenetic trees were constructed using neighbor-joining (NJ), maximum likelihood (ML), and Bayes algorithms. NJ with Kimura-2 and 1,000 bootstraps was used to construct the corresponding phylogenetic trees using the MEGA4 program package (Tamura & Dudley, 2007). DNA sequences were assessed for the best fit model to explain the sequence evolution using the test model (Posada & Crandall, 1998). The ML algorithms were constructed using PHYML version 3.5c (Guindon & Gascuel, 2003). One hundred bootstrap replicates were performed, and only bootstrap values above 50% were indicated at the nodes of the trees. Clade support was estimated using the general time-reversible (HKY) model. The parameters of the Ts/tv ratio and p-invar were set corresponding to the outputs from the test model (Posada & Crandall, 1998). The MrBayes program was used to execute the Bayes algorithms. The parameters in MrBayes were set to 5,000,000 generations, 50,000 trees, sampling at every 100th generation, use of the HKY model of DNA substitution, Nst = 6, and rates = gamma. The 16S rRNA sequence data were deposited in the GenBank under the following accession numbers: JQ004021–JQ004026.

Results

Morphological characteristics

The six *Limnothrix* isolates were morphologically examined,and they were all identified as *L. planctonica* (Woloszynska) Meffert based on the descriptions by Komárek & Anagostidis (2005). The trichomes of the six isolated strains were solitary, planktonic, or sometimes formed mats, settled to the bottom of the culture tubes, and they were unconstricted at the cross-wall (Fig. 1b, c). The color of the trichome was pale blue-green. The filaments contained PE because they were fluorescent through the PE-specific filter (490[Ex.]/580[Em.], Nikon, Japan) under a fluorescence light microscope. The trichomes were unsheathed, straight, or bent, consisting of cylindrical cells, and slowly glided with oscillation. The cells were 0.8–2.2 μm wide and 1.8–6.8 μm long, whereas the apical cells were rounded without calyptra. Gas vesicles were obviously observed in trichomes in the fresh samples from lake water (Fig. 1a). The ratios of the lengths to the widths of the cells were 2.5 to 3.2 on the average (Table 1). Based on the descriptions by Komárek & Anagostidis (2005), the six *Limnothrix* isolates were all identified as *L. planctonica* (Woloszynska) Meffert.

The six strains were examined for their ultrastructures. The cells have peripheral thylakoids annularly distributed at the cross section (Fig. 1d, e). However, the longitudinal thin sections of the trichomes showed that the thylakoids were only parietally distributed with 3–5 parallel layers. Gas vesicles were not found.

Phylogenetic analysis

The two strain pairs (CHAB751 and CHAB756; CHAB753 and CHAB759) of *L. planctonica* showed

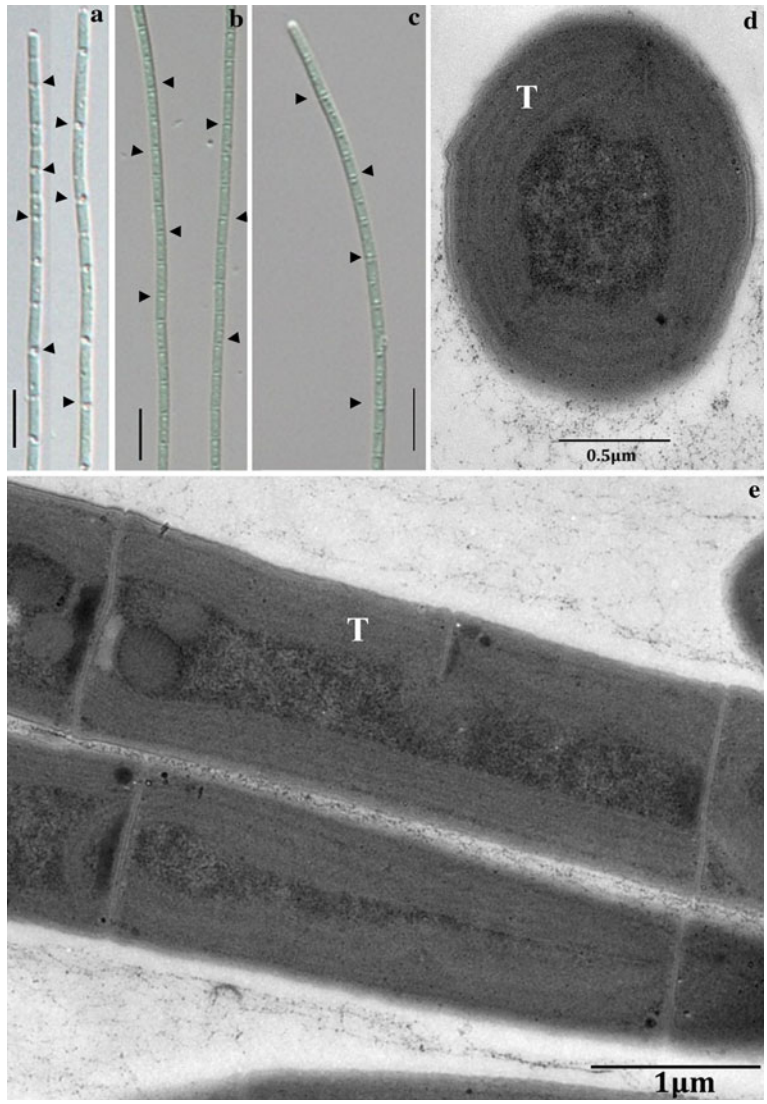

Fig. 1 Micrographs of *Limnothrix planctonica* strains in this study. **a** *L. planctonica* trichomes from a natural population. **b**, **c** Trichome of *L. planctonica* CHAB 709 strain; all *scale bars* = 10 μm, and the *arrows* indicate gas vacuoles. **d**, **e** Transmission electron micrographs of *L. planctonica* strain CHAB709, and thylakoids are marked with T

identical 16S rRNA gene sequences and had 99% similarity in 16S rRNA gene sequences with the other two strains. Six 16S rRNA gene sequences from this study and 52 previous 16S rRNA gene sequences from strains of *Limnothrix* and other Oscillatorean genera in Genbank were used to construct the phylogenetic trees using the NJ, ML, and Bayesian methods. All the *Limnothrix* strains form two distinct clusters, as shown in the Bayesian tree with the supporting values from the NJ, ML, and Bayesian methods (Fig. 2). The larger cluster II was formed mainly by *Limnothrix* strains from Asia, Europe, Africa, and America. However, the smaller cluster I was a mixture. The lowest similarity in 16S rRNA gene sequences among the *Limnothrix* strains within clusters I, II, and between Clusters I and II were 95.9%, 94.4%, and 86.2%, respectively, indicating large divergence in the *Limnothrix* strains reported so far.

Discussion

Taxonomic revisions have been continuously performed in Cyanobacteria during the last decades. In the order of Oscillatoriales, considerable treatments targeted on the genus *Oscillatoria* were performed, and the separation of several genera from *Oscillatoria*, such as *Planktothrix*, *Planktothricoides*, and *Limnothrix*,

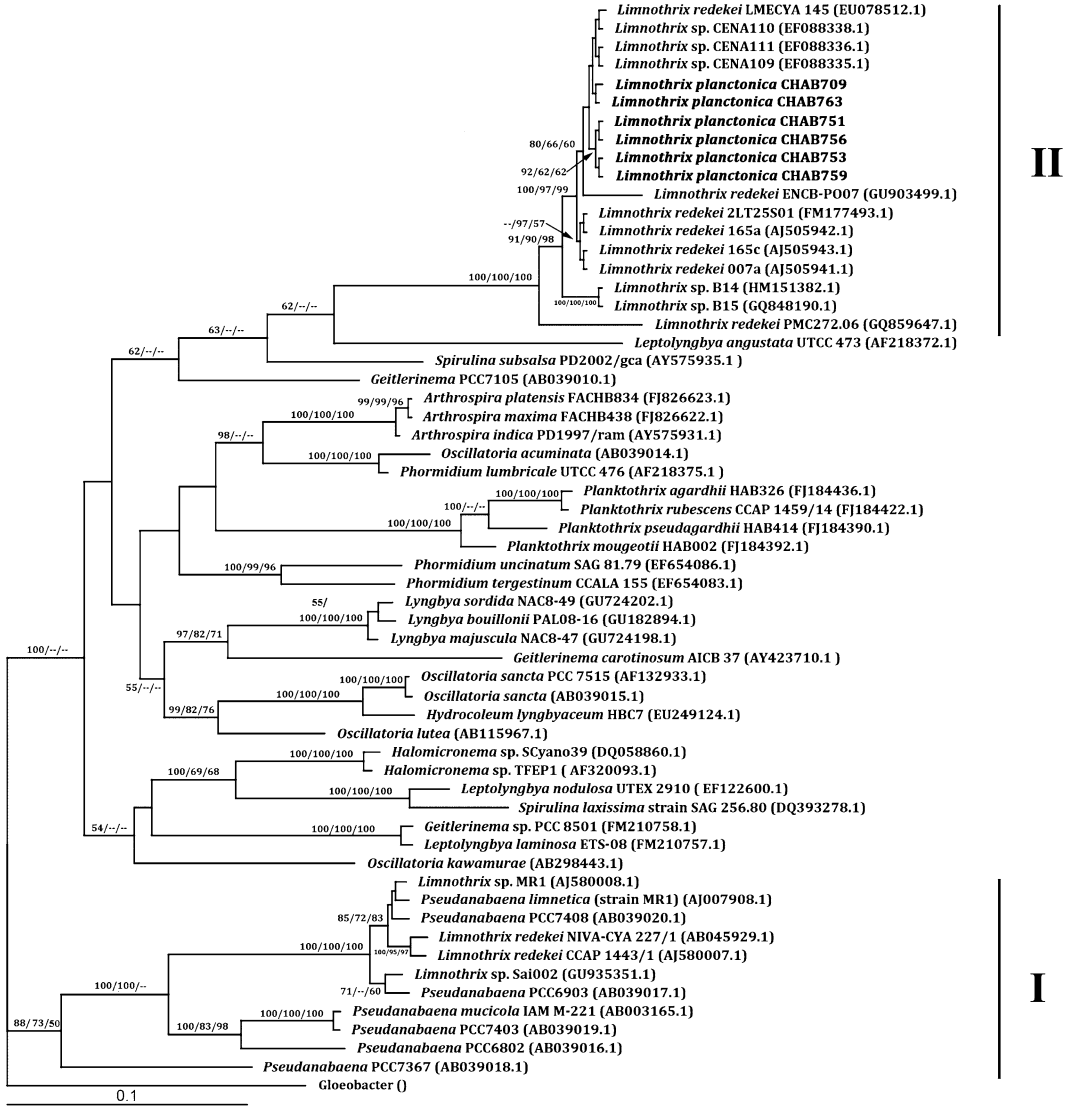

Fig. 2 Phylogenetic tree based on 16S rDNA region sequences (949 bp) of six 16S rRNA gene sequences from this study and 52 previous 16S rRNA gene sequences from strains of *Limnothrix* and other Oscillatorean genera in Genbank. Bootstrap values greater than 50% with Bayers/ML/NJ methods are indicated on the tree. Strains isolated from China are codes as "CHAB" strain number. *Gloeovacter violaceus* PCC7421 (BA000045) was used as the outgroup

successfully contributed to the taxonomic system of Oscillatorean organisms. The genus *Limnothrix* was established to distinguish from *Oscillatoria* based on several differences such as cellular shape and thylakoid arrangement. Suda et al. (2002) analyzed the 16S rDNA sequence of *L. redekei* NIVA-227 during the taxonomic studies on water-bloom-forming species of oscillatoroid Cyanobacteria and confirmed the molecular divergence among *Limnothrix*, *Oscillatoria*, and *Planktothrix*. Thus, they set *L. redekei* NIVA-227 as the strain type of the genus *Limnothrix*. However, three strains of *L. redekei* isolated from Lake Kastoria, Greece were shown to form a separate phylogenetic group from *L. redekei* NIVA-227, which clustered together with some *Pseudanabaena* strains (Gkelis et al., 2005), clearly exhibiting that *Limnothrix* is polyphyletic. Therefore, more strains isolated from more regions have to be examined to elucidate the detailed molecular divergence and phylogenetic relationship within the genus *Limnothrix*. This study is the first to examine

Table 2 Genetic distances of 16S rDNA sequences among the *Limnothrix* strains analyzed in this study (above 949 bp)

	CHAB 709	CHAB 751	CHAB 753	CHAB 756	CHAB 759	CHAB 763	B14	PMC 272.06	2LT 25S01	ENCB-PO07	LMECYA 145	CENA 110	CENA 111	CENA 109	B15	165c	165a	007a	NIVA 227	MR1	CCAP 1443
CHAB751	0.003																				
CHAB753	0.004	0.002																			
CHAB756	0.003	0	0.002																		
CHAB759	0.004	0.002	0	0.002																	
CHAB763	0.002	0.002	0.003	0.002	0.003																
B14	0.016	0.016	0.017	0.016	0.017	0.015															
PMC272.06	0.039	0.038	0.039	0.038	0.039	0.038	0.045														
2LT25S01	0.003	0.003	0.004	0.003	0.004	0.002	0.014	0.037													
ENCB-PO07	0.018	0.018	0.019	0.018	0.019	0.017	0.031	0.054	0.017												
LMECYA 145	0.003	0.003	0.004	0.003	0.004	0.002	0.016	0.039	0.003	0.018											
CENA110	0.002	0.002	0.003	0.002	0.003	0	0.015	0.038	0.002	0.017	0.002										
CENA111	0.002	0.002	0.003	0.002	0.003	0	0.015	0.038	0.002	0.017	0.002	0									
CENA109	0.002	0.002	0.003	0.002	0.003	0	0.015	0.038	0.002	0.017	0.002	0	0								
B15	0.016	0.016	0.017	0.016	0.017	0.015	0	0.045	0.014	0.031	0.016	0.015	0.015	0.015							
165c	0.004	0.004	0.005	0.004	0.005	0.003	0.015	0.036	0.002	0.018	0.004	0.003	0.003	0.003	0.015						
165a	0.003	0.003	0.004	0.003	0.004	0.002	0.014	0.037	0	0.017	0.003	0.002	0.002	0.002	0.014	0.002					
007a	0.004	0.004	0.005	0.004	0.005	0.003	0.015	0.036	0.002	0.018	0.004	0.003	0.003	0.003	0.015	0	0.002				
NIVA 227	*0.12*	*0.12*	*0.121*	*0.12*	*0.121*	*0.119*	*0.121*	*0.127*	*0.118*	*0.135*	*0.118*	*0.119*	*0.119*	*0.119*	*0.121*	*0.118*	*0.118*	*0.118*			
MR1	*0.122*	*0.122*	*0.123*	*0.122*	*0.123*	*0.121*	*0.123*	*0.129*	*0.12*	*0.137*	*0.12*	*0.121*	*0.121*	*0.121*	*0.123*	*0.12*	*0.12*	*0.12*	*0.02*		
CCAP 1443/1	*0.119*	*0.119*	*0.12*	*0.119*	*0.12*	*0.118*	*0.12*	*0.127*	*0.117*	*0.134*	*0.117*	*0.118*	*0.118*	*0.118*	*0.12*	*0.117*	*0.117*	*0.117*	*0.01*	*0.019*	
Sai002	*0.115*	*0.115*	*0.116*	*0.115*	*0.116*	*0.114*	*0.116*	*0.123*	*0.113*	*0.127*	*0.113*	*0.114*	*0.114*	*0.114*	*0.116*	*0.113*	*0.113*	*0.113*	*0.024*	*0.024*	*0.022*

Four strains in italics belong to Cluster II

Limnothrix based on cultivated strains from China, and the six strains were identified as *L. planctonica* by careful morphological examination. The *L. planctonica* populations from the Donghu Lake were shown to have small-sized gas vesicles, similar to the descriptions by Meffert (1988). However, the cultivated strains in this study tended to lose the gas vesicles, as shown in both the light microscope and TEM images (Fig. 1). Such loss of gas vesicles in the *Limnothrix* strains was also illustrated in the study by Gkelis et al. (2005). It was also shown that several strains encoded as *Limnothrix* sp. CENA isolated from Brazil had inconspicuous gas vesicles compared with *L. redekei* NIVA 227. The phylogenetic tree based on the 16S rDNA sequences, including most *Limnothrix* strains so far analyzed with longer lengths (above 949 bp) of the 16S rRNA gene, revealed that all *Limnothrix* strains form two distinct clusters, namely, cluster I (a mixture of *L. redekei* NIVA227 and *Pseudanabaena* strains) and cluster II (mostly *Limnothrix* strains). The 16S rDNA sequence similarity between clusters I and II was lower than 90% (Table 2). Hence, the reported *Limnothrix* strains should belong to at least two different genera given that 95% of 16S rRNA gene sequence is regarded as the cut-off for genus definition in bacteriological classification (Ludwig et al., 1998). The cluster considered as the relatively reasonable genus *Limnothrix* has to be determined. The *L. redekei* NIVA 227 was set as the strain type by Suda et al. (2002), and the *L. redekei* PCC9416 (SAG 3.89) was set as the reference strain for the *Limnothrix*-form genus (Castenholz, 2001). The *L. redekei* NIVA 227 was in cluster I based on the results of this study and that by Gkelis et al. (2005), whereas *L. redekei* SAG 3.89 was also shown to be in cluster I by Perkerson et al. (2010). By contrast, the *Limnothrix* strains in Cluster II, including three Greek strains of *L. redekei*, three Brazilian strains (*Limnothrix* sp. CENA 109-111), and the six strains of *L. planctonica* in this study, were all shown to lose gas vesicles or have small-sized gas vesicles under the cultivated conditions. Such difference in gas vesicles within *Limnothrix* corresponds well to the classified types of gas vesicles in the genus *Limnothrix* initially proposed by Meffert (1988) and it is very interesting to get that their morphological and molecular characteristics coincided. Hence, *L. redekei* NIVA 227 and the other *Limnothrix* strains in cluster I are proposed to represent the genus *Limnothrix*, but this proposal requires that the confusion among *Pseudanabaena* strains in this cluster be cleared. The *Limnothrix* strains in cluster II represent a monophyletic group, similar to the work by Perkerson et al. (2010) who taxonomically revised some *Geitlernema* strains, and they may be reorganized as a new cyanobacterial genus. All the findings in this study allowed us to reevaluate the taxonomic system related to *Limnothrix* at both genus and species levels. However, additional molecular evidence from *Limnothrix* strains, such as divergence in gas vesicles genes (*gvp*) in the strains between clusters I and II, are necessary, which will help fully understand the phylogenetic relationship among *Limnothrix*-related genera.

Conclusively, the morphological and phylogenetic characteristics of *Limnothrix* strains originating from China were studied for the first time in this study. This is also the first report to obtain *L. planctonica* strains and to elucidate their ultrastructure. Based on the results from this and previous studies showing *Limnothrix* as polyphyletic, taxonomy of *Limnothrix*, still needs further examination.

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