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Four newly recorded species of planktonic cyanobacteria (Oscillatoriales, Cyanobacteria) in Korea

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Four species of cyanobacteria that are unrecorded in Korea were isolated from freshwater and brackish water. These four species are *Laspinema thermale* of Laspinemaceae, *Planktothricoides raciborskii* and *Planktothrix spiroides* of Microcoleaceae, and *Cephalothrix lacustris* of Phormidiaceae, all belonging to the order Oscillatoriales. *Laspinema thermale* is morphologically characterized as apical cells that are longer than other cells. In this strain, the similarity of the 16S rRNA gene sequence with the previously reported *L. thermale* strains were 99.30–99.50%. *Planktothricoides raciborskii*, which is characterized by bluntly conical morphology of apical cells, showed 98.80–99.50% of similarity of the 16S rRNA gene sequence to the previously reported *P. raciborskii* strains. *Planktothrix spiroides* are characterized by floating due to gas vacuoles. In this strain, the similarity of the 16S rRNA gene sequence with the previously reported *P. spiroides* strains were 99.80–99.90%. *Cephalothrix lacustris*, characterized by having calyptra in apical cells, showed 99.80–99.90% similarity of the 16S rRNA gene sequence to previously reported *C. lacustris* strains. Also, these species were clustered in the same clade in phylogenetic analysis using 16S rRNA gene sequences with each corresponding species.

Keywords: 16S rRNA, brackish water, cyanobacteria, freshwater, polyphasic

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Introduction

Cyanobacteria inhabit various places on water and land, stabilizing sedimentary and soil layers by producing organic compounds needed by other living things. However, while having such positive action, they are also a problem worldwide because they can produce toxic substances or have negative impacts that cause harmful algal blooms (Graham *et al*., 2009). In the past, cyanobacteria were identified using morphological characteristics, but it is challenging to accurately identify cyanobacteria using only morphological characteristics, so the use of molecular data has been increasing recently (Anagnostidis and Komárek, 1985; Willame *et al*., 2006). Therefore, the identification of cyanobacteria is currently carried out through a polyphasic research method that combines the traditional research method using morphology and ecological characteristics and the modern research method using molecular data (Castenholz, 1992; Komárek, 2016). Since the 2000s, more species of cyanobacteria have been identified through molecular data analysis using the 16S rRNA gene sequence, and more than 5,000 species have been reported worldwide (Guiry and Guiry, 2022). In Korea, 393 taxa of cyanobacteria have been reported so far (NIBR, 2022). Recently, *Wilmottia koreana* (Lee *et al*., 2020), *Pinocchia daechunga* (Kim *et al*., 2021), and *Pseudoaliinostoc sejongens* (Lee *et al*., 2021) were reported as new species of cyanobacteria in Korea. Also, *Aerosakkonema funiforme* (Kim *et al*., 2020), *Microseira wollei* (Bae *et al*., 2020), and *Pantanalinema rosaneae* (Lee, 2022) were reported as newly recorded species of cyanobacteria in Korea.

The planktonic cyanobacteria *Laspinema thermale*, *Planktothricoides raciborskii*, *Planktothrix spiroides*, and *Cephalothrix lacustris* collected in this study were isolated from freshwater and brackish water. In the genus *Laspinema*, a total of three species (*L. etoshii*, *L. lumbricale*, and *L. thermale*) have been reported; they appear in thermal springs and small saline ponds, and some strains also appear in wet soils (Heidari *et al*., 2018). Only two species have been reported in the genus *Planktothricoides*, *P. attenuate* and *P. raciborskii*, and they appear in freshwaters such as lakes and reservoirs (Suda *et al*., 2002; Komárek and Komárková-Legnerová, 2007). A

total of 16 species, including *P. spiroides*, have been reported in the genus *Planktothrix*. They appear in seawater as well as freshwaters such as lakes, reservoirs, and ponds, and some strains also appear in thermal springs (Anagnostidis and Komárek, 1988; Suda *et al*., 2002; Komárek and Komárková, 2004; Liu *et al*., 2013). In the genus *Cephalothrix*, a total of three species (*C. alaskaensis*, *C. komarekiana*, and *C. lacustris*) have been reported; they appear in freshwaters such as alkaline lakes and ponds, and some strains also appear in plants around lakes (Malone *et al*., 2015; Strunecky *et al*., 2020).

In this study, cyanobacteria of four strains isolated from freshwater and brackish water in Korea were cultured. After that, they were identified as *Laspinema thermale*, *Planktothricoides raciborskii*, *Planktothrix spiroides*, and *Cephalothrix lacustris* using morphological traits and molecular data with 16S rRNA sequences. As a result, in this study, these cyanobacteria of four species were added to the Korean flora as unrecorded species in Korea.

Materials and Methodes

Sample collection and cultures

The cyanobacteria strains for this study were collected from freshwater and brackish water located in Asan-si, Gongju-si, Nonsan-si, and Seocheon-gun in Chungcheongnam-do from February 2019 to May 2020 (Table 1). The planktonic cyanobacteria were collected using a plankton net with a mesh diameter of 25 μm (Sournia, 1978). The collected samples were sealed up in the icebox at 4°C and transported to the laboratory.

For the unialgal culture, only one trichome was picked using a Pasteur's pipette under a light microscope and then transferred to a 12-well plate (SPL, Pocheon, Korea) containing BG-11 medium (Stanier *et al*., 1971). After culturing in a 12-well plate for 1−2 weeks, the presence of contamination was checked, and for mass culture, the cyanobacteria with an unialgal culture were transferred to a 50 mL cell culture flask (SPL, Pocheon, Korea) containing BG-11 medium. Cultures conditions were 20−25°C, a photoperiod 16 : 8, and illumination of 25 μmol/m^{2}s (Lee *et al*., 2019). The mass-cultured cyanobacteria were deposited in the Freshwater Bioresources Culture Collection (FBCC) of the Nakdonggang National Institute of Biological Resources of Korea.

Morphological analysis and characterization

Morphological traits of cyanobacteria were observed for using a light microscope (Olympus BX53, Olympus, Japan) under 100−1,000× magnification, and photographs were taken under 200−1,000× magnification (Olympus UC-90, Olympus, Japan). The identification of cyanobacteria was performed with reference to Suda *et al*. (2002), Liu *et al*. (2013), Malone *et al*. (2015), and Heidari *et al*. (2018).

DNA extraction and sequencing

For genomic DNA (gDNA) extraction, 1 mL of mass-cultured cyanobacteria were transferred to a 1.5 mL microcentrifuge tube and centrifuged at 13,000 rpm for 5−10 minutes. After centrifugation, the supernatant was removed, and the concentrated cells were ground using a homogenizer. Then, gDNA was extracted using the i-genomic Plant DNA Extraction Mini Kit (iNtRON, Korea).

PCR was performed using bacterial primers to secure the 16S rRNA gene sequence, and the bacterial primers used were 27F1 (5′-AGAGTTTGATCCTGGCTCAG-3′) (Neilan *et al*., 1997) and 23S30R (5′-CTTCGCCTCTGT GTGCCTAGGT-3′) (Taton *et al*., 2003). The PCR reaction was prepared by putting sterile tertiary distilled water (17 μL), extracted gDNA (1 μL), 27F1, and 23S30R (10 pmole, 1 μL) in Maxime™ PCR PreMix Kit (i-StarTaq™ GH) (iNtRON, Korea) so that the total volume was 20 μL, and the PCR reaction was performed in Mastercycler® NEXUS GRADIENT 6331 model (Eppendorf, Germany). The PCR reaction was initially denatured ant 94°C for

Table 1. Sampling sites in Korea from July 2019 to August 2020.

Strain	Location	GPS	Source
FBCC-A1475	Hakseong Stream, Daeheung-ri, Seonjang-myeon, Asan-si, Chungcheongnam-do	36°48′48.1″N, 126°52′35.2″E	Freshwater
FBCC-A1472	Noseong Stream, Hangwol-ri, Gwangseok-myeon, Nonsan-si, Chungcheongnam-do	36°14′09.4″N, 127°08′02.3″E	Freshwater
SJH-1	Keum River estuary, Dosam-ri, Maseo-myeon, Seocheon-gun, Chungcheongnam-do	36°01′22.4″N, 126°44′34.2″E	Brackish water
FBCC-A1473	Keum River, Ungjin-dong, Gongju-si, Chungcheongnam-do	36°27′49.3″N, 127°06′01.8″E	Freshwater

5 minutes, and then repeated 33 times at 94°C for 1 minutes, 55°C for 2 minutes and 72°C for 3 minutes to amplify the target 16S rDNA gene region. After gene amplification was completed, the reaction was terminated after maintaining at 72°C for 10 minutes. The amplified PCR product was observed through electrophoresis on an agarose gel at a concentration of 1%. The PCR products were purified using MEGAquick-spin™ Plus DNA Purification Kit (iNtRON, Korea) and sequenced at Bionics Co., Ltd. (Seoul, Korea). The individual gene sequences were assembled using DNASTAR Lasergene® SeqMan Pro™ ver. 7.1.0 (DNASTAR Inc., WI, USA).

Data analyses

To analyze the similarity and genetic distance of the 16S rRNA gene sequence, the previously reported gene sequences relatively closely related to the gene sequence of cyanobacteria to be identified in this study were confirmed and collected from Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of NCBI (National Center for Biotechnology Information). The collected 16S rRNA gene sequence data were aligned with BioEdit ver. 7.2.5 (Hall, 1999), then MEGA X (Kumar *et al*., 2018) was used to calculate gene sequence similarity and genetic distance with Kimura 2-parameter model.

Phylogenetic analyses

For the phylogenetic analysis, the Maximum Likelihood (ML) phylogeny was estimated from RAxML ver. 7.0.3 (Stamatakis, 2006) using General Time Reversible (GTR), and then TreeView ver. 1.6.6 was used to visualize the phylogenetic tree. Also, MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001) was used to infer the Bayesian tree, with Markov Chain Monte Carlo (MCMC) performed 5 million generations.

Results and Discussion

Morphological characterization

Order Oscillatoriales Schaffner, 1922
Family Laspinemataceae Zimba *et al*., 2020

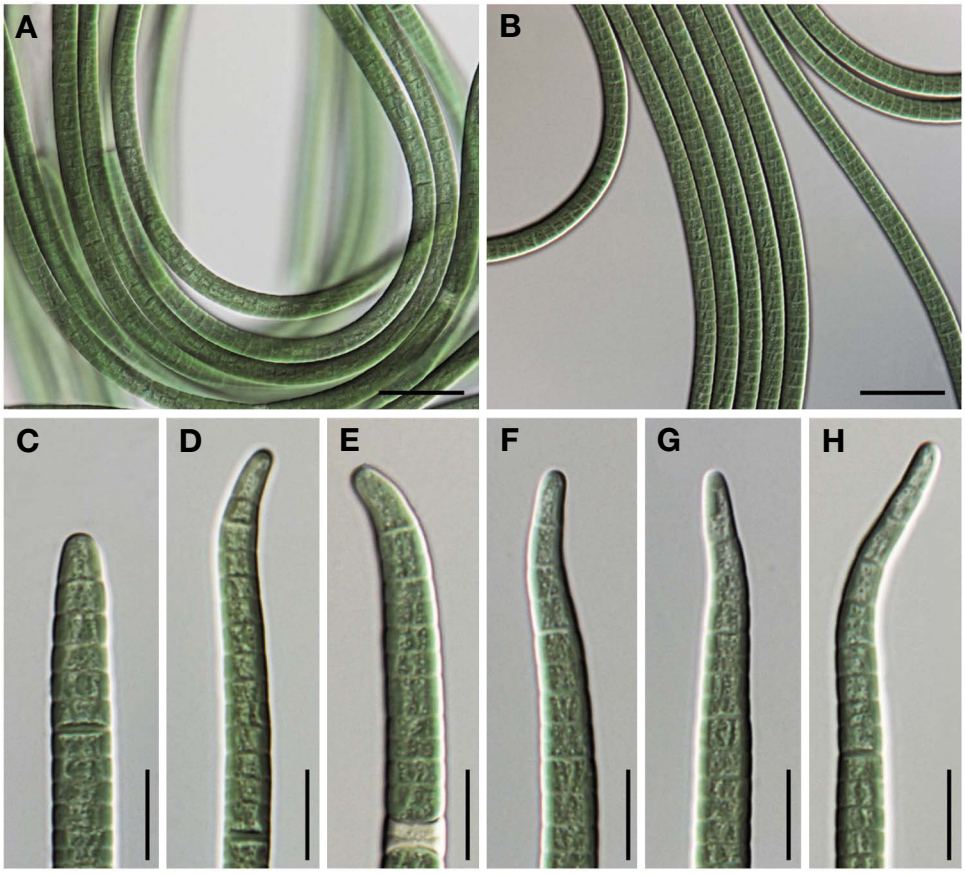

Fig. 1. Microscopic photographs of *Laspinema thermale* FBCC-A1475. (A, B) Arrangement of filament in the colony, (C–H) Apical cell of trichomes, (E) Necridic cell. Scale bars (A, B) 20 μm, (C–H) 10 μm.

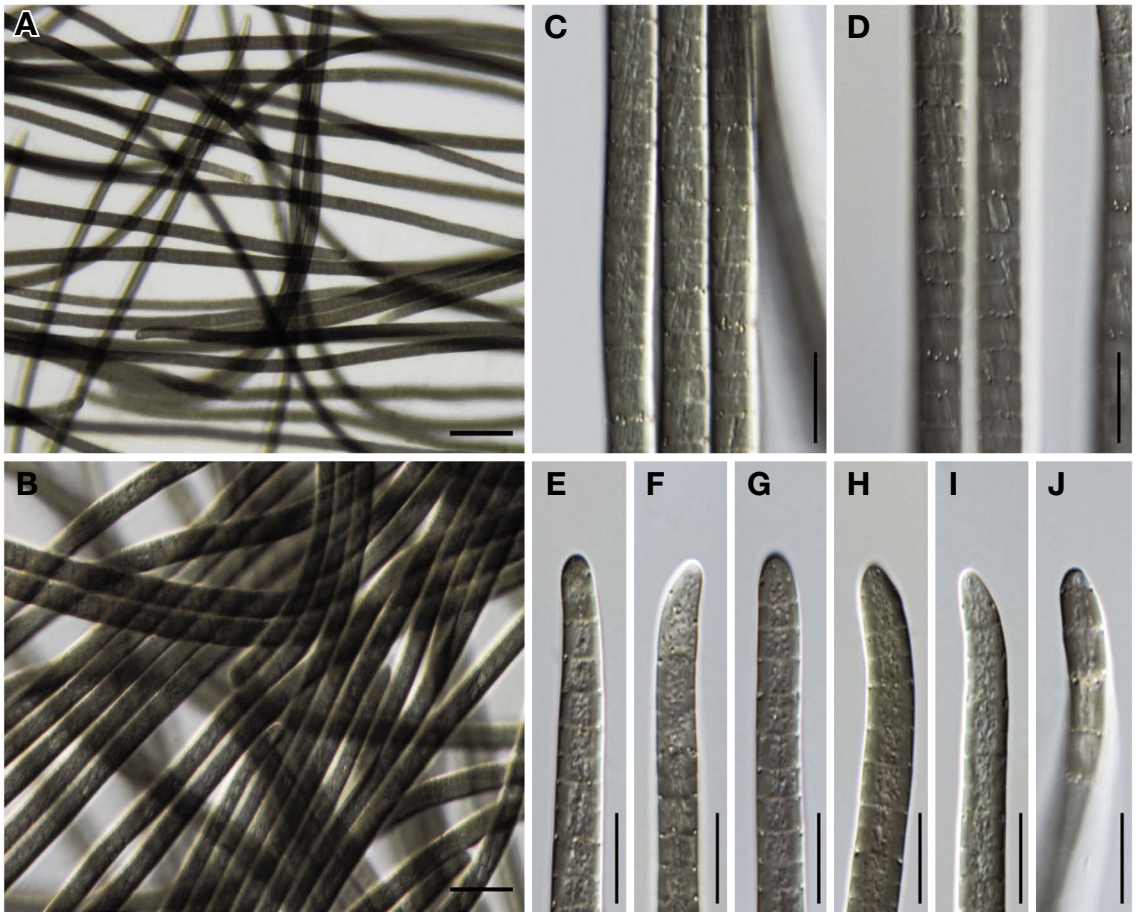

Fig. 2. Microscopic photographs of *Planktothricoides raciborskii* FBCC-A1472. (A, B) Arrangement of filament in the colony, (C, D) Surface of trichomes, (E–J) Apical cell of trichomes. Scale bars (A) 50 μm, (B) 20 μm, (C–J) 10 μm.

Genus *Laspinema* Heidari and Hauer, 2018

***Laspinema thermale* Heidari and Hauer, 2018 (Fig. 1)**

Filaments straight, blue-green or olive green in color. Trichomes cylindrical, unbranched, motile, slightly constricted at the cross-walls. Apical cells longer than other vegetative cells, conical and rounded at the apex, curved or straight, without calyptra. Cells 1.29–2.28 μm long, 3.88–4.75 μm wide.

Ecology. This species appeared in thermal springs (Heidari *et al*., 2018) and was isolated from freshwater in this study.

Distribution. Iran (Heidari *et al*., 2018).

Site of collection. 483-1, Daeheung-ri, Seonjang-myeon, Asan-si, Chungcheongnam-do (37°01′07.5″N, 127°55′11.4″E).

Date of collection. May 25, 2020.

Specimen deposit No. FBCC-A1475.

Family Microcoleaceae Strunecky *et al*., 2013

Genus *Planktothricoides* Suda and Watanabe, 2002

***Planktothricoides raciborskii* Suda and Watanabe, 2002 (Fig. 2)**

Trichomes solitary, floating, straight or slightly bent at the end, attenuated towards ends, pale blue-green or yellow-green in color. Cross-walls slightly constricted or not constricted, ungranulated. Sheaths rarely, thin, clear. Apical cells rounded, bluntly conical, more or less tapered, bent, not pointed. Cells with small gas vacuoles, 2.39–2.97 μm long, 6.88–7.98 μm wide.

Ecology. This species appeared in an island pond (Suda *et al*., 2002) and was isolated from freshwater in this study.

Distribution. Indonesia (Suda *et al*., 2002).

Site of collection. 80-1, Hangwol-ri, Gwangseok-myeon, Nonsan-si, Chungcheongnam-do (36°14′09.4″N, 127°08′02.3″E).

Date of collection. August 06, 2019.

Specimen deposit No. FBCC-A1472.

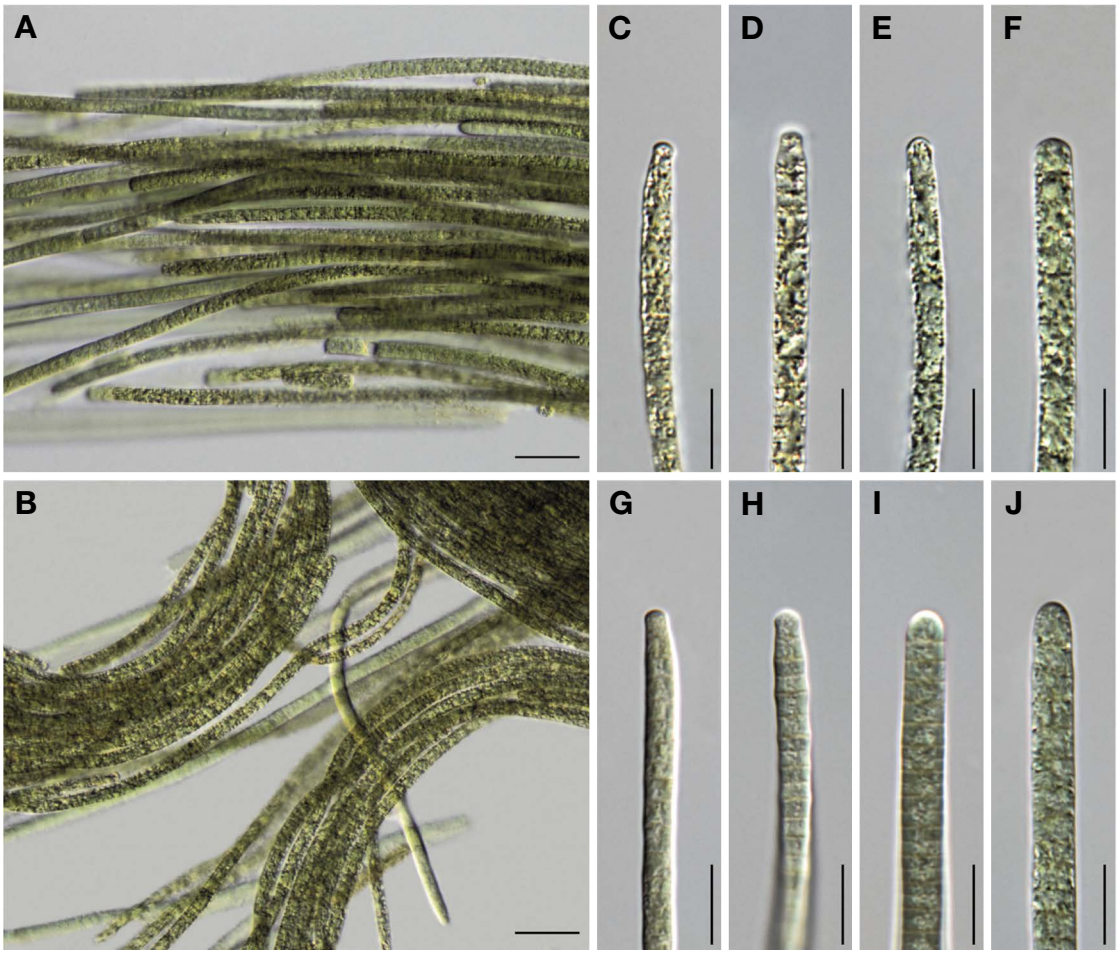

Fig. 3. Microscopic photographs of *Planktothrix spiroides* SJH-1. (A, B) Arrangement of filament in the colony, (C–J) Apical cell of trichomes. Scale bars (A, B) 20 μm, (C–J) 10 μm.

Genus *Planktothrix* (Anagnostidis and Komárek, 1988)

***Planktothrix spiroides* Wang and Li, 2013 (Fig. 3)**

Filaments solitary, floating, blue-green or olive green, rarely yellowish blue-green in color. Trichomes non-heterocystous, straight or slightly curved, regularly loose screw-like coils, become irregularly coiled with prolonged cultivation, attenuated or not attenuated towards ends, rarely motile, without false branching. Cross-walls rarely or not constricted, ungranulated. Mucilaginous sheaths rare. Apical cells rounded, slightly or not narrowing towards the apex, sometimes with calyptra. Cells cylindrical or rarely barrel-shaped, usually slightly shorter than wide, with gas vacuoles, 1.91–2.63 μm long, 3.97–6.14 μm wide. Reproduction by fragmentation into hormocytes.

Ecology. This species appeared in ponds (Liu *et al*., 2013) and was isolated from brackish water in this study.

Distribution. China (Liu *et al*., 2013).

Site of collection. 59, Dosam-ri, Maseo-myeon, Seocheon-gun, Chungcheongnam-do (36°01′22.4″N, 126°44′34.2″E).

Date of collection. June 27, 2019.

Specimen deposit No. SJH-1.

Family Phormidiaceae Anagnostidis and Komárek, 1988

Genus *Cephalothrix* Malone *et al*., 2015

***Cephalothrix lacustris* Malone *et al*., 2015 (Fig. 4)**

Filaments fasciculated, blue-green in color. Trichomes cylindrical, straight, slightly attenuated towards ends, sometimes bent at the end, constricted at the cross-walls. Sheaths hyaline and firm. Apical cells strongly capitate, sometimes with conical calyptra. Cells shorter than wide, with facultative aerotopes, 2.12–3.43 μm long, 4.92–6.12 μm wide. Hormogonia formation by necridic cells.

Ecology. This species appeared in freshwater ponds (Malone *et al*., 2015) and was isolated from freshwater in this study.

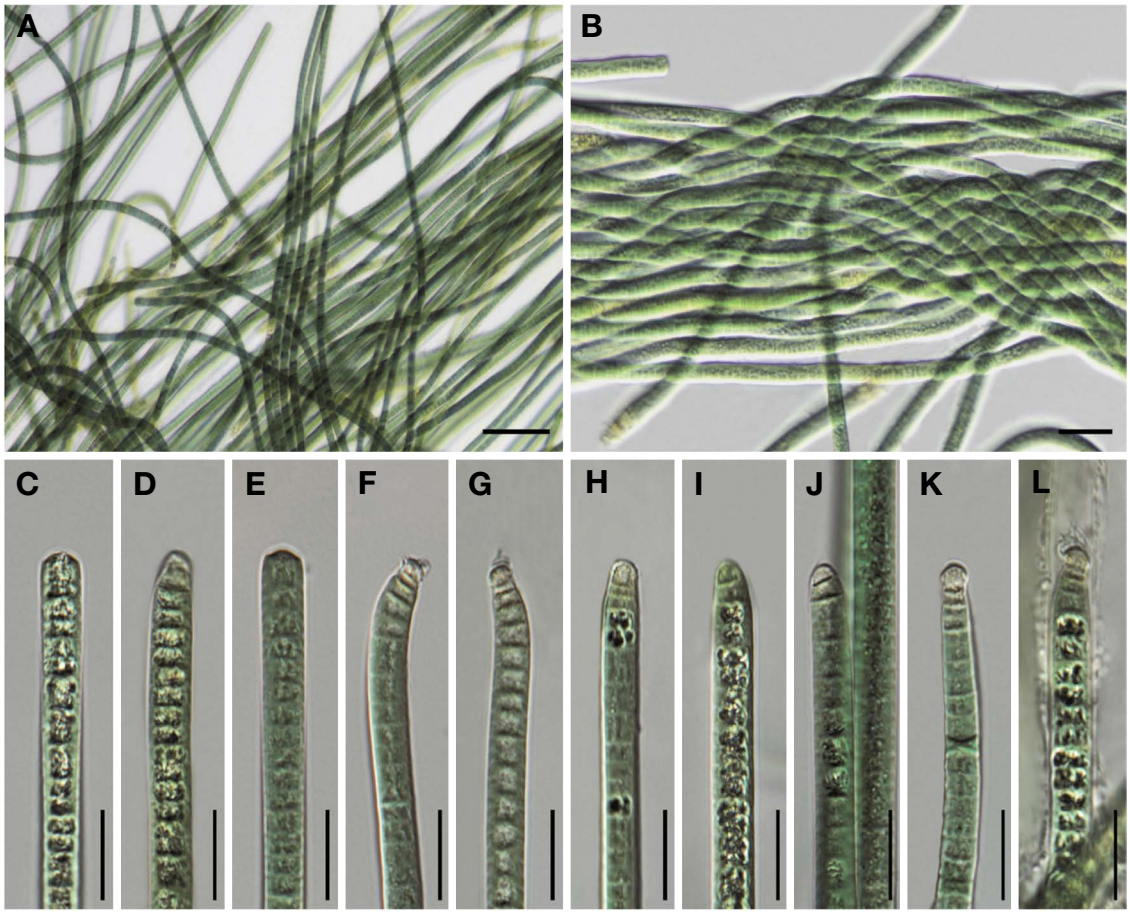

Fig. 4. Microscopic photographs of *Cephalothrix lacustris* FBCC-A1473. (A, B) Arrangement of filament in the colony, (C–H) Apical cell of trichomes, (C, D, H, I, L) Aerotopes, (F–H, J–L) Apical cell strongly capitate with calyptra, (K) Necridic cell. Scale bars (A) 50 μm, (B) 20 μm, (C–L) 10 μm.

Table 2. The 16S rRNA gene sequence similarity and *p*-distance between four strains of this study and each corresponding species.

Strain	Order	Family	Most closely related species	16S rRNA similarity (%)	*p*-distance (%)
FBCC-A1475	Oscillatoriales	Laspinemaceae	*Laspinema thermale*	99.30-99.50 (99.40)	0.42–0.63 (0.53)
FBCC-A1472		Microcoleaceae	*Planktothricoides raciborskii*	98.80–99.50 (99.13)	0.38–1.08 (0.74)
SJH-1			*Planktothrix spiroides*	99.80–99.90 (99.83)	0.08–0.15 (0.10)
FBCC-A1473		Phormidiaceae	*Cephalothrix lacustris*	99.80–99.90 (99.83)	0.1–0.19 (0.13)

Distribution. Brazil (Malone *et al*., 2015).
Site of collection. 472, Woongjin-dong, Gongju-si, Chungcheongnam-do (36°27′47.9″N, 127°06′05.4″E).
Date of collection. August 26, 2019.
Specimen deposit No. FBCC-A1473.

16S rRNA and phylogenetic affiliation

In this study, ML and Bayesian phylogenetic analysis were performed for more accurate phylogenetic analysis. In addition, if the 16S rRNA gene sequence similarity is 98.65% or more, it can be determined as the same species (Kim *et al*., 2014), so the 16S rRNA gene sequence similarity and genetic distance were also analyzed.

In *Laspinema thermale* (FBCC-A1475), a phylogenetic analysis was performed by comparing *L. thermale*, *L. etoshii*, *L. lumbricale*, and other species, previously reported in NCBI, and as a result, all trees showed similar branching patterns. Also, *L. thermale* (FBCC-A1475) was tied to the same cluster as previously reported *L. thermale*

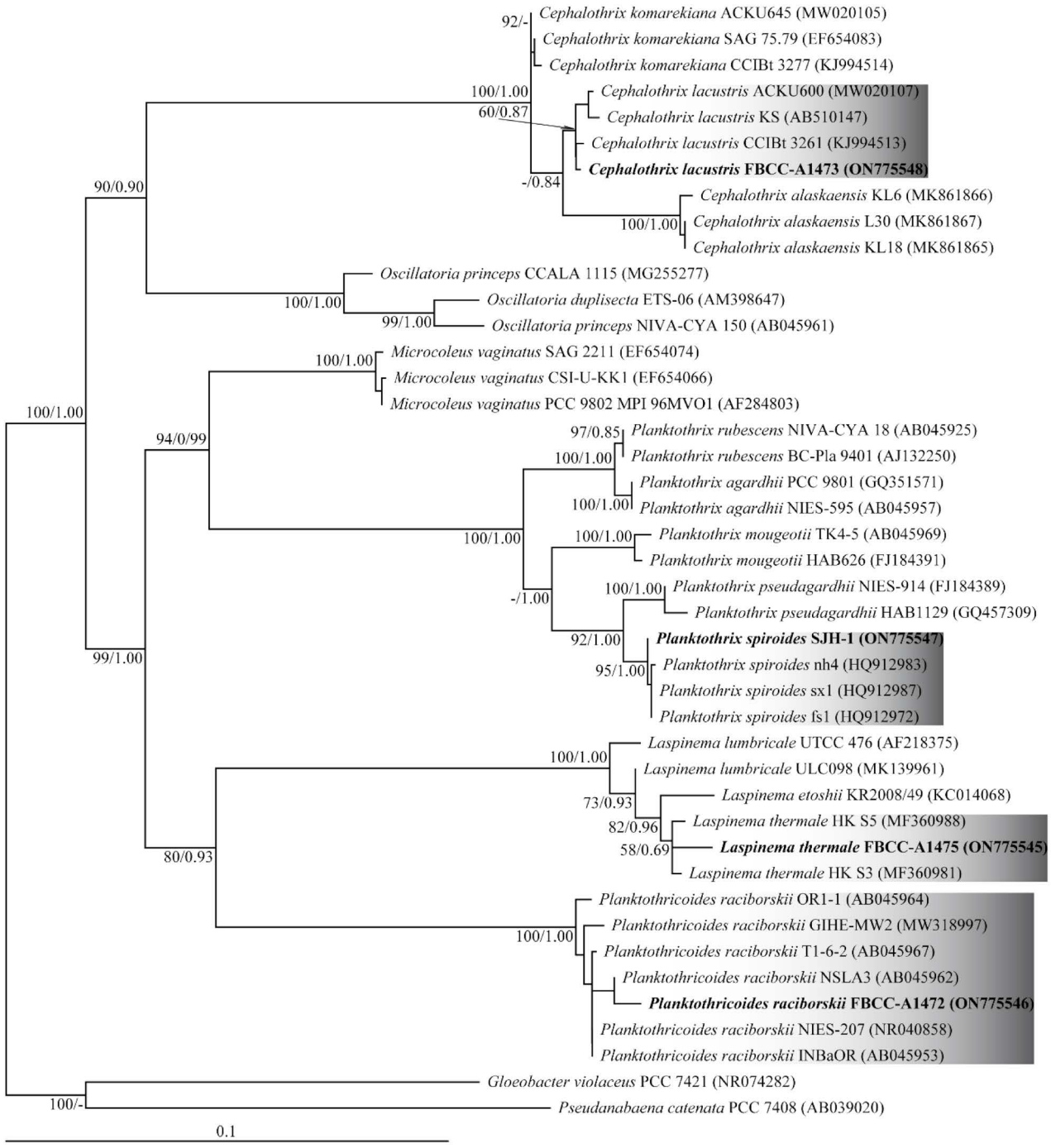

Fig. 5. Maximum-Likelihood (ML) phylogenetic tree based on 16S rRNA gene sequences of *Laspinema thermale*, *Planktothricoides raciborskii*, *Planktothrix spiroides*, *Cephalothrix lacustris*, and other cyanobacterial strains. A 16S rRNA gene sequences of *Gloeobacter violaceus* (Gloeobacteraceae), *Pseudanabaena catenata* (Pseudanabaenaceae) were included as the outgroups. The support values at the nodes are written as follows: ML/Bayesian. Support values are displayed at nodes for >50% ML bootstrap proportions and >0.5 Bayesian posterior probability. The branch lengths are proportional to the scale given. Bold represents data obtained in this study.

(two strains including HK S5), and it was confirmed that it formed a cluster distinctly different from *L. etoshii* and *L. lumbricale* included in the genus *Laspinema* (Fig. 5). Additionally, as a result of analyzing the 16S rRNA gene sequence similarity and genetic distance, it showed a similarity of 99.30−99.50% and a genetic distance of 0.42−0.63% with the previously reported *L. thermale* (Table 2).

For *Planktothricoides raciborskii* (FBCC-A1472), anal-

ysis was performed on *P. raciborskii* and other species, previously reported in NCBI. All trees showed similar branching patterns, and *P. raciborskii* (FBCC-A1472) was tied to the same cluster as previously reported *P. raciborskii* (six strains including NIES-207), forming a cluster distinctly different from other species (Fig. 5). In the case of *P. attenuata* belonging to the same genus as *P. raciborskii*, there was no previously reported genetic information, so it was excluded from the phylogenetic analysis. However, *P. raciborskii* exhibits narrowing from apical cell of trichome, whereas *P. attenuata* showed a distinct morphological difference because trichome gradually narrowed from the central part to apical cell (Komárek and Komárková-Legnerová, 2007). Additionally, as a result of analyzing the 16S rRNA gene sequence similarity and genetic distance, it showed a similarity of 98.80–99.50% and a genetic distance of 0.38–1.08% with the previously reported *P. raciborskii* (Table 2).

The phylogenetic analysis of *Planktothrix spiroides* (SJH-1) was performed by comparing *P. spiroides*, *P. agardhii*, *P. mougeotii*, *P. pseudagardhii*, *P. rubescens*, and other species, previously reported in NCBI, and as a result, all trees showed similar branching patterns. Also, *P. spiroides* (SJH-1) was tied to the same cluster as previously reported *P. spiroides* (three strains including fs1), and it was confirmed that it formed a cluster distinctly different from *P. agardhii*, *P. mougeotii*, *P. pseudagardhii*, and *P. rubescens* included in the genus *Planktothrix* (Fig. 5). Additionally, as a result of analyzing the 16S rRNA gene sequence similarity and genetic distance, it showed a similarity of 99.80–99.90% and a genetic distance of 0.08–0.15% with the previously reported *P. spiroides* (Table 2).

For *Cephalothrix lacustris* (FBCC-A1473), a phylogenetic analysis was performed by comparing *C. lacustris*, *C. alaskaensis*, *C. komarekiana*, and other species, previously reported in NCBI. As a result, all trees showed similar branching patterns, and *C. lacustris* (FBCC-A1473) was tied to the same cluster as previously reported *C. lacustris* (three strains including CCIBt 3261), forming a cluster distinctly different from *C. alaskaensis* and *C. komarekiana* included in the genus *Cephalothrix* (Fig. 5). Additionally, as a result of analyzing the 16S rRNA gene sequence similarity and genetic distance, it showed a similarity of 99.80–99.90% and a genetic distance of 0.1–0.19% with the previously reported *C. lacustris* (Table 2).

Through the above results, *Laspinema thermale*, *Planktothricoides raciborskii*, *Planktothrix spiroides*, and *Cephalothrix lacustris* are proposed as unrecorded species of cyanobacteria in Korea.

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