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Six newly recorded species of epilithic cyanobacteria isolated in Korea

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In this study, 11 strains of epilithic cyanobacteria belonging to six unrecorded species in Korea were isolated from gravel submerged in freshwater of seven collection sites in Korea. The morphological characteristics of the six species isolated in this study were consistent with the type strain of each species, and the similarity of the 16S rRNA gene sequences with the type strain of each species were 98.8−100%. In the phylogenetic tree using the 16S rRNA gene sequences, the 11 strains of these six species formed the same cluster as the strains of each species. The habitat of each previously reported species is mainly the soil surface, but all Korean strains appeared from the gravel submerged in freshwater. As a result of the morphological, ecological, and molecular analyses, these six species of cyanobacteria were identified as *Geminocystis papuanica*, *Allocoleopsis franciscana*, *Ancylothrix terrestris*, *Klisinema persicum*, *Scytolyngbya timoleontis*, and *Shackletoniella antarctica*, which were added as newly recorded species in Korea.

Keywords: 16S rRNA, cyanobacteria, epilithic, freshwater, polyphasic

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Introduction

Cyanobacteria occurs in most light-filled underwater and land areas on Earth (Whitton and Potts, 2012), and also lives in salt pans, hot springs, dry soil surfaces in deserts, and glaciers in polar regions (Muhlsteinova *et al*., 2014; Segawa *et al*., 2017; Jaiswal *et al*., 2021; Lee *et al*., 2023). Cyanobacteria is a major group that causes green tide in eutrophic water bodies, which can have a serious impact on water quality deterioration along with the release of toxins (Kaplan *et al*., 2012; Rastogi *et al*., 2014).

Classification of cyanobacteria began mainly using morphological features, such as cell shape, characteristics of the mucilage sheath, branching shape, and presence or absence of heteromorphic cells, but later, cell microstructures such as thylakoid arrangements by electron microscopy were used (Komárek *et al*., 2014). Since the late 20th century, molecular traits have been introduced into the classification of cyanobacteria, and polyphasic research methods combined with morphology and ecological characteristics have been used (Johansen and Casamatta, 2005; Dvořák *et al*., 2015; Komárek, 2016). There have been difficulties in identifying cyanobacteria because their morphological characteristics are simple and variable, but the introduction of molecular data has given clarity to the identification of cyanobacteria. As a result, many new genera and species have been added to each taxonomic group (Sciuto and Moro, 2016; Mai *et al*., 2018). Among cyanobacteria established using 16S rRNA gene sequencing worldwide, 273 species belonging to at least 140 genera were newly described between 2014 and 2021 (Strunecký *et al*., 2023). Recently, Strunecký *et al*. (2023) proposed 10 new orders and 15 new families based on the 16S rRNA gene phylogeny of overall cyanobacteria.

The genera that are covered in this study are *Allocoleopsis* Fernandes *et al*. (2021: 1575), Coleofasciculaceae of Oscillatoriales, was transferred to Coleofasciculaceae of Coleofasciculales, and *Klisinema* Heidari and Hauer (Heidari *et al*., 2018: 145), Microcoleaceae of Oscillatoriales, was changed to Desertifilaceae of Desertifilales (Strunecký *et al*., 2023). In addition, genera *Scytolyngbya* Song and Li (Song *et al*., 2015: 74) and *Shackletoniella* (West and West) Strunecký, Raabová and Bernardová (Strunecký *et al*., 2020: 16), were each modified from Leptolyngbyaceae and Oculatellaceae of Synechococcales to Leptolyngbyaceae of Leptolyngbyales and Oculatellaceae of Oculatellales, respectively (Strunecký *et al*., 2023). Each of the four genera mentioned above (*Allocoleopsis*, *Klisinema*, *Scytolyngbya*, and *Shackletoniella*) have one spe-

cies described, and these species are found in the soils around greenhouses, soils around hot spring water of radioactive regions, stones of wells in a water treatment facility, and freshwater in low temperatures (Song *et al*., 2015; Heidari *et al*., 2018; Strunecký *et al*., 2020; Fernandes *et al*., 2021). Additionally, two species of the genus *Geminocystis* Korelusová *et al*. (2009: 934), including *G. papuanica*, were reported, which were collected from wet soil surfaces of oligotrophic lakes and tropical rainy forests (Korelusová *et al*., 2009). The genus *Ancylothrix* Martins and Branco (Martins *et al*., 2016: 2403), in which the two species including *A. terrestris* have been reported, was isolated from benthos, shadowed soils, and tree bark (Martins *et al*., 2016).

To date, more than 5,600 species of cyanobacteria have been described worldwide (Guiry and Guiry, 2023), and 397 species of cyanobacteria have been reported in Korea (NIBR, 2023). Research on unrecorded species of domestic cyanobacteria is continuously being conducted, and recently, a total of 11 species, including *Alkalinema pantanalense*, were newly reported in Korea (Kim *et al*., 2022b; Lee, 2022; Song *et al*., 2022).

In this study, epilithic cyanobacteria were collected from gravel of seven freshwater sites in Korea, and 11 strains were isolated and cultured, which were identified by analyzing morphological characteristics, ecological characteristics, and 16S rRNA sequences. From this research, we sought to secure the cultures of unrecorded cyanobacteria in Korea and contribute by adding to the domestic cyanobacterial flora.

Materials and Methods

Sample collection and cultures

Samples in this study were collected from gravel submerged in seven freshwater sites in the Republic of Korea from October 2018 to May 2022 (Table 1). Attached cyanobacteria were collected using a soft brush or spatula (Kiel and Gaylarde, 2006), and samples were sealed on site, stored in an ice box at 4°C, and transported to the laboratory.

For monospecies cultures of cyanobacteria, only one cyanobacterial trichome was isolated using a Pasteur pipette under an optical microscope, and then was cultured in a 12-well plate (SPL, Pocheon, Korea) for 2−3 weeks. Monospecies cultured cyanobacteria were transferred to a 50 mL cell culture flask (SPL, Pocheon, Korea) (Lee *et al*., 2019). BG-11 liquid medium (Stanier *et al*., 1971) was used to culture cyanobacteria, and the culture was performed at a temperature of 20−25°C, 16 : 8 h light : dark cycle, and illuminance 25 μmol m^{-2} s^{-1} (Lee *et al*., 2019). Monospecies cultured cyanobacterial strains were depo-

Table 1. Strain numbers and sampling sites information from gravel submerged in freshwater.

Strain	Species	Site No.	Location	Sampling date	GPS
FBCC-A202	*Geminocystis papuanica*	Site 1	Jiam-ri, Sabuk-myeon, Chuncheon-si, Gangwon-do	2018-10-31	37°58′44.6″N, 127°36′07.4″E
ACKU679	*Allocoleopsis franciscana*	Site 2	Bujang-ri, Ugang-myeon, Dangjin-si, Chungcheongnam-do	2021-05-18	36°51′06.1″N, 126°49′37.0″E
ACKU673 ACKU674 ACKU675 ACKU676	*Ancylothrix terrestris*	Site 3	Wongil-ri, Bongpyeong-myeon, Pyeongchang-gun, Gangwon-do	2022-05-23	37°37′26.0″N, 128°22′37.0″E
ACKU678	*Klisinema persicum*	Site 4	Munbang-ri, Inju-myeon, Asan-si, Chungcheongnam-do	2021-05-18	36°52′45.2″N, 126°51′42.9″E
ACKU680		Site 5	Boseong-ri, Pungse-myeon, Dongnam-gu, Cheonan-si, Chungcheongnam-do	2021-05-17	36°43′58.2″N, 127°07′30.9″E
ACKU677	*Scytolyngbya timoleontis*	Site 6	Dulhyeon-ri, Juksan-myeon, Anseong-si, Gyeonggi-do	2021-09-23	37°04′22.3″N, 127°24′04.0″E
ACKU671 ACKU672	*Shackletoniella antarctica*	Site 7	Samsan-ri, Yeongok-myeon, Gangneung-si, Gangwon-do	2022-05-23	37°50′49.1″N, 128°41′12.3″E

sited in the Freshwater Bioresources Culture Collection (FBCC) of the Nakdonggang National Institute of Biological Resources of Korea (FBCC-A202) or the Algal Culture Collection of Kyonggi University (ACKU) (ACKU 671–680).

Morphological analysis and characterization

For morphological observation and photographing of cyanobacteria, an optical microscope (Olympus BX53, Olympus, Japan) equipped with a digital camera (Olympus UC-90, Olympus, Japan) was used, and morphological images were taken at 200–1000× magnification. In this study, we used the overall taxonomic classification system of cyanobacteria of Strunecký *et al.* (2023) and referred to Komárek *et al.* (2014) and AlgaeBase (Guiry and Guiry, 2023). Also, for the identification of cyanobacteria, Komárek and Anagnostidis (1998; 2005) were referenced and the original papers of each species were used: Korelusová *et al.* (2009), Song *et al.* (2015), Martins *et al.* (2016), Heidari *et al.* (2018), Strunecký *et al.* (2019), and Fernandes *et al.* (2021).

Molecular methods

For genomic DNA (gDNA) extraction, 1 mL of the cultured cyanobacteria was transferred to a 1.5 mL microcentrifuge tube, and the cells were concentrated by centrifugation at 13,000 rpm for 10 minutes. Subsequently, gDNA was extracted using an i-genomic Plant DNA Extraction Mini Kit (iNtRON, Daejeon, Korea).

16S rRNA gene sequences were amplified by polymerase chain reaction (PCR), using primers 27F1 (Neilan *et al.*, 1997) and CY-23R600 (Lee *et al.*, 2019). The PCR was carried out in a total volume of 20 μL, containing a Maxime™ PCR PreMix Kit (i-StarTaq™ GH) (iNtRON, Daejeon, Korea), sterilized distilled water (17 μL), gDNA (10–30 μg, 1 μL), 27F1, and CY-23R600 (10 pmole, 1 μL). The PCR was performed on a Mastercycler gradient (Eppendorf, Hamburg, Germany) with the following program: denatured at 94°C for the initial 5 minutes, followed by 35 cycles of 94°C for 20 seconds, 55°C for 30 seconds, and 72°C for 90 seconds, with a final extension 72°C for an additional 10 minutes. The resulting PCR products were observed by electrophoresis on a 1.2% agarose gel. The PCR product was purified using the MEGAquick-spin™ Plus DNA Fragment Purification Kit (iNtRON, Daejeon, Korea), and then the purified PCR product was analyzed by Bionics Co., Ltd. (Seoul, Korea) to secure the 16S rRNA gene sequence. Editing the 16S rRNA gene sequence fragments were performed using DNASTAR Lasergene® SeqMan Pro™ ver.7.1.0 (DNASTAR Inc., WI, USA), and the determined sequences were deposited at the GenBank (https://www.ncbi.nlm.nih.gov/genbank) (Table 2).

We used Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for related species of the 11 strains. For phylogenetic analysis, we assembled the 16S rRNA gene sequence data sets of the studied species, which grouped two species with relatively close genetic distances and divided them into the following three groups: *Geminocystis papuanica* and *Allocoleopsis franciscana* (total 22 sequences), *Ancylothrix terrestris* and *Klisinema persicum* (total 39 sequences), *Scytolyngbya timoleontis* and *Shackletoniella antarctica* (total 38 sequences) (Tables S1–3). The collected data were aligned by BioEdit ver. 7.2.5 (Hall, 1999). Nucleotide sequence similarity and pairwise genetic distance (*p*-distance) were calculated using Kimura 2-parameter model of MEGA X (Kumar *et al.*, 2018).

Maximum-Likelihood (ML) analysis was implemented in RAxML ver. 7.0.3 (Stamatakis, 2006), using the General Time Reversible (GTR) + MIX model. Additionally, Bayesian inference (BI) analysis was carried out using the MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001) with the same data as ML analysis. The Markov chain Monte Carlo (MCMC) process was set to four chains, and 5,000,000 generations were performed with a sampling frequency of one per 100 generations. After this analysis, the first 200 trees were deleted as burn-in and a consensus tree was constructed. TreeView 1.6.6 (Page, 1996) was used to visualize the phylogenetic trees.

Results and Discussion

In this study, 11 strains of cyanobacteria were isolated from seven sites in Korea and these were cultured as monospecies for identification. The identification process was based on morphological, ecological, and molecular characteristics of 16S rRNA gene sequences. As a result, the 11 strains were identified as six species belonging to six orders, six families, and six genera based on the classification system of Strunecký *et al.* (2023). The six species identified in this study were *Geminocystis papuanica*, *Allocoleopsis franciscana*, *Ancylothrix terrestris*, *Klisinema persicum*, *Scytolyngbya timoleontis*, and *Shackletoniella antarctica*, which were newly recorded species in Korea. Among these, five genera (*Allocoleopsis*, *Ancylothrix*, *Klisinema*, *Scytolyngbya*, and *Shackletoniella*) are newly recorded genera in Korea.

1. Morphological and ecological characterization

Order Chroococcales Schaffner, 1922

Family Geminocystaceae A.Tuji, H.Yamaguchi, and Y.Niiyama, 2021

Genus *Geminocystis* Korelusová, Kaštovský, and Komárek, 2009

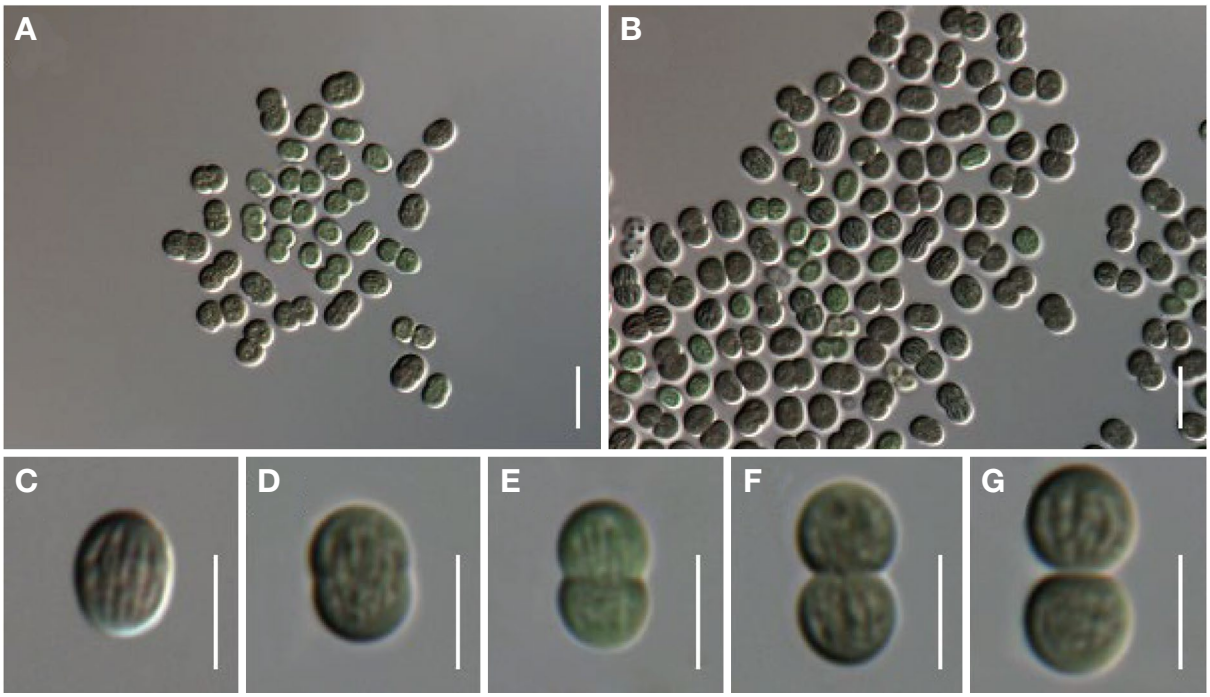

Fig. 1. Microscopic photographs of *Geminocystis papuanica* from the strain FBCC-A202. (A, B) Colony and cell colors, (C−G) Keritomic content and cell division. Scale bars = 10 μm.

***Geminocystis papuanica* Korelusová, Kaštovský, and Komárek, 2009 (Fig. 1)**

Cells form a small colony or solitary, often double, blue green or dark grey in color. Cell cyanoprokaryotic, spherical or oval, irregularly or parallelly striated, slightly keritomized, 3.9−5.8 μm in diameter. Divided by binary fission, hemispherical and a short time two together after division.

Ecology. This species was isolated on the wet soil surface of tropical forests (Korelusová *et al*., 2009), and in this study from gravel submerged in freshwater.
Distribution. Papua-New Guinea (Korelusová *et al*., 2009).
Site of collection. Site 1.
Specimen locality: FBCC-A202.

Order Coleofasciculales Strunecký and Mareš, 2023
Family Coleofasciculaceae J.Komárek, J.Kaštovský, Mareš, and J.R.Johansen, 2014
Genus *Allocoleopsis* Moreira-Fernandes, Giraldo-Silva, D.Roush, and Garcia-Pichel, 2021

***Allocoleopsis franciscana* Moreira-Fernandes, Giraldo-Silva, D.Roush, and Garcia-Pichel, 2021 (Fig. 2)**

Filaments fasciculated, heteropolar, blue green in color. Sheaths colorless, firm and thin, commonly open at the end. Trichomes straight, slightly constricted at cross-walls. Cells isodiametric or longer than wide or occasionally slightly shorter than wide, 4.1−6.8 μm long, 4.6−5.4 μm wide. Apical cells rounded or rounded to conical. Hormogonia are separated by necridic cells.

Ecology. This species was isolated in greenhouse soil and biocrusts (Fernandes *et al*., 2021), and in this study from gravel submerged in freshwater.
Distribution. San Francisco, California, United States of America (Fernandes *et al*., 2021).
Site of collection. Site 2.
Specimen locality: ACKU679.

Order Oscillatoriales Schaffner, 1922
Family Microcoleaceae O.Strunecký, J.R.Johansen, and J.Komárek, 2013
Genus *Ancylothrix* M.D.Martins and L.H.Z.Branco, 2016

***Ancylothrix terrestris* M.D.Martins and L.H.Z.Branco, 2016 (Fig. 3)**

Filaments fasciculated, bent at the ends, bright green in color. Sheath thin, firm, sometimes widen, colorless, rarely two trichomes in a single sheath. Trichomes straight, entangled, slightly or not constricted at cross-walls, attenuated at the end, without calyptra. Cells shorter than wide, often isodiametric, 2.1−6.2 μm long, 5.0−6.7 μm wide.

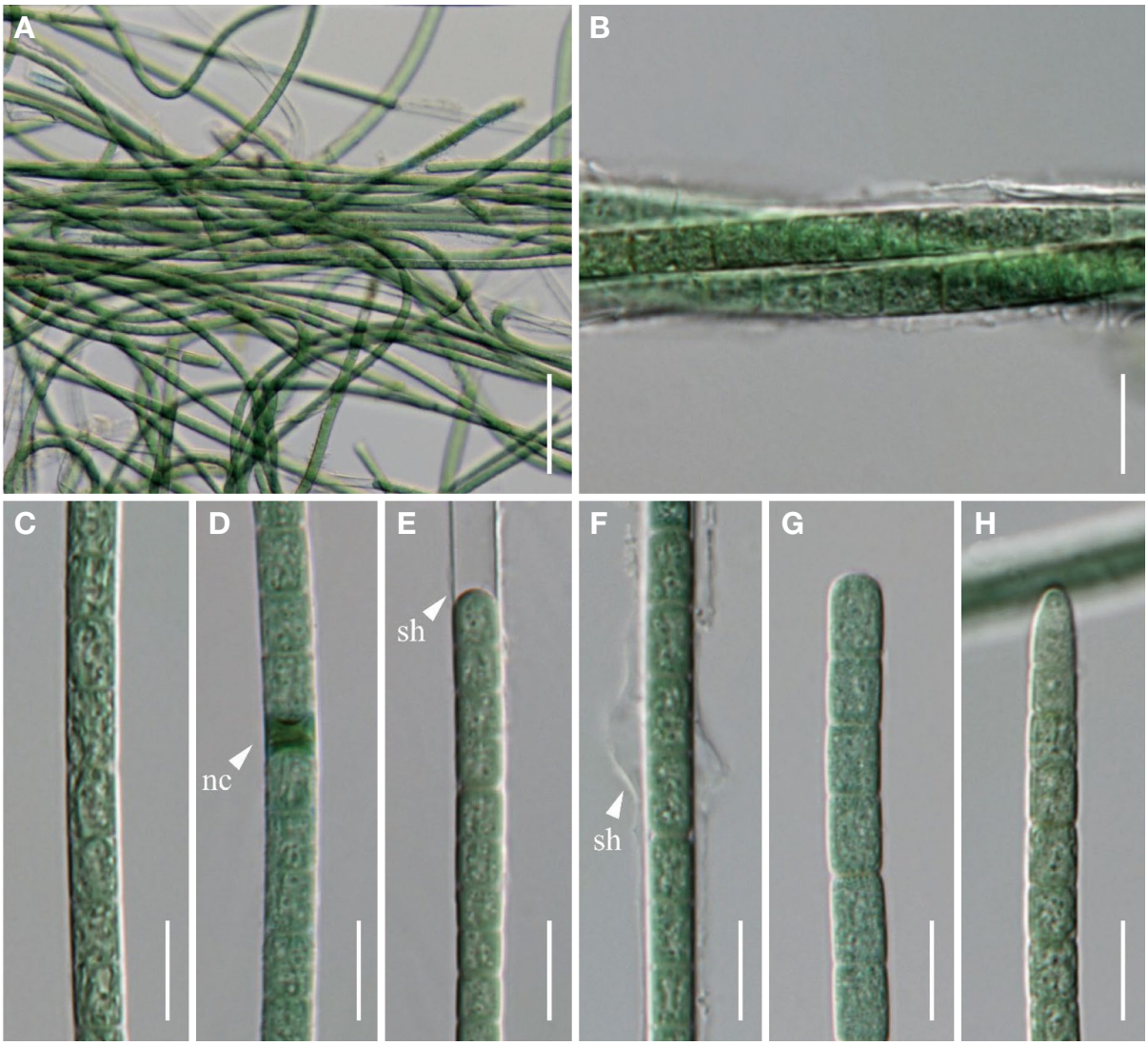

Fig. 2. Microscopic photographs of *Allocoleopsis franciscana* from the strain ACKU679. (A, B) Arrangement of filament, (C) Morphology of trichome, (D) Necridic cell (nc), (E, F) Sheaths (sh), (G, H) Apical cell of trichomes. Scale bars = (A) 50 μm, (B–H) 10 μm.

Apical cells conical rounded or rounded.

Ecology. This species was isolated on the soil of decidual forest and bark of trees (Martins *et al*., 2016), and in this study from gravel submerged in freshwater.
Distribution. Cachoeira do Sul, Rio Grande do Sul, Brazil (Martins *et al*., 2016).
Site of collection. Site 3.
Specimen locality: ACKU673–676.

Order Desertifilales Strunecký and Mareš, 2023
Family Desertifilaceae Hašler, Casamatta, Dvořák, and Poulícová, 2017
Genus *Klisinema* Heidari and Hauer, 2018

***Klisinema persicum* Heidari and Hauer, 2018 (Fig. 4)**

Filaments solitary, wavy or straight, blue-green or pale blue green in color. Sheaths thin, firm, colorless. Trichomes slightly constricted at cross-walls or not. Cells isodiametric or longer than wide or sometimes shorter than wide, 2.4–4.0 μm long, 3.1–4.0 μm wide. Apical cells conical, elongated up to 7 μm. Hormogonia are formed by necridic cells.

Ecology. This species was isolated on the soil around thermal springs (50°C) with high radiation background (Heidari *et al*., 2018), and in this study from gravel submerged in freshwater.
Distribution. Mahallat, Markazi, Iran (Heidari *et al*., 2018).
Site of collection. Sites 4, 5.
Specimen locality: ACKU678, ACKU680.

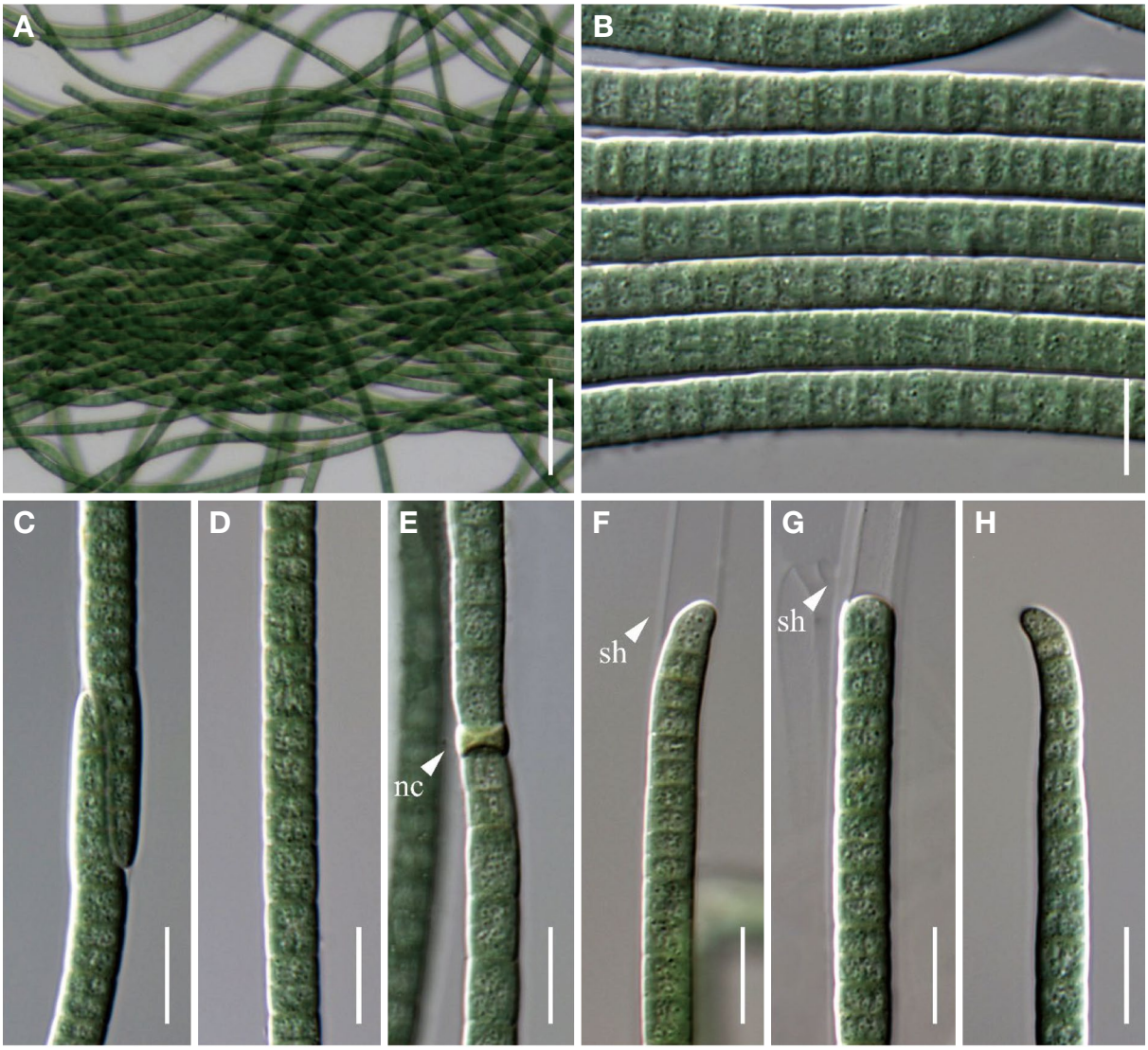

Fig. 3. Microscopic photographs of *Ancylothrix terrestris* from the strain ACKU673. (A, B) Arrangement of filament, (C) Containing two trichomes within a sheath, (D) Morphology of trichome, (E) Necridic cell (nc), (F, G) Sheaths (sh) and apical cell of trichomes, (H) Apical cell of trichome. Scale bars = (A) 50 μm, (B−H) 10 μm.

Order Leptolyngbyales Strunecký and Mareš, 2023
Family Leptolyngbyaceae J.Komárek, J.Kaštovský, Mareš, and J.R.Johansen, 2014
Genus *Scytolyngbya* Gao-Fei Song, Yong-Guang Jiang, and Ren-Hui Li, 2015

Scytolyngbya timoleontis Gao-Fei Song, Yong-Guang Jiang, and Ren-Hui Li, 2015 (Fig. 5)

Filaments wavy or straight, entangled, attenuated at the end, yellow-brown or dark green in color. Sheath initially thin, firm or diffluent, colorless, commonly multiple trichomes in a single sheath. Trichomes constricted at cross-walls, rarely false branched in old culture. Cells longer than wide, 2.7−5.2 μm long, 1.5−2.3 μm wide. Apical cells rounded and cylindrical. Hormogonia are separated by necridic cells.

Ecology. This species was isolated on the stone surfaces of wells at a water treatment facility (Song *et al*., 2015), and in this study from gravel submerged in freshwater.
Distribution. Xishui, Hubei, China (Song *et al*., 2015).
Site of collection. Site 6.
Specimen locality: ACKU677.

Order Oculatellales Strunecký and Mareš, 2023
Family Oculatellaceae Mai and J.R.Johansen, 2018
Genus *Shackletoniella* (W.West and G.S.West) Strunecký, Raabová, and Bernardova, 2020

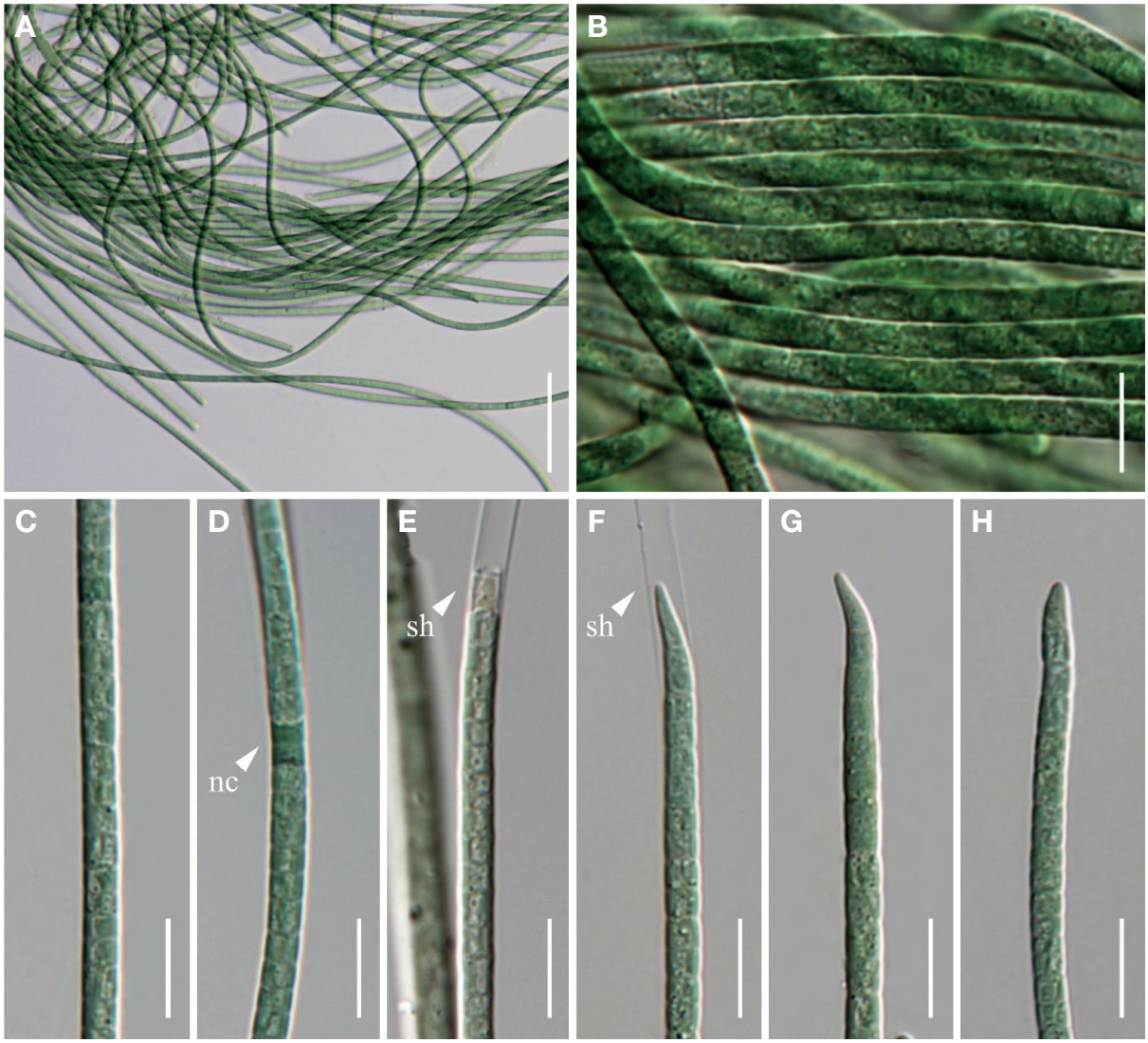

Fig. 4. Microscopic photographs of *Klisinema persicum* from the strain ACKU678. (A, B) Arrangement of filament, (C) Morphology of trichome, (D) Necridic cells (nc), (E, F) Sheaths (sh) and apical cell of trichomes, (G, H) Apical cell of trichomes. Scale bars = (A) 50 μm, (B–H) 10 μm.

***Shackletoniella antarctica* (W.West and G.S.West) Strunecký, Raabová, and Bernardova, 2020 (Fig. 6)**

Basionym: *Phormidium antarcticum* W.West and G.S.West, 1911

Synonym: *Leptolyngbya antarctica* (W.West and G.S.West) Anagnostidis and Komárek, 1988

Filaments straight or wavy, commonly coiled, occasionally twisted, pale blue green or olive green in color, sometimes false branched in old culture. Sheath colorless, thin, firm, sometimes diffluent, widened. Trichomes not or slightly constricted at the cross-walls. Cells longer than wide or occasionally quadratic, 1.4–3.1 μm long, 1.2–1.7 μm wide. Apical cells rounded, distinct granules at the apex. Hormogonia are formed by necridic cells.

Ecology. This species was isolated from microbial mat in an Antarctic lake (Strunecký *et al*., 2020), and in this study from gravel submerged in freshwater.

Distribution. Prydz Bay, East Antarctica (Strunecký *et al*., 2020).

Site of collection. Site 7.

Specimen locality: ACKU671, 672.

2. 16S rRNA and phylogenetic affiliation

In this study, the 16S rRNA gene sequences of 11 cultured strains and related species registered in NCBI were used to analyze data for species identification (Tables S1–3). Through these three datasets, three phylogenetic trees were obtained, and the nucleotide sequence similarity

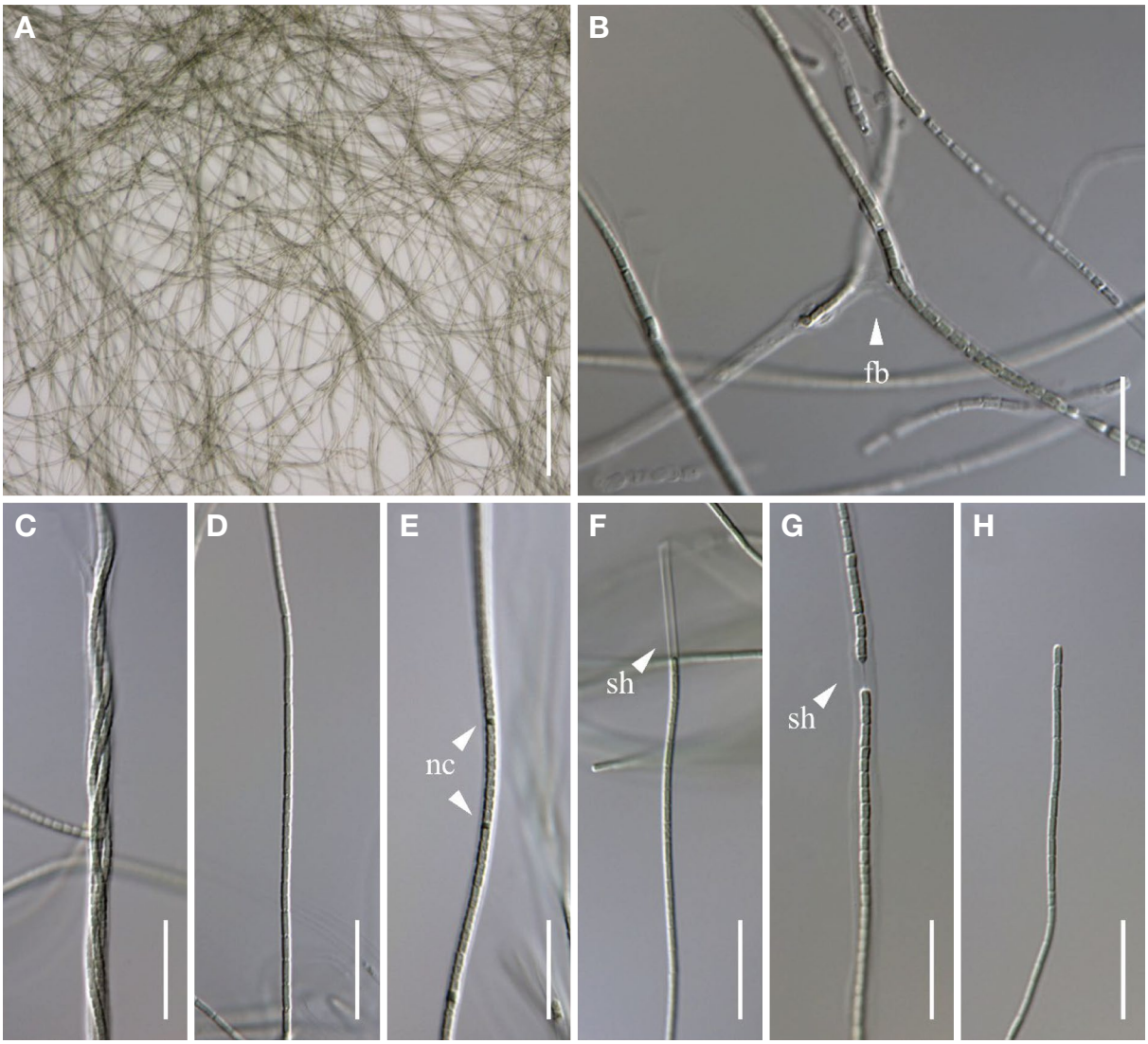

Fig. 5. Microscopic photographs of *Scytolyngbya timoleontis* from the strain ACKU677. (A) Arrangement of filament, (B) False branching (fb) in old culture, (C) Several trichomes within a sheath, (D) Morphology of trichome, (E) Necridic cells (nc), (F, G) Sheaths (sh), (H) Apical cell of trichome. Scale bars = (A) 50 μm, (B–H) 10 μm.

and genetic distance with each type strains were calculated (Figs. 7–9). All phylogenetic trees showed a similar branch pattern in ML and BI phylogenetic trees.

In the phylogenetic tree, *Geminocystis papuanica* (FBCC-A202) formed a clade with *G. papuanica* (PAP1), and was distinct from *Geminocystis herdmanii* (PCC 6308) in the same genus (Korelusová *et al*., 2009) (Fig. 7). The genus *Geminocystis* was clearly separated from other genera. *Allocoleopsis franciscana* (ACKU679) clustered with *A. franciscana* (PCC 7113) (Fernandes *et al*., 2021) (Fig. 7). *G. papuanica* (FBCC-A202) showed 99.4% nucleotide sequence similarity and 0.30% genetic distance with a previously reported *G. papuanica* (PAP1) (Korelusová *et al*., 2009) (Table 2). Korean *A. franciscana* (ACKU679), the nucleotide sequence similarity with the type strain of *A. franciscana* (PCC 7113) was 99.7%, and the genetic distance was confirmed as 0.14% (Fernandes *et al*., 2021) (Table 2).

As a result of the analysis, genus *Ancylothrix* formed a monophyletic clade. *Ancylothrix terrestris* (four strains including ACKU673) clustered with *A. terrestris* (10PC, 12PC), but it was clearly separated from *Ancylothrix rivularis* (7PC, 9PC) (Martins *et al*., 2016) (Fig. 8). In addition, *Klisinema persicum* (ACKU678, ACKU680) clustered with *K. persicum* (SHAFA S10) (Heidari *et al*., 2018). Four strains collected in Korea, including ACKU673 of *A. terrestris*, had 100% nucleotide sequence similarity and 0.00% genetic distance with the type strain *A. terrestris*

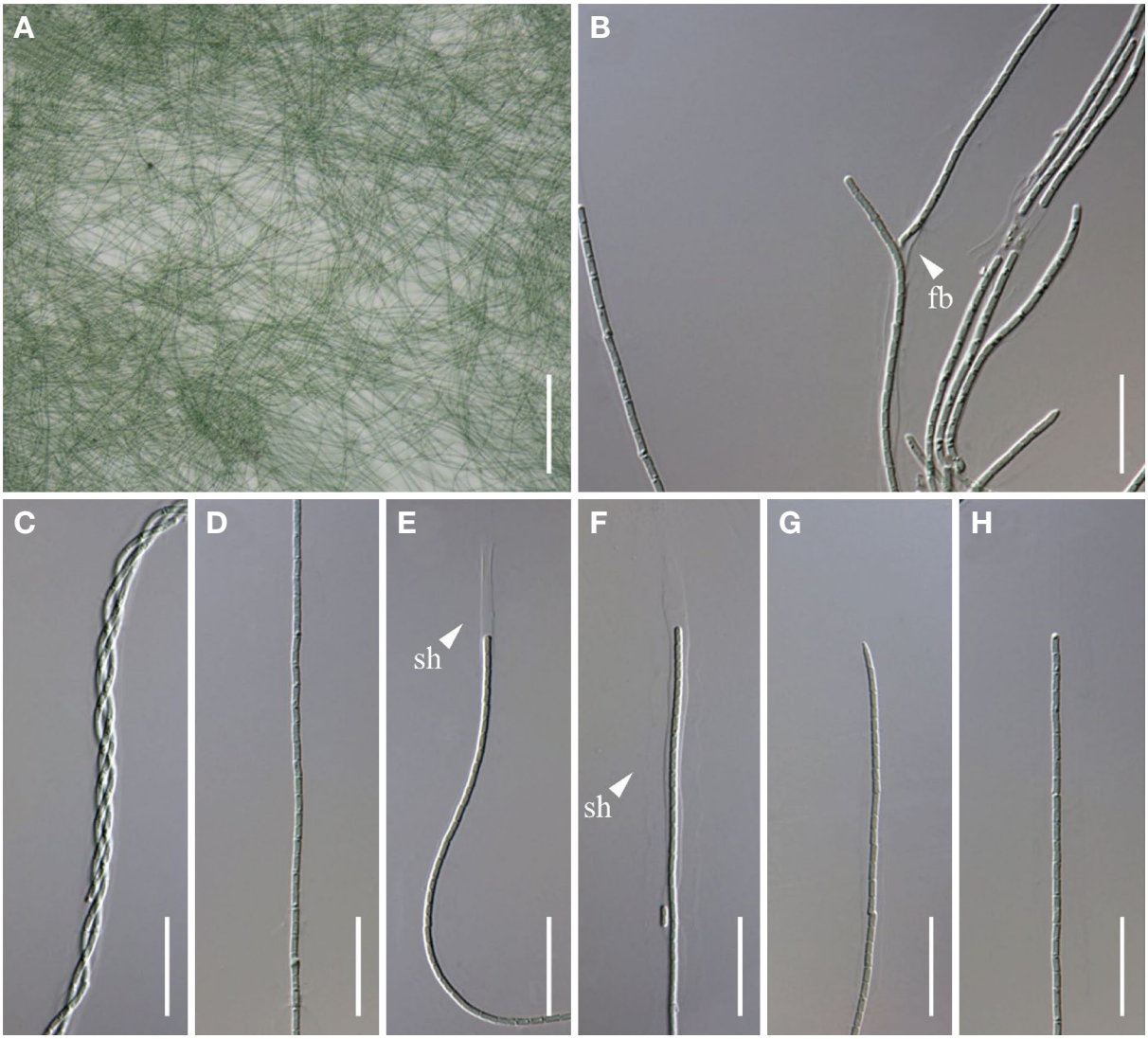

Fig. 6. Microscopic photographs of *Shackletoniella antarctica* from the strain ACKU671. (A) Arrangement of filament, (B) False branching (fb), (C) Trichomes twisted into a rope, (D) Morphology of trichome, (E, F) Sheaths (sh), (G, H) Apical cell of trichomes. Scale bars = (A) 50 μm, (B–H) 10 μm.

(10PC) (Martins *et al*., 2016) (Table 2). *Klisinema persicum* (ACKU678, ACKU680) showed 99.5–99.7% nucleotide sequence similarity and 0.27–0.54% genetic distance with type strain of *K. persicum* (SHAFA S10) (Heidari *et al*., 2018) (Table 2).

In the phylogenetic tree, Korean *Scytolyngbya timoleontis* (ACKU677) formed a clade with three strains of *Scytolyngbya timoleontis* (three strains including XSP1) (Song *et al*., 2015) (Fig. 9). *Shackletoniella antarctica* (ACKU 671, ACKU672) formed a clade with two strains of *Shackletoniella antarctica* (ANT.L18.2, KL8) (Strunecký *et al*., 2020). *Scytolyngbya timoleontis* (ACKU677) showed 99.7–99.8% nucleotide sequence similarity and 0.14–0.21% genetic distance with previously reported *Scytolyngbya timoleontis* (three strains including XSP1) (Song *et al*., 2015). *Shackletoniella antarctica* (ACKU671, ACKU672) of two strains showed 98.8–99.2% nucleotide sequence similarity and 0.74–1.12% genetic distance with *Shackletoniella antarctica* (ANT.L18.2, KL8) (Table 2).

The morphological characteristics of the six species of cyanobacteria in this study were mostly consistent with each type strain, including filaments, branching, sheath characteristics, cell width and length, and apical cell shape (Table 3). An open sheath at the end was observed in *Allocoleopsis franciscana* (ACKU679), but the sheath of cyanobacteria is considered a feature that can change depending on culture conditions and ecological characteristics (Casamatta *et al*., 2003; Komárek and Anagnostidis,

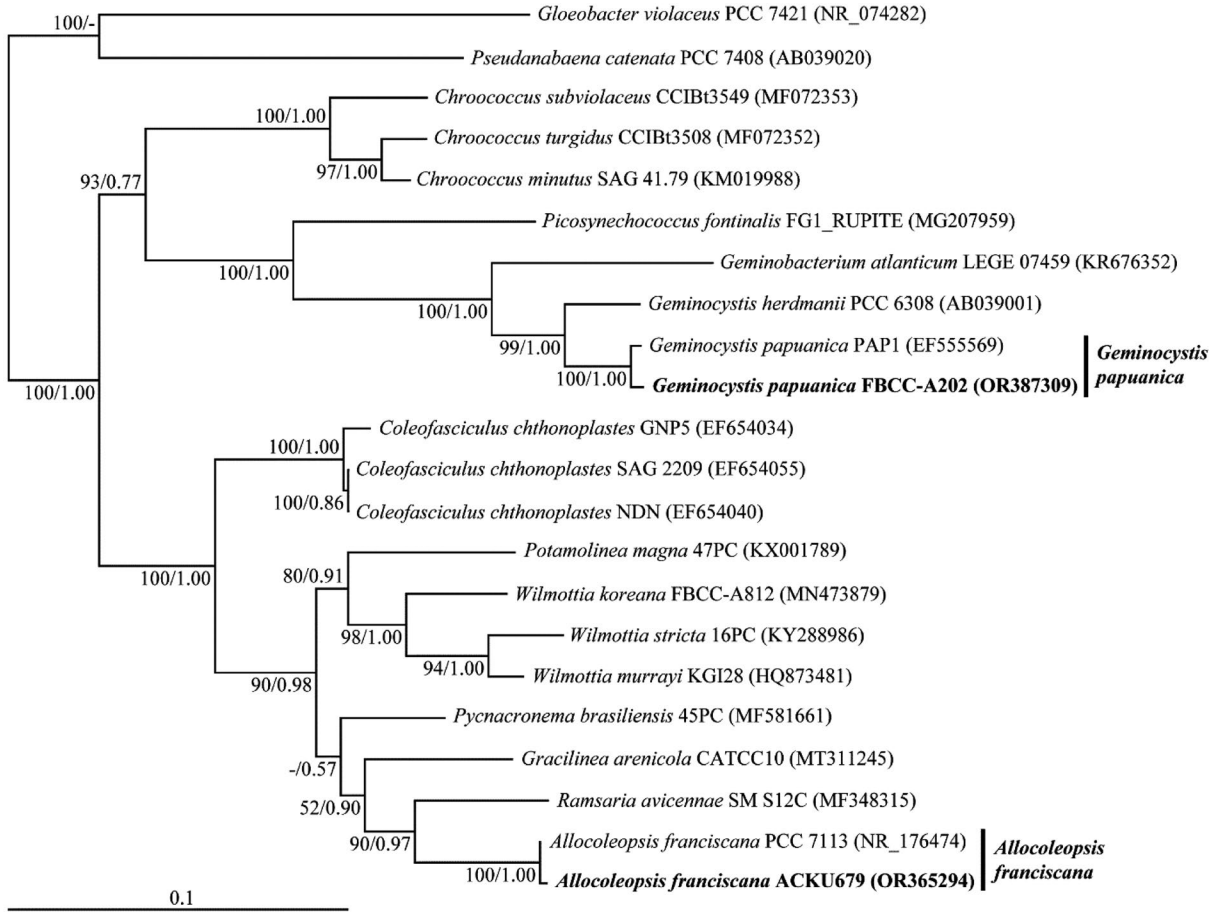

Fig. 7. Maximum-Likelihood (ML) phylogenetic tree based on 16S rRNA gene sequences of *Geminocystis papuanica*, *Allocoleopsis franciscana*, and other cyanobacterial strains. 16S rRNA gene sequences of species (*Gloeobacter violaceus* PCC 7421, *Pseudanabaena catenata* PCC 7408) were included as the 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.

Table 2. Similarity and *p*-distance between six species of this study and each corresponding species of the partial 16S rRNA gene sequences.

Order	Family	Most closely related species	Similarity (%)	*p*-distance (%)	Strain	Accession No.
Chroococcales	Geminocystaceae	*Geminocystis papuanica*	99.4	0.30	FBCC-A202	OR387309
Coleofasciculales	Coleofasciculaceae	*Allocoleopsis franciscana*	99.7	0.14	ACKU679	OR365294
Oscillatoriales	Microcoleaceae	*Ancylothrix terrestris*	100	0.00	ACKU673 ACKU674 ACKU675 ACKU676	OR367307 OR367308 OR367309 OR367310
Desertifilales	Desertifilaceae	*Klisinema persicum*	99.5–99.7	0.27–0.54	ACKU678 ACKU680	OR387310 OR387311
Leptolyngbyales	Leptolyngbyaceae	*Scytolyngbya timoleontis*	99.7–99.8	0.14–0.21	ACKU677	OR393053
Oculatellales	Oculatellaceae	*Shackletoniella antarctica*	98.8–99.2	0.74–1.12	ACKU671 ACKU672	OR393054 OR393055

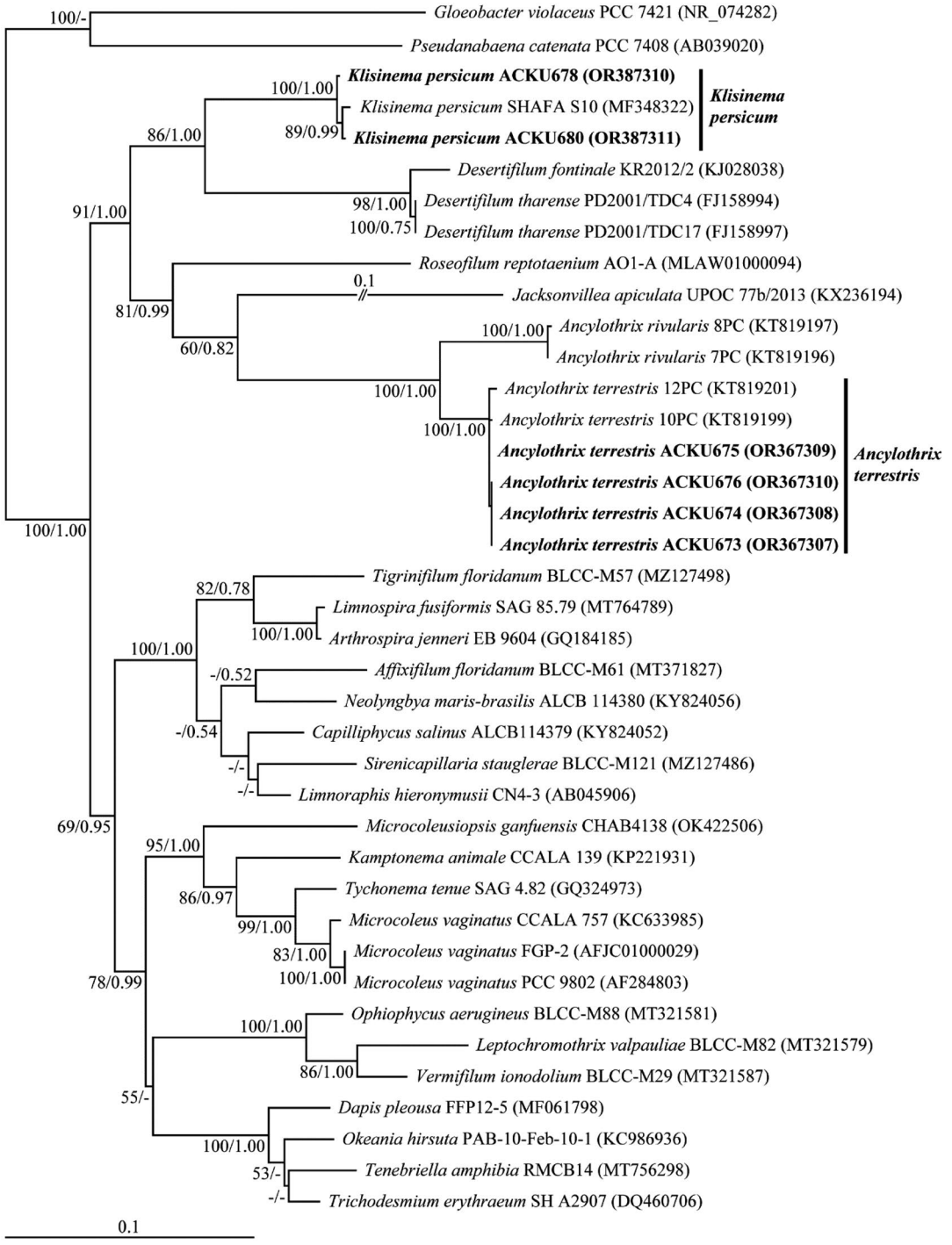

Fig. 8. Maximum-Likelihood (ML) phylogenetic tree based on 16S rRNA gene sequences of *Ancylothrix terrestris*, *Klisinema persicum*, and other cyanobacterial strains. 16S rRNA gene sequences of species (*Gloeobacter violaceus* PCC 7421, *Pseudanabaena catenata* PCC 7408) were included as the outgroups. 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.

2005; Hašler *et al*., 2012). In addition, the type strain of *Shackletoniella antarctica* was described as having unbranched filaments (Strunecký *et al*., 2019), but false branching was commonly observed in strains cultured for more than 4 weeks in this study (Fig. 6). Such variability of false branching was observed in other strains that were

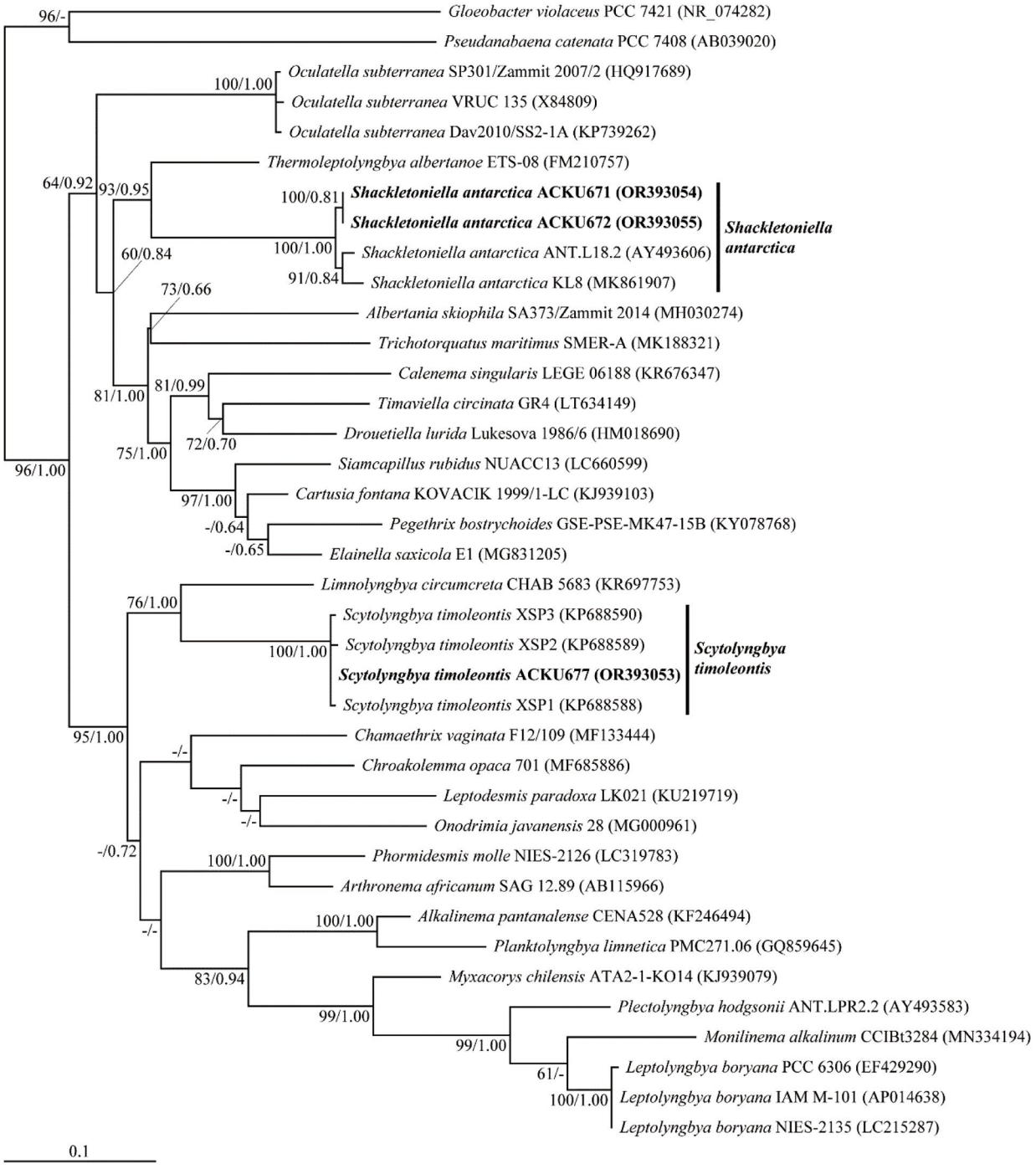

Fig. 9. Maximum-Likelihood (ML) phylogenetic tree based on 16S rRNA gene sequences of *Scytolyngbya timoleontis*, *Shackletoniella antarctica*, and other cyanobacterial strains. 16S rRNA gene sequences of species (*Gloeobacter violaceus* PCC 7421, *Pseudanabaena catenata* PCC 7408) were included as the 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.

isolated in Korea, although there was no false branching in the type strain of *Drouetiella lurida* (Gomont) Mai, Johansen and Pietrasiak (Mai *et al*., 2018: 28) belonging to the same family Oculatellaceae (Kim *et al*., 2023). Likewise, false branching has been reported as a feature that facultatively exhibits in various species (Stam, 1978; 1980;

Table 3. The morphological characteristics of six unrecorded species in Korea.

Species	Strain	Trichome	Branching	Sheath	Cell length (μm)	Cell width (μm)	Apical cell
Geminocystis papuanica	PAP1	−	−	−	4−5		spherical or slightly oval
	FBCC-A202	−	−	−	3.9−5.8		spherical or oval
Allocoleopsis franciscana	PCC 7113	entangled	−	thin or diffluent, do not open at the end	6−7.5	5−6	rounded to slightly conical
	ACKU679	entangled, straight	−	thin, firm, commonly open at the end	4.1−6.8	4.6−5.4	rounded or rounded to conical
Ancylothrix terrestris	10PC	entangled	−	thin, homogenous	2.5−5.5	4.0−6.5	conical rounded, narrowed
	ACKU673−676	straight, entangled	−	thin, firm	2.1−6.2	5.0−6.7	conical rounded or rounded, narrowed
Klisinema persicum	SHAFA S10	straight	−	−	3−5	3−5	conical, elongated
	ACKU678, ACKU680	wavy or straight	−	thin, firm	2.4−4.0	3.1−4.0	conical, elongated
Scytolyngbya timoleontis	XS01, XSP1−3	entangled, wavey	false branching	initially thin, later widened	3.5−10.8	1.9−2.6	rounded
	ACKU677	wavy or straight	false branching	thin, firm or diffluent	2.7−5.2	1.5−2.3	rounded
Shackletoniella antarctica	ANT.LH18.2	wavy or coiled	−	thin	1.7−7	1−1.5	rounded
	ACKU671, ACKU672	wavy or straight, occasionally twisted	false branching	thin, firm, sometimes diffluent, widened	1.4−3.1	1.2−1.7	rounded

Komárek and Anagnostidis, 2005).

The type strains of *Geminocystis papuanica*, *Allocoleopsis franciscana*, *Ancylothrix terrestris*, and *Klisinema persicum* appeared mainly as aerial algae on soil surface, especially *G. papuanica* in tropical forests soil and *K. persicum* in soil near thermal springs with high radioactivity background (Korelusová *et al*., 2009; Heidari *et al*., 2018). *Shackletoniella antarctica* was collected as planktonic algae from Antarctic lakes (Strunecký *et al*., 2020). However, all Korean strains appeared as epilithic algae collected from gravel surfaces in temperate freshwater and exhibited characteristics of cyanobacteria that occur in various types of habitats (Engene *et al*., 2012; Bagchi *et al*., 2017; Radzi *et al*., 2021; Kim *et al*., 2022a).

In the 16S rRNA gene sequence analysis, the 11 strains isolated in this study showed high nucleotide sequence similarity with each type strain than 98.65%, which is the standard for the same species (Kim *et al*., 2014). In addition, they were confirmed to belong to the same species by forming clades with each respective type strain in phylogenetic trees (Table 2, Figs. 7−9).

In this study, 11 strains of Korean cyanobacteria isolated from gravels in freshwaters were identified *Geminocystis papuanica*, *Allocoleopsis franciscana*, *Ancylothrix terrestris*, *Klisinema persicum*, *Scytolyngbya timoleontis*, and *Shackletoniella antarctica* based on morphological, ecological, and molecular analyses, and they were found to be newly recorded species in Korea.

Conflicts of Interest

The author of this paper has no affiliation with any interests and is solely responsible for the paper.

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Supplementary Table 1. For species list of group 1, including *Geminocystis papuanica* and *Allocoleopsis franciscana*, strain numbers and their GenBank accession numbers were used in this study.

Species	Strain No.	Accession No.	Reference
Allocoleopsis franciscana	ACKU679	OR365294	This study
Allocoleopsis franciscana	PCC 7113	NR_176474	Fernandes *et al.* (2021)
Chroococcus minutus	SAG 41.79	KM019988	Gama *et al.* (2019)
Chroococcus subviolaceus	CCIBt3549	MF072353	Gama *et al.* (2019)
Chroococcus turgidus	CCIBt3508	MF072352	Gama *et al.* (2019)
Coleofasciculus chthonoplastes	GNP5	EF654034	Siegesmund *et al.* (2008)
Coleofasciculus chthonoplastes	NDN	EF654040	Siegesmund *et al.* (2008)
Coleofasciculus chthonoplastes	SAG 2209	EF654055	Siegesmund *et al.* (2008)
Geminobacterium atlanticum	LEGE 07459	KR676352	Brito *et al.* (2017)
Geminocystis herdmanii	PCC 6308	AB039001	Korelusová *et al.* (2009)
Geminocystis papuanica	FBCC-A202	OR387309	This study
Geminocystis papuanica	PAP1	EF555569	Korelusová *et al.* (2009)
Gloeobacter violaceus	PCC 7421	NR_074282	Nakamura *et al.* (2003)
Gracilinea arenicola	CATCC10	MT311245	Machado-de-Lima and Branco (2020)
Picosynechococcus fontinalis	FG1 Rupite	MG207959	Komárek *et al.* (2020)
Potamolinea magna	47PC	KX001789	Martins *et al.* (2016)
Pseudanabaena catenata	PCC 7408	AB039020	Ishida *et al.* (2001)
Pycnacronema brasiliensis	45PC	MF581661	Martins *et al.* (2019)
Ramsaria avicennae	SM S12C	MF348315	Heidari *et al.* (2018)
Wilmottia koreana	FBCC-A812	MN473879	Lee *et al.* (2020)
Wilmottia murrayi	KGI28	HQ873481	Strunecký *et al.* (2011)
Wilmottia stricta	16PC	KY288986	Machado-de-Lima *et al.* (2017)

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Supplementary Table 2. For species list of group 2, including *Ancylothrix terrestris* and *Klisinema persicum*, strain numbers and their GenBank accession numbers were used in this study.

Species	Strain No.	Accession No.	Reference
Affixifilum floridanum	BLCC-M61	MT371827	Lefler *et al*. (2021)
Ancylothrix rivularis	7PC	KT819196	Martins *et al*. (2016)
Ancylothrix rivularis	8PC	KT819197	Martins *et al*. (2016)
Ancylothrix terrestris	10PC	KT819199	Martins *et al*. (2016)
Ancylothrix terrestris	12PC	KT819201	Martins *et al*. (2016)
Ancylothrix terrestris	ACKU673	OR367307	This study
Ancylothrix terrestris	ACKU674	OR367308	This study
Ancylothrix terrestris	ACKU675	OR367309	This study
Ancylothrix terrestris	ACKU676	OR367310	This study
Arthrospira jenneri	EB 9604	GQ184185	Papapanagiotou *et al*. (2019)
Capilliphycus salinus	ALCB114379	KY824052	Caires *et al*. (2018a)
Dapis pleousa	FFP12-5	MF061798	Engene *et al*. (2018)
Desertifilum fontinale	KR2012/2	KJ028038	Dadheech *et al*. (2014)
Desertifilum tharense	PD2001/TDC4	FJ158994	Dadheech *et al*. (2012)
Desertifilum tharense	PD2001/TDC17	FJ158997	Dadheech *et al*. (2012)
Gloeobacter violaceus	PCC 7421	NR_074282	Nakamura *et al*. (2003)
Jacksonvillea apiculata	UPOC 77b/2013	KX236194	Hašler *et al*. (2017)
Kamptonema animale	CCALA 139	KP221931	Strunecký *et al*. (2014)
Klisinema persicum	SHAFA S10	MF348322	Heidari *et al*. (2018)
Klisinema persicum	ACKU678	OR387310	This study
Klisinema persicum	ACKU680	OR387311	This study
Leptochromothrix valpauliae	BLCC-M82	MT321579	Berthold *et al*. (2021)
Limnoraphis hieronymusii	CN4-3	AB045906	Suda *et al*. (2002)
Limnospira fusiformis	SAG 85.79	MT764789	Hauerová *et al*. (2021)
Microcoleus vaginatus	CCALA 757	KC633985	Strunecký *et al*. (2013)
Microcoleus vaginatus	FGP-2	AFJC01000029	Starkenburg *et al*. (2011)
Microcoleus vaginatus	PCC 9802	AF284803	Garcia-Pichel *et al*. (2001)
Microcoleusiopsis ganfuensis	CHAB4138	OK422506	Geng *et al*. (2021)
Neolyngbya maris-brasilis	ALCB 114380	KY824056	Caires *et al*. (2018b)
Okeania hirsuta	PAB-10-Feb-10-1	KC986936	Engene *et al*. (2013)
Ophiophycus aerugineus	BLCC-M88	MT321581	Berthold *et al*. (2021)
Pseudanabaena catenata	PCC 7408	AB039020	Ishida *et al*. (2001)
Roseofilum reptotaenium	AO1-A	MLAW01000094	Buerger *et al*. (2016)
Sirenicapillaria stauglerae	BLCC-M121	MZ127486	Berthold *et al*. (2022)
Tenebriella amphibia	RMCB14	MT756298	Hauerová *et al*. (2021)
Tigrinifilum floridanum	BLCC-M57	MZ127498	Berthold *et al*. (2022)
Trichodesmium erythraeum	SH A2907	DQ460706	Palinska *et al*. (2006)
Tychonema tenue	SAG 4.82	GQ324973	Arp *et al*. (2010)
Vermifilum ionodolium	BLCC-M29	MT321587	Berthold *et al*. (2021)

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Supplementary Table 3. For species list of group 3, including *Scytolyngbya timoleontis* and *Shackletoniella antarctica*, strain numbers and their GenBank accession numbers were used in this study.

Species	Strain No.	Accession No.	Reference
Albertania skiophila	SA373/Zammit 2014	MH030274	Zammit (2018)
Alkalinema pantanalense	CENA528	KF246494	Vaz *et al*. (2015)
Arthronema africanum	SAG 12.89	AB115966	Covarrubias *et al*. (2016)
Calenema singularis	LEGE 06188	KR676347	Brito *et al*. (2017)
Cartusia fontana	KOVACIK 1999/1-LC	KJ939103	Mai *et al*. (2018)
Chamaethrix vaginata	F12/109	MF133444	Dvořák *et al*. (2017)
Chroakolemma opaca	701	MF685886	Becerra-Absalon *et al*. (2018)
Drouetiella lurida	Lukesova 1986/6	HM018690	Mai *et al*. (2018)
Elainella saxicola	E1	MG831205	Jahodářová *et al*. (2018)
Gloeobacter violaceus	PCC 7421	NR_074282	Nakamura *et al*. (2003)
Leptodesmis paradoxa	LK021	KU219719	Raabová *et al*. (2019)
Leptolyngbya boryana	IAM M-101	AP014638	Hiraide *et al*. (2015)
Leptolyngbya boryana	NIES-2135	LC215287	Kim *et al*. (2022)
Leptolyngbya boryana	PCC 6306	EF429290	Johansen *et al*. (2011)
Limnolyngbya circumcreta	CHAB 5683	KR697753	Li and Li (2016)
Monilinema alkalinum	CCIBt3284	MN334194	Malone *et al*. (2021)
Myxacorys chilensis	ATA2-1-KO14	KJ939079	Pietrasiak *et al*. (2019)
Oculatella subterranea	Dav2010/SS2-1A	KP739262	Christodoulou *et al*. (2015)
Oculatella subterranea	SP301/Zammit 2007/2	HQ917689	Zammit *et al*. (2012)
Oculatella subterranea	VRUC 135	X84809	Zammit *et al*. (2012)
Onodrimia javanensis	E28	MG000961	Jahodářová *et al*. (2017)
Pegethrix bostrychoides	GSE-PSE-MK47-15B	KY078768	Mai *et al*. (2018)
Phormidesmis molle	NIES-2126	LC319783	Davydov and Vilnet (2022)
Planktolyngbya limnetica	PMC271.06	GQ859645	Thomazeau *et al*. (2010)
Plectolyngbya hodgsonii	ANT.LPR2.2	AY493583	Taton *et al*. (2011)
Pseudanabaena catenata	PCC 7408	AB039020	Ishida *et al*. (2001)
Scytolyngbya timoleontis	XSP1	KP688588	Song *et al*. (2015)
Scytolyngbya timoleontis	XSP2	KP688589	Song *et al*. (2015)
Scytolyngbya timoleontis	XSP3	KP688590	Song *et al*. (2015)
Scytolyngbya timoleontis	ACKU677	OR393053	This study
Shackletoniella antarctica	ACKU671	OR393054	This study
Shackletoniella antarctica	ACKU672	OR393055	This study
Shackletoniella antarctica	ANT.L18.2	AY493606	Strunecký *et al*. (2020)
Shackletoniella antarctica	KL8	MK861907	Strunecký *et al*. (2020)
Siamcapillus rubidus	NUACC13	LC660599	Tawong *et al*. (2022)
Thermoleptolyngbya albertanoe	ETS-08	FM210757	Moro *et al*. (2010)
Timaviella circinata	GR4	LT634149	Sciuto *et al*. (2017)
Trichotorquatus maritimus	SMER-A	MK188321	Pietrasiak *et al*. (2021)

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