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# *Limnothece alkaliphila* gen. et sp. nov. (Chroococcales, Cyanobacteria) from an alkaline lake in Mongolia Plateau, China

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**Abstract**: A coccoid cyanobacterial strain FACHB–3576 was isolated from Lake Hamatai, an alkaline–saline lake located near the Ordos *Spirulina* Industrial Park, Ordos, Inner Mongolia, China. This strain exhibited morphological similarities to the genus *Cyanobacterium*. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain FACHB–3576 formed a unique clade, distinctly separate from the other genera within the family Geminocystaceae. The genetic identity of 16S rRNA sequence between our strain and all other currently described genera in Geminocystaceae, including *Cyanobacterium*, was less than 95%. Moreover, this strain was further differentiated by its morphology and ecological characteristics. The strain is herein classified as a new cyanobacterial taxon *Limnothece alkaliphila* gen. et sp. nov. based on a polyphasic approach including data on morphology, ultrastructure, ecology, 16S rRNA phylogeny and secondary structure of the 16S–23S ITS region. The description of this novel taxon contributes to a better understanding of the diversity of coccoid cyanobacteria in alkaline–saline habitats.

**Keywords**: alkaline–saline lake, Cyanobacteria, *Limnothece*, morphology, phylogeny, polyphasic approach, taxonomy

## Introduction

Coccoid cyanobacteria are widely distributed in freshwater, marine and terrestrial habitats worldwide, and they play an important role in many aquatic and terrestrial communities for their substantial biomass, primary production and $N_2$ fixation, etc. (Komárek & Johansen 2015; Zhang et al. 2018). The taxonomic classification of cyanobacteria has undergone extensive restructuring and revision by a polyphasic approach using combined molecular, cytomorphological and ecological criteria in recent years (Komárek et al. 2014). Coccoid cyanobacteria, once previously grouped under Chroococcales, have now been reclassified into at least nine major groups, such as the Chroococcales, the Gloeobacterales, the Synechococcales, and the Chroococcidiopsidales (Komárek et al. 2014; Komárek & Johansen 2015; Mareš 2018; Strunecký et al. 2023). Cyanobacteria are typically the primary producers in saline environments, and several halophilic species belong to coccoid genera, such as *Aphanothece* Nägeli, *Chroococcidiopsis* Geitler, *Cyanothece* Komárek, *Euhalothece* Garcia–Pichel, Nübel et Muyzer, *Halothece* Margheri, Ventura, Kaštovský et Komárek, *Merismopedia* Meyen, *Pleurocapsa* Thuret, *Stanieria* Komárek et Anagnostidis, *Synechococcus* Nägeli and *Synechocystis* Sauvageau (Komárek & Johansen 2015; Oren 2015).

The non–baeocyte producing coccoids with fasciculated thylakoids mostly belong to the Chroococcales, which are probably the least studied major group of cyanobacteria (Mareš et al. 2019). Korelusová et al. (2009) confirmed the diversity and heterogeneity of traditional coccoid genus *Synechocystis* and described a new genus *Geminocystis* Korelusová, Kaštovský et Komárek, which is phylogenetically closely related to *Cyanobacterium* Rippka et Cohen–Bazire. The *Pseudanabaena*–like filamentous genus *Annamia* Nguyen with microcystin–producing type species *A. toxica* Nguyen was described by Nguyen et al. (2013), and assigned to the family Microcoleaceae in Oscillatoriales by Komárek et al. (2014). Brito

et al. (2017) proposed the new unicellular genus *Geminobacterium* Ramos, Brito et Kaštovský to accommodate to marine species *G. atlanticum* Ramos, Brito et Kaštovský. Komárek et al. (2020) established the *Synechococcus*–like genus *Picosynechococcus* Komárek, Johansen et Strunecký and classified it in the family Aphanothecaceae within Chroococcales. Tuji et al. (2021) proposed the family Geminocystaceae for a monophyletic clade within Chroococcales, including the genera *Annamia*, *Geminocystis*, *Geminobacterium* and *Cyanobacterium*, based on 16S rRNA phylogenetical analysis. Strunecký et al. (2023) transferred *Picosynechococcus* and *Microcrocis* Richter into the family Geminocystaceae based on phylogenomic and polyphasic analysis, although the genus *Microcrocis* lacks reliable sequencing data. Pokorný et al. (2023) proposed a novel epitype of the type species *M. geminata* (Lagerheim) Geitler, and confirmed the position of the genus *Microcrocis* within Geminocystaceae based on 16S rRNA phylogeny. The genus *Cyanobacterium*, with the type species *C. stanieri* Rippka et Cohen–Bazire, was initially proposed by Rippka & Cohen–Bazire (1983), but the names were not validly published under any code of nomenclature. Oren et al. (2022) proposed the names *Cyanobacterium* and *Cyanobacterium stanieri* for valid publication under the provisions of the ICN (International Code of Nomenclature for algae, fungi, and plants, then named the International Code of Botanical Nomenclature), and also assigned the genus to the family Geminocystaceae. Both coccoid and filamentous morphotypes are found in the family Geminocystaceae.

Lake Hamatai, located in the arid region of the Mongolia Plateau, China, is recognized as one of the most representative alkaline–saline lakes in the Ordos Desert. With an area of approximately 2.5 km$^2$ and a depth ranging from 2 to 3 meters in the central area, the lake exhibits a pH range of 8.63 to 10.30 and salinity levels between 0.71% and 4.46% (Liu et al. 2021). The ecological balance of this lake has been significantly affected by human activities. Notably, it is situated near the Ordos *Spirulina* Industrial Park, which serves as the largest *Limnospira* ("*Spirulina*") production base in China. The park contributes to about one–third (4000 t.year$^{-1}$) of China's total *Limnospira* production (Yang et al. 2022). The presence of sodium bicarbonate in the culture medium used for *Limnospira* production is easily obtained from the natural alkaline lake, and the lake also receives wastewater from commercial *Limnospira* production. The dominant phytoplankton in the lake is the filamentous cyanobacterium *Limnospira* Nowicka–Krawczyk, Mühlsteinová et Hauer and the flagellate green alga *Chlamydomonas* Ehrenberg. Furthermore, we have isolated some algal strains from Lake Hamatai belonging to *Chlamydomonas* sp., *Picocystis salinarum* R.A. Lewin, *Nitzschia* sp., *Limnospira* sp., *Nodosilinea* sp. and some coccoid cyanobacteria, etc.

In this study, a coccoid cyanobacterial strain was isolated from the planktonic community of Lake Hamatai. We have characterized this strain as a novel cyanobacterium, which we propose to name *Limnothece alkaliphila* gen. et sp. nov. This taxonomic assignment has been determined through a comprehensive polyphasic approach within the order Chroococcales.

## Materials and Methods

**Sampling and cultivation.** Water samples were collected from Lake Hamatai (39°06' N, 108°02' E) in Otog Banner, Ordos, Inner Mongolia, China. Cyanobacterial strain was isolated from the colonies by means of the micropipette washing method, and cultivated on Zarrouk medium (Zarrouk 1966) at 25 °C under a 12h:12h light–dark regime, with illumination of 30 μmol.m$^{-2}$.s$^{-1}$. The strain is maintained in the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB), National Aquatic Biological Resource Center.

**Light and transmission electron microscopy.** Morphological observation was performed for this cultured strain under an Olympus BX 53 light microscope (Olympus, Tokyo, Japan) with differential interference contrast. Micrographs were taken with an Olympus DP80 digital camera (Olympus, Tokyo, Japan) and Olympus software cellSens Standard (v. 1.14).

Cells were fixed with 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for transmission electron microscopy (TEM). The fixed materials were washed with 0.05 M phosphate buffer (pH 7.2), and postfixed with 2% osmium tetroxide in the same buffer at room temperature for 2 hours. They were dehydrated using a sequential ethanol gradient, and then embedded in Spurr's resin via propylene oxide (Spurr 1969). Ultrathin sections were cut using a diamond knife on Leica UC–7 (Leica, Wetzlar, Germany) and poststained with 1% uranyl acetate solution and lead citrate. The sections were observed using a Hitachi HT–7700 transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV.

**DNA extraction, amplification and sequencing.** Cyanobacterial genomic DNA was extracted using the NuClean Plant Genomic DNA Kit (CWBIO, Taizhou, China). The 16S rRNA gene and the associated 16S–23S ITS region were amplified using the two sets of primers CYA106F/23S5'R (Nübel et al. 1997; Baurain et al. 2002) and F322/R340 (Iteman et al. 2000). Amplification was performed in a BioRad PCR Thermocycler T100 (Bio–Rad Laboratories, Inc., USA) using the Ex Taq DNA Polymerase (Takara, Dalian, China). Reactions were cycled with an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of DNA denaturation at 94 °C for 40 s, primer annealing at 55 °C for 40 s, strand extension at 72 °C for 1.5 min, and a final extension step at 72 °C for 4 min. PCR products were sequenced with the same primers as used for PCR amplification. Both forward and reverse strands were sequenced on an ABI 3700 sequencer (Applied Biosystems, CA, USA). The assembled results were checked manually after automatic assembling by Chromas PRO.

**Phylogenetic analyses.** Phylogenetic analyses were based on 16S rRNA gene obtained in this study and representative sequences retrieved from GenBank using BLAST. All the sequences were aligned using Clustal X (v1.8) (Thompson

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et al. 1997) and MEGA7 (Kumar et al. 2016). The alignment was refined to a matrix of 106 sequences containing 1238 nucleotides, with ambiguous gap sites removed. The GTR+I+G was the best fit model under the Akaike Information Criterion (AIC) using the program jModelTest (v2.1.5) (Darriba et al. 2012). Phylogenetic analysis based on Maximum likelihood method (ML) was conducted in RAxML (v8.1.2) (Stamatakis 2014) with 1000 bootstrap repetitions. The software MrBayes (v3.2.2) was used for Bayesian inference (BI) (Ronquist et al. 2012). Seven chains of Bayesian Markov Chain Monte Carlo analyses, including six heated chains and one cold chain, were run for $5\times10^6$ generations. Log likelihood values and trees were sampled every 100 generations. The final average standard deviation of split frequencies was < 0.01. Bootstrap probability (BP) in ML tree and Bayesian posterior probability (PP) in BI tree of each clade were calculated. Uncorrected p–distance in 16S rRNA was used to calculate sequence identity (100*(1–p)). 16S–23S rRNA ITS secondary structures of D1–D1' and Box B helices were determined using RNA Folding Form version 2.3 energies at the Mfold server (Zuker 2003; http://unafold.rna.albany.edu/?q=mfold/RNA–Folding–Form2.3) and drawn by Adobe Illustrator CS5.

## Results

### Taxonomic description

***Limnothece* Q. Zhang et L. Song gen. nov. (Figs 1, 2)**

**Description:** Cells solitary or in pairs, aggregated into irregular groups. Cells oval or cylindrical with rounded ends, blue–green, usually surrounded by individual mucilaginous envelopes. Thylakoids arranged irregularly, usually more or less parietal. Cell division by binary fission in one plane in successive generations, perpendicular to the long axis.

**Etymology:** *Límni–* (Gr.) = lake; *thece* (Gr.) = chest.
**Type species:** *Limnothece alkaliphila* Q. Zhang et L. Song sp. nov.

***Limnothece alkaliphila* Q. Zhang et L. Song sp. nov. (Figs 1, 2)**

**Description:** Cells solitary or in pairs, aggregated into irregular groups. Cells oval or cylindrical with rounded ends, measuring 5.9–9.6 µm (–13.7 µm) long, 3.5–5.8 µm in diameter, 1.4 to 2.7(–3.9)× longer than wide (mean, 1.7×). Mucilage fine, homogeneous, colourless and diffluent surrounding individual cells, usually measuring about 0.1–2.2 µm width. Cell contents blue–green, usually with separation of centro– and chromatoplasma. Thylakoids distributed irregularly, usually arranged in small fascicles (with 3–8 thylakoids), and more concentrated towards the periphery of the cells (± parietal). Cell division by binary fission in one plane in successive generations, perpendicular to the long axis.

**Holotype:** HBI! Preserved material number GZ20191016 deposited in Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China.

**Type locality:** Lake Hamatai (39°06' N, 108°02' E), Ordos, Inner Mongolia, China, collected on 25th October 2019 by Qi Zhang.
**Habitat:** Free–living in an alkaline–saline lake.
**Etymology:** The species epithet '*alkaliphila*' reflects to the species loving alkaline environments.
**Reference strain:** Strain FACHB–3576 deposited in Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB–collection), National Aquatic Biological Resource Center, Wuhan, Hubei, China.
**GenBank accession numbers:** The 16S–23S rRNA gene sequence deposited as GenBank accession number OR162557.

### Molecular and phylogenetic analyses

In the molecular phylogenetic analyses based on the 16S rRNA gene, ML and Bayesian analyses produced similar tree topologies. The ML phylogenetic tree with indication of Bayesian posterior probabilities was shown in Fig. 3. The results showed that the genus *Limnothece* was closely related to the genera *Annamia*, *Geminocystis*, *Geminobacterium*, *Cyanobacterium*, *Microcrocis* and *Picosynechococcus* in Geminocystaceae with strong BP and PP support. However, it was distantly related to other halotolerant coccoid genera such as *Halothece* and "*Euhalothece*". The phylogenetic trees revealed that strain FACHB–3576 formed a separate genus–level clade, distinct from other genera other genera in Geminocystaceae. The phylogenies supported the monophyly of certain genera in Geminocystaceae, such as *Geminocystis* and *Picosynechococcus*. However, several strains of "*Cyanobacterium*" (PCC 10605, ETS–03 and CHAB TP201717.1) which fell outside of the clade with *C. stanieri* PCC 7202 rendered the genus *Cyanobacterium* polyphyletic.

FACHB–3576 exhibited relatively high 16S rRNA gene sequence similarities with representative strains of the genera *Annamia* (91.29%), *Geminocystis* (89.62–90.38%), *Geminobacterium* (89.35%), *Cyanobacterium* (89.10–89.69%), *Microcrocis* (89.10%) and *Picosynechococcus* (92.29–92.30%) (Table 1). Phylogenetic analyses further revealed that FACHB–3576 was closely related to three undescribed strains of "*Cyanobacterium*" (MT488169–171), with relatively high similarities in the 16S rRNA gene (96.57–96.72%). Additionally, three 16S rRNA sequences from uncultured cyanobacteria (JN397959, KX589496 and HM445952) exhibited similarities of 93.43–95.65% with FACHB–3576.

### Analyses of ITS secondary structures

In the analysis of ITS secondary structures, the secondary structures of the D1–D1' and Box–B regions from FACHB–3576 and its related taxa were compared (Fig. 4). The D1–D1' helix of FACHB–3576 consisted of 65 nt, with basal bilateral bulge at position 5–8/53–61, two internal loops in the mid–helix region at positions 15–16/43–46 and 20–23/35–39, and terminal loop

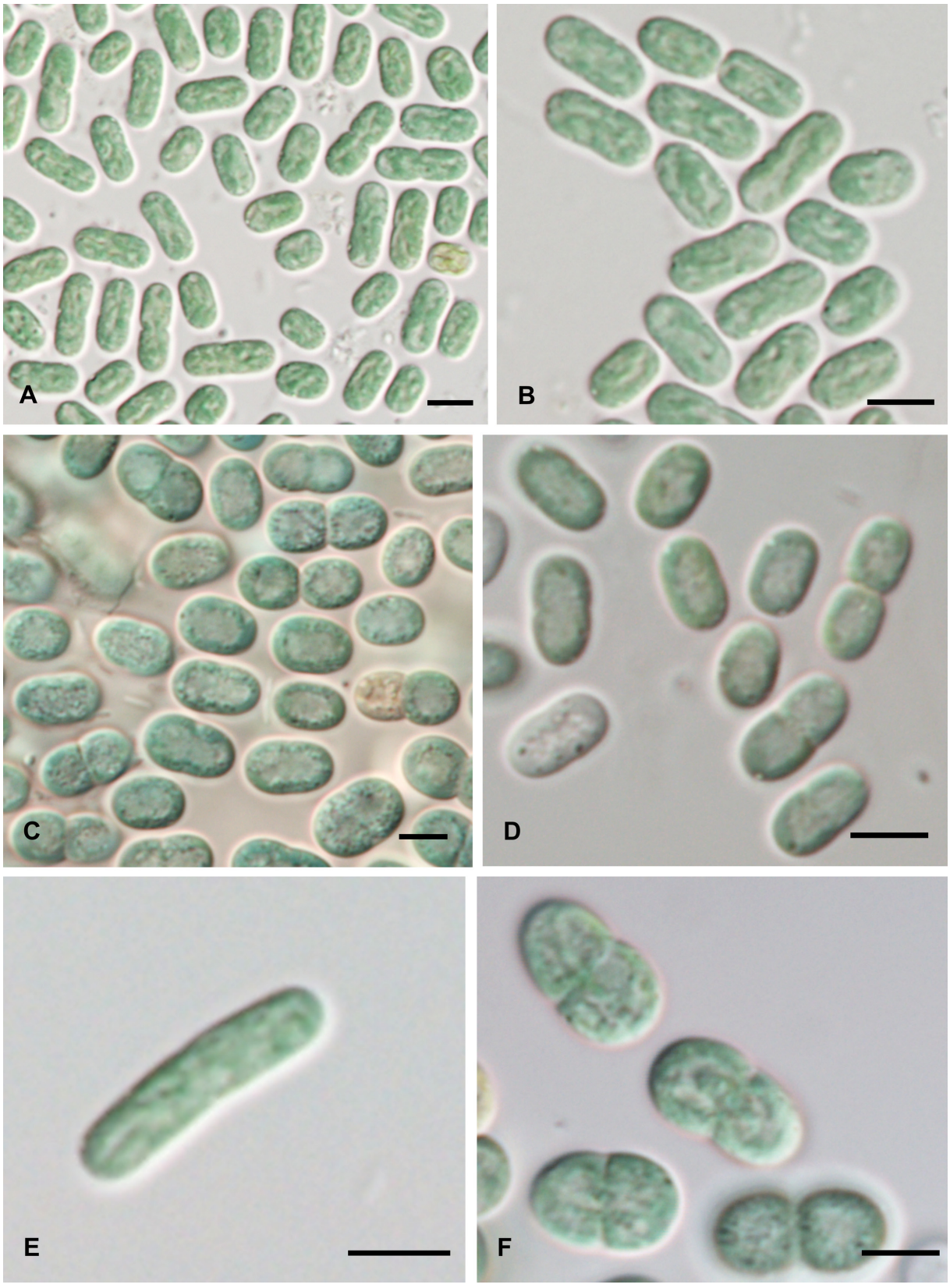


Fig. 1. Light micrographs of *Limnothece alkaliphila*: (A, B) cylindrical cells with rounded ends; (C, D) oval cells; (E) rare long cylindrical cells; (F) oval cells in binary fission. Scale bars 5 μm.

(5'–AGAAA–3'). In contrast, the D1–D1' helices in *Cyanobacterium stanieri* PCC 7202, *Geminocystis herdmanii* PCC 6308 and *Geminobacterium atlanticum* LEGE 07459 were considerably short and lacked an internal loop in the middle portion. The terminal loop in the D1–D1' helix consisted of five nucleotides

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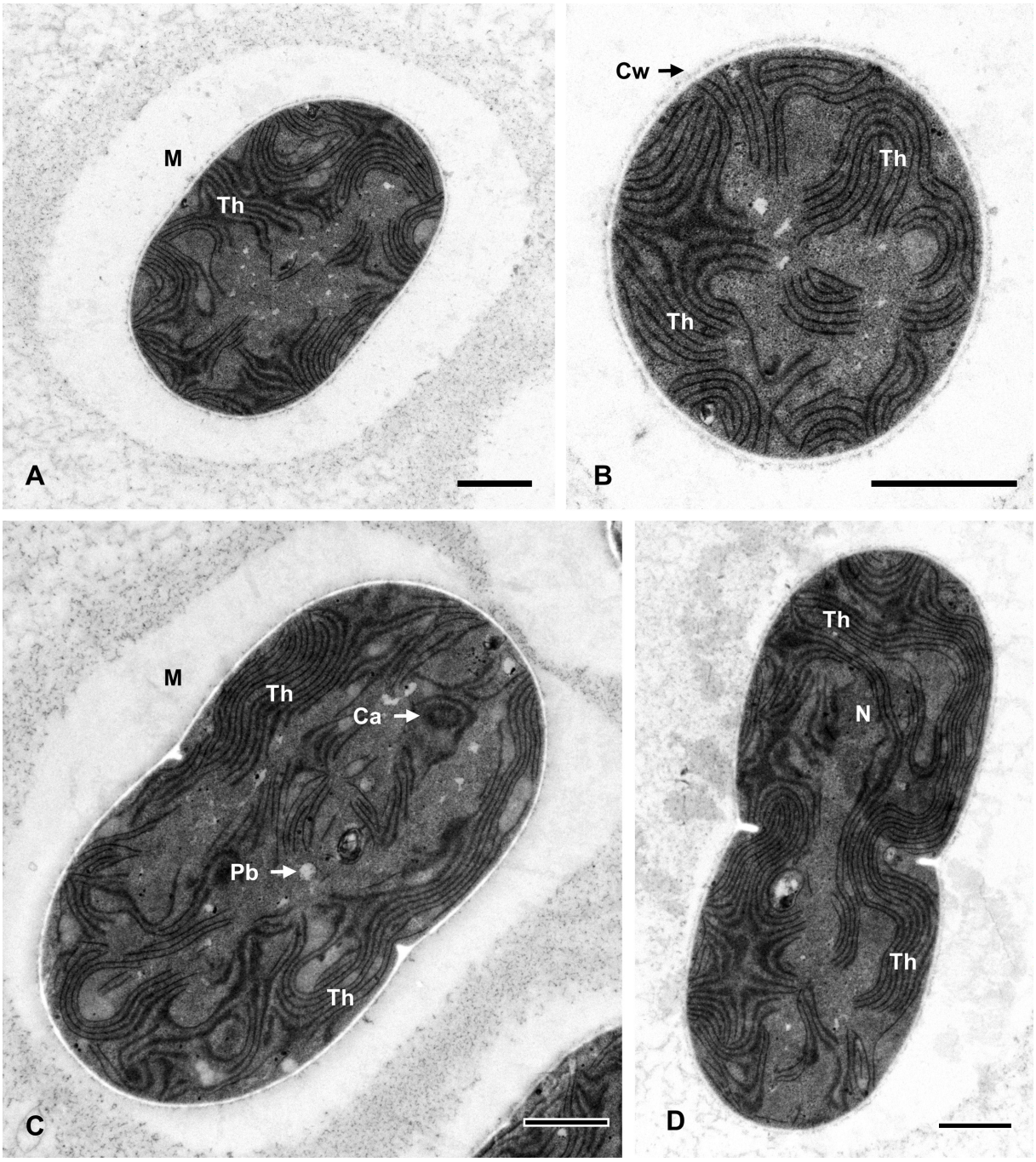


Fig. 2. Transmission electron micrographs of *Limnothece alkaliphila*: (A) section of cylindrical cell surround by mucilaginous envelopes in longitudinal view; (B) section of cell in cross–sectional view; (C, D) section of cell in binary fission. Scale bars 1 μm. Abbreviations: (Ca) = carboxysome; (CW) = cell wall; (M) = mucilage; (N) = nucleoplasmic region; (Pb) = polyphosphate body; (TH) = thylakoids.

in strain FACHB–3576, *C. stanieri* PCC 7202 and *Picosynechococcus* sp. PCC 7002, six nucleotides in *Annamia dubia* NIES–4383 and *G. atlanticum* LEGE 07459, four nucleotides in *G. herdmanii* PCC 6308, and ten nucleotides in *M. geminata* MC10. The Box–B helix was quite variable in structure. The Box–B helix of FACHB–3576 consisted of 34 nt, with a bilateral bulge at position 7–8/24–28, one unpaired adenine residue at position 20 of the 3' strand and a terminal loop (5'–GAAG–3'). The Box–B helix in *C. stanieri* PCC 7202 was shorter than FACHB–3576, and lacked an internal loop. The terminal loop in the Box–B helix had four nucleotides in FACHB–3576, *C. stanieri* PCC 7202, *G. herdmanii* PCC 6308 and *M. geminata* MC10. However, a larger terminal loop with eight nucleotides was present in *Picosynechococcus* sp. PCC 7002, nine nucleotides in *A. dubia* NIES–4383, and ten nucleotides in *G. atlanticum* LEGE 07459. The secondary structures of the D1–D1' and Box–B helices in FACHB–3576 were dissimilar compared with those from other related taxa used in this study.


Fig. 3. Maximum likelihood phylogenetic tree based on 16S rRNA gene. Numbers at nodes represent bootstrap support values (BP)/posterior probabilities (PP) from maximum likelihood and Bayesian inference, respectively. Only support values above 0.50 are shown. The sequence obtained from our study is shaded grey.

Table 1. 16S rRNA gene sequence similarities between *Limnothece alkaliphila* FACHB–3576 and its closely related taxa.

| | Strain | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Limnothece alkaliphila* OR162557 | | | | | | | | | | | | | | | | | | |
| 2 | "*Cyanobacterium* sp." MT488170–171 | 96.72 | | | | | | | | | | | | | | | | | |
| 3 | "*Cyanobacterium* sp." MT488169 | 96.57 | 99.85 | | | | | | | | | | | | | | | | |
| 4 | Uncultured bacterium JN397959 | 95.65 | 95.49 | 95.34 | | | | | | | | | | | | | | | |
| 5 | Uncultured bacterium HM445952 | 93.57 | 93.80 | 93.64 | 93.94 | | | | | | | | | | | | | | |
| 6 | Uncultured bacterium KX589496 | 93.43 | 92.90 | 92.74 | 93.66 | 91.41 | | | | | | | | | | | | | |
| 7 | *Picosynechococcus* sp. AJ000716 | 92.30 | 91.30 | 91.15 | 91.21 | 89.82 | 89.69 | | | | | | | | | | | | |
| 8 | *Picosynechococcus fontinalis* DQ455751 | 92.29 | 91.67 | 91.51 | 91.73 | 89.79 | 89.51 | 97.86 | | | | | | | | | | | |
| 9 | *Annamia dubia* NR_177026 | 91.29 | 91.67 | 91.52 | 91.60 | 90.95 | 89.30 | 90.53 | 90.96 | | | | | | | | | | |
| 10 | *Geminocystis* sp. AP014815 | 90.38 | 90.53 | 90.37 | 90.21 | 89.80 | 88.22 | 90.45 | 89.82 | 90.51 | | | | | | | | | |
| 11 | *Geminocystis herdmanii* AB039001 | 89.89 | 89.96 | 89.81 | 89.49 | 88.54 | 87.80 | 90.34 | 89.95 | 89.95 | 97.55 | | | | | | | | |
| 12 | *Geminocystis* sp. MH094654 | 89.62 | 89.38 | 89.23 | 88.98 | 88.65 | 87.45 | 89.76 | 89.36 | 90.21 | 98.17 | 97.86 | | | | | | | |
| 13 | *Geminobacterium atlanticum* KR676352 | 89.35 | 89.49 | 89.34 | 89.48 | 88.45 | 88.02 | 89.34 | 89.71 | 89.09 | 93.95 | 93.16 | 93.10 | | | | | | |
| 14 | *Cyanobacterium* sp. KM502966 | 89.69 | 89.84 | 89.69 | 89.36 | 88.50 | 87.30 | 88.24 | 88.75 | 89.36 | 93.51 | 93.72 | 93.51 | 92.03 | | | | | |
| 15 | *Cyanobacterium aponinum* NR_102443 | 89.59 | 89.51 | 89.36 | 89.72 | 88.70 | 88.11 | 89.82 | 89.65 | 90.41 | 95.18 | 94.78 | 94.87 | 93.79 | 93.50 | | | | |
| 16 | *Cyanobacterium aponinum* AM238427 | 89.56 | 89.48 | 89.32 | 89.69 | 88.67 | 88.23 | 89.78 | 89.62 | 90.38 | 95.17 | 94.77 | 94.78 | 93.69 | 93.32 | 99.69 | | | |
| 17 | *Cyanobacterium* sp. KF246493 | 89.54 | 89.76 | 89.61 | 88.98 | 88.11 | 87.61 | 88.39 | 88.37 | 88.91 | 93.66 | 93.57 | 93.51 | 91.88 | 97.48 | 93.11 | 93.02 | | |
| 18 | *Cyanobacterium stanieri* NR_102450 | 89.24 | 89.69 | 89.53 | 89.44 | 88.50 | 87.76 | 88.85 | 88.68 | 89.21 | 94.05 | 94.10 | 93.59 | 91.95 | 97.48 | 93.42 | 93.40 | 99.16 | |
| 19 | *Microcrocis geminata* ON426371 | 89.10 | 88.43 | 88.33 | 89.26 | 88.37 | 86.80 | 88.05 | 88.14 | 89.35 | 90.98 | 89.92 | 90.88 | 89.05 | 89.76 | 90.29 | 90.16 | 89.19 | 89.00 |

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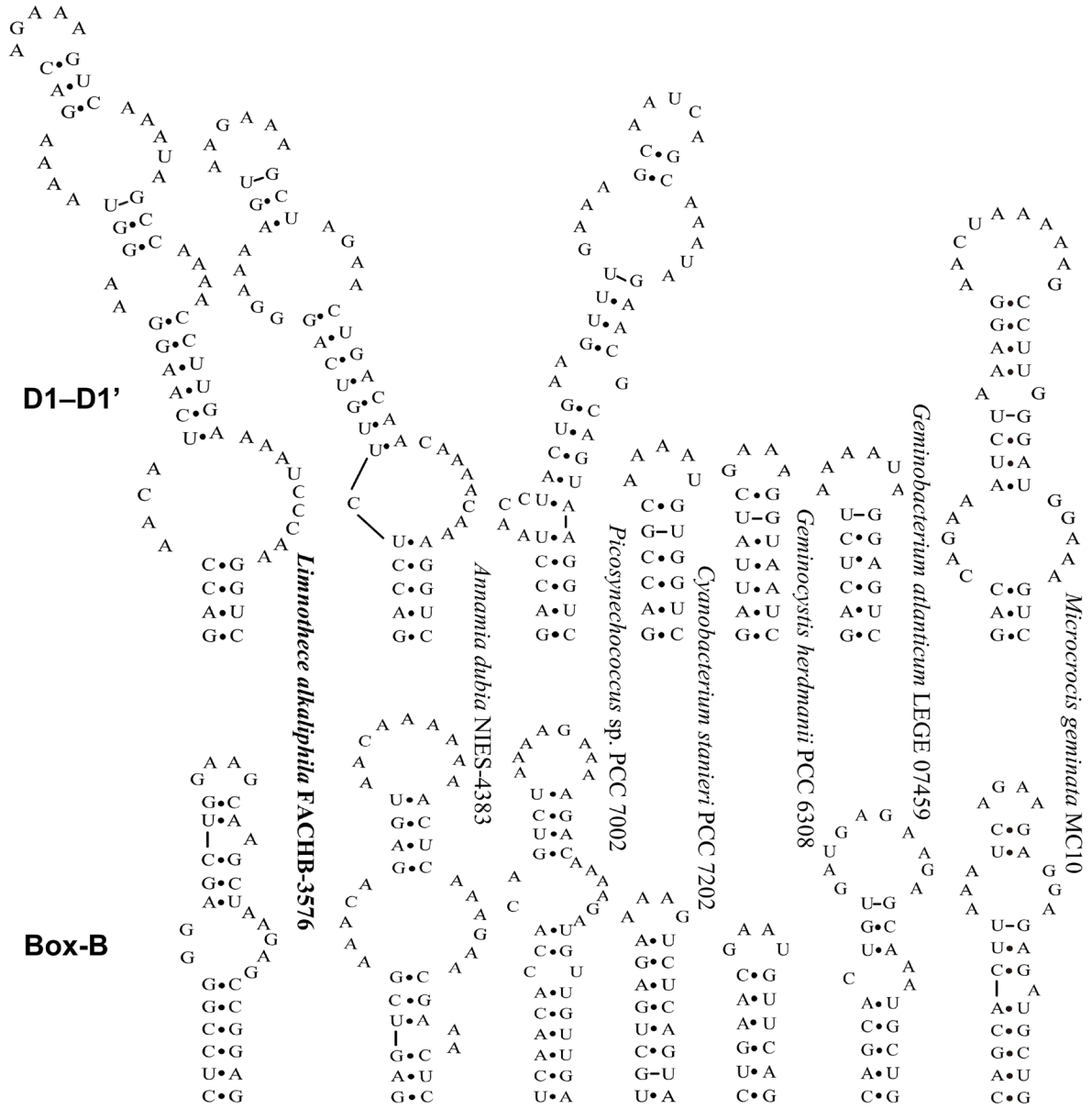


Fig. 4. Predicted secondary structure for D1–D1' and Box–B helices of 16S–23S rRNA intergenic spacer of representative strains in family Geminocystaceae.

## Discussion

In the family Geminocystaceae, the newly described genus *Limnothece* can be distinguished from other cyanobacterial genera, including *Annamia*, *Cyanobacterium*, *Geminobacterium*, *Geminocystis*, *Microcrocis* and *Picosynechococcus*, mainly by cell size, mucilage and colonial characteristic, thylakoid arrangement and ecological niche (Table 2). Most genera within the family Geminocystaceae consist of coccoid cyanobacteria, with the exception of the unbranched filamentous genus *Annamia* (Nguyen et al. 2013). The coccoid cells in Geminocystaceae are typically spherical to rod–shaped with rounded ends, with irregularly or parallely distributed thylakoids, lack sheaths, and undergo binary fission in one or two planes (Tuji et al. 2021).

The coccoid cells in Geminocystaceae can exist as solitary cells or in aggregated groups. In particular, the genus *Microcrocis* usually forms a typical tabular colony with irregular cells arrangement (Pokorný et al. 2023). The cells of *Geminobacterium* and *Geminocystis* are spherical or nearly spherical, while those of *Limnothece*, *Cyanobacterium*, *Microcrocis* and *Picosynechococcus* are oval, cylindrical or rod–like. Notably, long and abundant pilus–like structures have been observed only in *Geminobacterium* (Brito et al. 2017). The cells of *Picosynechococcus* are smaller compared to other coccoid genera in Geminocystaceae (Komárek et al. 2020). Furthermore, the cells of *Limnothece*, *Cyanobacterium*, *Geminobacterium* and *Picosynechococcus* are divided by

Table 2. Morphological comparison of *Limnothece* with other closely related genera in family Geminocystaceae.

| | *Limnothece* | *Cyanobacterium* | *Geminobacterium* | *Geminocystis* | *Picosynechococcus* | *Microcrocis* |
|---|---|---|---|---|---|---|
| Thallus structure | solitary or in pairs, aggregated in groups | solitary or in pairs, rarely aggregated in groups | solitary or aggregated in groups | solitary | solitary or rarely short pseudofilaments | microscopic or rarely macroscopic, flat, with irregularly aggregated cells |
| Mucilage | fine, homogeneous, colourless and diffluent surrounding cells | very fine, formless | with mucilage | narrow, fine, colorless, or rarely without mucilage | without prominent mucilage | fine, colorless colonial mucilage, and without individual mucilaginous envelopes |
| Cell content | separation of centro and chromatoplasma | homogeneous | — | homogeneous | homogeneous | homogeneous |
| Cell shape | oval to cylindrical with rounded ends | widely oval to rod–like curved | spherical | spherical, slightly oval or hemispherical after division | slightly elongated and oval with rounded ends, sometimes slightly curved | widely oval to rod–like with rounded ends |
| Cell size | 5.9–9.6 (–13.7) × 3.5–5.8 µm | 2.5–7 (–12) × 1.7–4.5 µm | 2.0–3.0 µm in diameter | 3–10 µm in diameter | 1.2–3.0 × 0.8–2.0 µm | (2–) 5–16 (–19) × 1.5–6 (–8) µm |
| Thylakoid arrangement | irregular, usually slightly fasciculate and ± parietal | ± parallel and usually in entire cell | irregular | irregular or ± parallel | ± parietal, slightly irregular and waved | parallel |
| Colour | blue–green | blue–green | blue–green or olive–green | pale blue–green, bright–green, grey or pinkish | blue–green or greenish | pale to bright blue–green |
| Cell division | one plane in successive generations | one plane in successive generations | one plane in successive generations | two perpendicular planes in successive generations | one plane in successive generations | two perpendicular planes in successive generations |
| Habitat | free–living in alkaline–saline lake | euryhaline or alkaline water | epizoic on shells of marine gastropod mollusc | planktic in lakes, on surface of wet soils | planktic or periphytic in mineral springs with high salt concentration, rarely in the littoral of seas | free–living, metaphytic or epipelic in freshwater and marine environments |
| References | this study | Oren et al. 2022 | Brito et al. 2017 | Korelusová et al. 2009 | Komárek et al. 2020 | Wehr et al. 2015; Pokorný et al. 2023 |

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simple binary cell division in a single plane perpendicular to the longitudinal axis, while *Geminocystis* and *Microcrocis* have two perpendicular planes for division. The longitudinal cell division in *Microcrocis* is rather specific (Pokorný et al. 2023). The cells of *Limnothece* are morphologically similar to *Cyanobacterium*, but mainly differ in the arrangement of thylakoids. The thylakoids of *Cyanobacterium* are arranged in more or less parallel throughout the entire cell, whereas *Limnothece* exhibits thylakoids usually irregularly arranged in small fascicles and parietally distributed in the longitudinal and cross–section of cell, leaving an irregular central area (Table 2). The chromatoplasm and centroplasm in *Limnothece* are usually recognizable under light microscopy; however, the cell content of *Cyanobacterium* is usually homogeneous. Habitat specificity is suggested to be a taxonomic informative character for cyanobacteria (Řeháková et al. 2007; Dadheech et al. 2012). *Geminocystis* and planktonic *Annamia* are commonly found in freshwater lakes or on the surface of wet soil. *Picosynechococcus* and *Microcrocis* species extensively exist in freshwater, brackish, saline and marine environments. On the other hand, the only known species of *Geminobacterium*, *G. atlanticum*, is rarely found as epizoic on the shells of marine gastropod mollusks (Brito et al. 2017). It is worth noting that both the strain FACHB–3576 and the reference strain of *Cyanobacterium* (*C. stanieri* strain PCC 7202) were isolated from alkaline water environments, further highlighting their ecological similarity.

Genera are best defined as monophyletic clades containing one or more species which have at least some morphological characters separating them from other genera, so phylogenetic analysis is conducted as a prerequisite to identifying genus–level clusters (Mai et al. 2018). Our phylogenetic analyses support the classification of the genera *Annamia*, *Cyanobacterium*, *Geminobacterium*, *Geminocystis*, *Microcrocis* and *Picosynechococcus* within the monophyletic family Geminocystaceae in the order Chroococcales (Tuji et al. 2021; Strunecký et al. 2023). The 16S rRNA gene phylogeny also supports that strain FACHB–3576 is phylogenetically placed in the family Geminocystaceae. The genera *Halothece*, *Myxobactron* and invalidly published "*Euhalothece*" form a halophilic cluster distinctively separated from strain FACHB–3576 in our phylogenetic analyses. Most bacteriologists currently suggest that the strains with 16S rRNA sequence similarity less than 94.5% should be considered as separate different genera (Stackebrandt 2006; Yarza et al. 2014). In the case of strain FACHB–3576, it has low 16S rRNA gene sequence identity (< 94.5%) with other genera, providing strong evidence that it represents a separate genus–level lineage in the molecular phylogenies. The establishment of the genus *Limnothece* is further supported by the unique characteristics observed in D1–D1' and Box–B helices of the 16S–23S ITS secondary structure. These features indicate that this strain does not belong to any of the related genera. The polyphasic approach, including data on morphology, ultrastructure, ecology, 16S rRNA phylogeny and secondary structure of the 16S–23S ITS region, provides comprehensive support for proposing the new genus *Limnothece* within the family Geminocystaceae.

The genus *Cyanobacterium* was initially proposed by Rippka & Cohen–Bazire (1983) with type species *C. stanieri* based on strain PCC 7202, and validly published by Oren et al. (2022). Strain PCC 7202 was isolated by M. Lefèvre from an alkaline pond or lake in Chad in 1963. Strain CENA527 and IPPAS B–1200 were isolated from saline–alkaline Lake Grande in Brazil and saline Lake Balkhash in Kazakhstan, respectively (Andreote et al. 2014; Sarsekeyeva et al. 2014), and they exhibit morphological and ecological similarities to *C. stanieri* PCC 7202. Our phylogenies based on 16S rRNA sequences indicate that CENA527 and IPPAS B–1200 form a highly supported clade with *C. stanieri* PCC 7202, confirming their placement within the genus *Cyanobacterium*. These three strains undoubtedly represent the true lineage of *Cyanobacterium*. Moro et al. (2007) described a new species *Cyanobacterium aponinum* from microbial mats of Euganean thermal springs in Italy, with strains ETS–03 and PCC 10605. However, our phylogenetic analyses indicate that *C. aponinum* is phylogenetically distant from the type species *C. stanieri* and is closely related to *Geminobacterium atlanticum*, suggesting that it should be removed from the genus *Cyanobacterium*. Our analyses also reveal that the reference strain of the genus *Limnothece* is morphologically and ecologically similar to *Cyanobacterium*, but phylogenetically separated from all previously described genera within the Geminocystaceae. In addition, *L. alkaliphila* strain FACHB–3576 clusters together with some taxonomically uncertain cyanobacteria within the phylogeny analyses based on 16S rRNA gene. Three "*Cyanobacterium*" strains (CHAB TP201717.1 clone 03–05) have relatively high similarities (96.57–96.72%) of the 16S rRNA sequence to *L. alkaliphila*. However, due to the lack of sufficient information, we cannot formally describe these strains as new species within the genus *Limnothece* at this time

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