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Newly recorded genera and species, *Pantanalinema rosaneae* and *Alkalinema pantanalense* (Leptolyngbyaceae, Cyanobacteria) isolated in Korea

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Two strains of cyanobacteria were isolated from the soil of Seodaemun-gu, Seoul and from the gravel of the Ansung Stream, Gyeonggi Province, Korea, respectively. They were identified as *Pantanalinema rosaneae* and *Alkalinema pantanalense* under the Leptolyngbyaceae through the morphological, ecological, and molecular analyses and first reported in Korea. Belonging to the *Leptolyngbya* morphotypes, they are thin filamentous cyanobacteria and morphologically indistinguishable cryptic species. The strains of *P. rosaneae* and *A. pantanalense* isolated in Korea revealed the same cluster as their type species in the phylogenetic analysis using the 16S rRNA gene sequences, and similarities in the secondary structures of 16S−23S ITS sequences. Although both *P. rosaneae* and *A. pantanalense* were collected from water samples in the Pantanal wetland of Brazil, the *P. rosaneae* obtained in Korea, was soil-dwelling subaerophytic species whereas *A. pantanalense* was epilithic species living on gravel in the freshwater. Therefore, they are considered to have an extensive habitat.

Keywords: *Alkalinema pantanalense*, cryptic, *Pantanalinema rosaneae*, saline-alkaline lake, subaerophytic

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Introduction

Cyanobacteria are distributed in most habitats worldwide such as freshwater, seawater, and aerial regions. As prokaryotes, they are so small in size and simple in morphology that they are rarely classified with the morphological traits alone. In particular, new genera and species are reported by a recent attempt of the molecular analysis using 16S rRNA and 16S−23S ITS sequences as well as TEM ultrastructure so as to overcome the morphological similarities of *Leptolyngbya* Anagnostidis and Komárek (1988) morphotype which are thin filamentous homocytous strains (Komárek *et al*., 2014; Malone *et al*., 2021).

The genus *Leptolyngbya* is recognized as clearly polyphyletic (Zammit *et al*., 2012), reinforcing the notion that it requires a taxonomic re-evaluation that takes into account more determining features such as molecular and ecophysiological data (Vaz *et al*., 2015).

In the phylogenetic study on the *Leptolyngbya sensu stricto* using the 16S rRNA gene sequences, numerous novel genera such as *Amazoninema* Genuário, de Souza, Monteiro, Sant'Anna and Melo 2018, *Nodosilinea* Perkerson et Casamatta 2011, *Oculatella* Zammit *et al*. 2012, *Onodrimia* Jahodářová, Dvořák and Hašler 2017, *Phormidesmis* Turicchia, Ventura, Komárková and Komárek 2009, *Plectolyngbya* Taton *et al*. 2011, *Scytolyngbya* Song et Li 2015, *Stenomitos* Miscoe et Johansen 2016, *Thermoleptolyngbya* Sciuto and Moro 2016 *Timaviella* Sciuto and Moro 2016, *Toxifilum* Zimba *et al*. 2017 are described (Turicchia *et al*., 2009; Perkerson *et al*., 2011; Taton *et al*., 2011; Zammit *et al*., 2012; Song *et al*., 2015; Miscoe *et al*., 2016; Sciuto and Moro, 2016; Jahodářová *et al*., 2017; Zimb *et al*., 2017; Genuário *et al*., 2018; Genuário *et al*., 2020).

The cyanobacterial communities were discovered in several saline-alkaline lakes of the Brazilian Pantanal wetland (Vaz *et al*., 2015). In particular, a number of homocytous cyanobacterial strains were isolated. *Pantanalinema* Vaz *et al*. 2015, *Alkalinema* Vaz *et al*. 2015, Monilinema Malone, Genuário, Vaz, Fiore and Sant'Anna 2021 among them were described based upon the phylogeny using the 16S rRNA gene sequences and using the 16S−23S rRNA intergenic spacer (ITS) secondary structure (Vaz *et al*., 2015; Malone *et al*., 2021).

Such genera, called cryptic taxa, are clearly different and separated clades in the phylogenetic tree but morphologically difficult to separate (Komárek, 2016).

In this study, two strains of cyanobacteria living on gravel in the Ansung Stream, Ansung City, Gyeonggi Province, and the other strains of soil-dwelling cyanobacteria on the Chungjung-ro, Seodaemun-gu, Seoul were collected. The strains of thin filamentous homocytous cyanobacteria were isolated and then cultured. The identification of above cyanobacteria based upon the morphological traits, 16S rRNA gene sequences, and 16S−23S rRNA ITS secondary structures proved them to be unreported Korean genera and species belonging to the *Pantanalinema rosaneae* Vaz *et al*. 2015 and *Alkalinema pantanalense* Vaz *et al*. 2015.

Materials and Methods

Sampling, culture, and morphological analysis

Two strains of subaerial cyanobacteria were collected from the soil located at 3-148, Chungjung-ro-3-ga, Seodaemun-gu, Seoul, on July 24, 2020, and another two strains of cyanobacteria were collected from the gravel in the Ansung Stream, 58-4 Dogi-Dong, Ansung Si, Gyeonggi Province, on September 20, 2020 (conductivity 264 μS/cm, water temperature 16.14°C and pH 7.04). Also, Quantum Geographical Information System (QGIS 3.16.4) was used in order to generate the map (Fig. 1).

The soil of Chungjung-ro, Seoul was moist, which was obtained in the conical tube using a spatula. The gravel was collected in the Ansung Stream which had a shallow depth of less than 30 centimeters and slow flow velocity. Samples were obtained by scratching the surface with a soft brush (Kiel and Gaylarde, 2006).

For a single-species culture, a single trichome was picked using a Pasteur pipette under a microscope and then transferred to a 24-well plate (SPL, Pocheon, Korea) containing BG-11 medium (Rippka *et al*., 1979). After the trichome were moved onto the 24-well plate and then incubated for two weeks, formed filaments were transferred to a flask of the 50 mL cell culture (SPL, Pocheon, Korea) for the mass culture. The synthetic culture was then carried out under the condition of 25°C ± 1, photoperiod 16 : 8 h (light : dark) and illumination of 25 μmol photons·m-2s-1 in the Algal Culture Collection of Kyonggi University (ACKU).

The four isolated strains were deposited in the Nakdonggang National Institute of Biological Resources (NNIBR) of Korea with the following accession numbers: FBCC-A1468, FBCC-A1469, FBCC-A1470 and FBCC-A1471.

Morphological characteristics of fresh field samples and cultured cyanobacteria strains were discerned at 100−1000× magnification with a light microscope (Olympus BX41, Olympus, Tokyo, Japan) equipped with a digital camera (Olympus UC-90, Olympus, Tokyo, Japan). Image analysis was performed with the Olympus CellSens entry software (Olympus, Japan) to take images in the 200−1000× magnification. The shapes and sizes of vegetative cells were observed using at least more than 30 cells.

The taxonomic classification system of cyanophytes was followed by Komárek *et al*. (2014) and AlgaeBase (Guiry and Guiry, 2021), and the identification of cyanobacteria was conducted according to Komárek (2013), Vaz *et al*. (2015) and Malone *et al*. (2021).

Molecular methods

Centrifugation for 10 minutes at 13,000 rpm after transferring 1 mL to a 1.5-mL e-tube in order to extract the genomic DNA (gDNA) from the cultured sample. Total genomic DNA (gDNA) was extracted using the i-genomic Plant DNA Extraction Mini Kit (iNtRON, Daejeon, Korea).

We used the following primers to amplify 16S rRNA: 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) (Neilan *et al*., 1997) and 23S600R (5′-CGG CTC ATT CTT CAA CAG GCA C-3′) (Lee *et al*., 2019). The Maxime™ PCR PreMix Kit (i-StarTaq™ GH) (iNtRON, Daejeon, Korea) was used for the DNA amplification, in a total volume of 20 μL, including the extracted gDNA (1 μL) with 17 μL the sterilized distilled water. PCR cycling was done on an i-Cycler (Bio-Rad Laboratories, Hercules, CA) with the following process: initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 20 sec., annealing at 55°C for 30 sec., and extension at 72°C for 90 sec.; with a final extension at 72°C for 10 minutes. The resulting PCR products were subjected to electrophoresis in 1.2% agarose gel stained with ethidium bromide and viewed under ultraviolet light on a transilluminator.

The PCR products were purified using the MEGAquick-spinTM Plus DNA Fragment Purification Kit (iNtRON, Daejeon, Korea) for the DNA sequencing, which was analyzed using an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA). The sequences were viewed and edited using the SeqMan program (DNASTAR, Madison, WI). All the sequences identified here were deposited in the GenBank database (MZ567050, MZ567052, MZ567048, MZ567049).

Phylogenetic analyses

We used BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify closely allied species. Likewise, we calculated the genetic distance using the Kimura 2-parameter on MEGA 7.0 (Kumar *et al*., 2016).

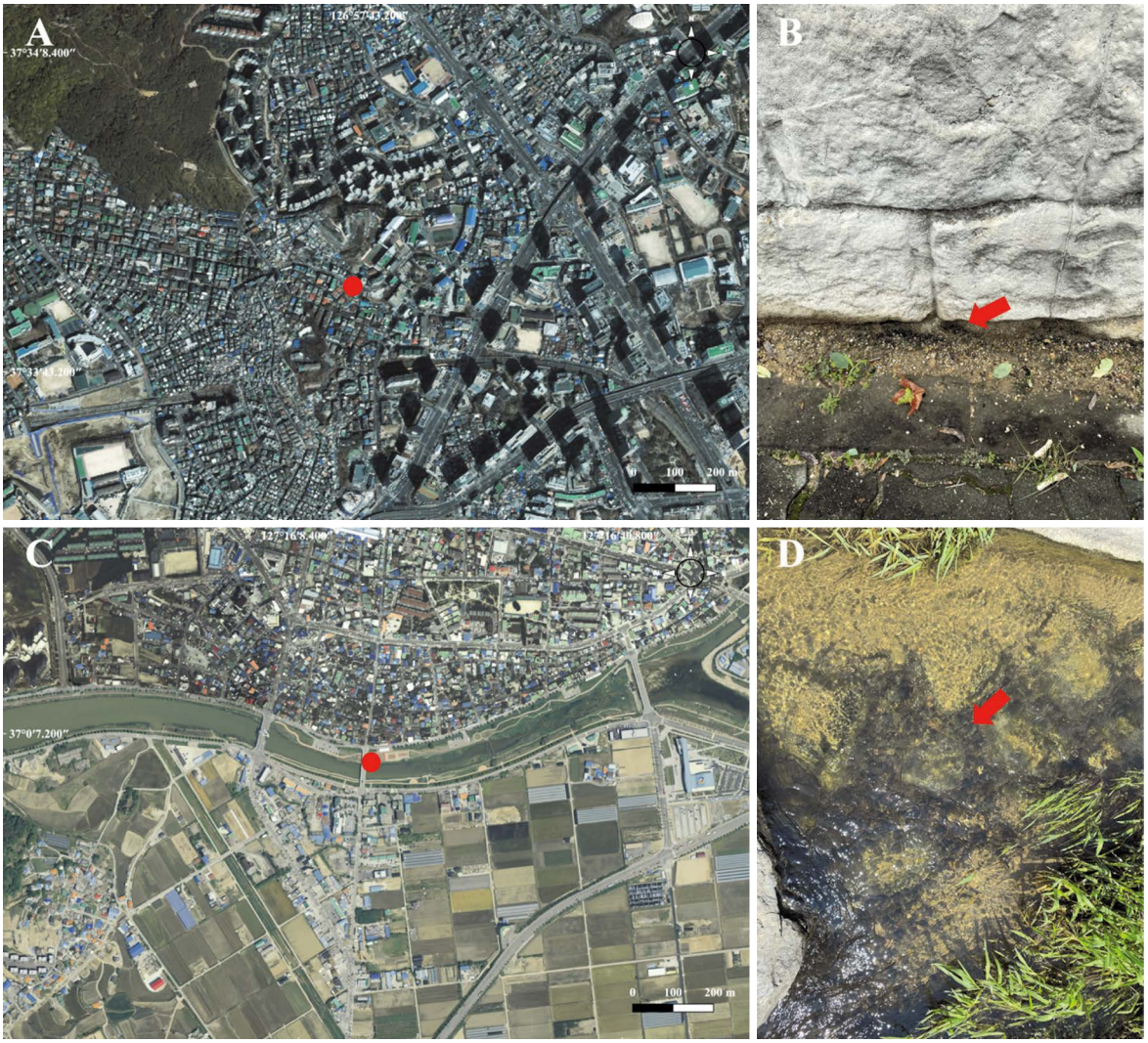

Fig. 1. Photographs showing the collection sites of *Pantanalinema rosaneae* (A, B) and *Alkalinema pantanalense* (C, D). (A) The red circle is Chungjeong-ro 3-ga, Seodaemun-gu, Seoul (37°33′51.1″N / 126°57′38.6″E), (C) The red circle is Anseongcheon, Anseong-si, Gyeonggi-do (37°00′04.2″N / 127°16′13.5″E), and the habitat views of collection sites (red arrow) of (B) *P. rosaneae* and (D) *A. pantanalense*.

For phylogenetic analysis, we used 16S rRNA gene sequences of our four strains and previously reported 10 strains belonging to *Pantanalinema rosaneae* and *Alkalinema pantanalense*, and its 13 relative genera, belonging to the order Synechococcales (including family Leptolyngbyaceae and Pseudanabaenaceae). These relatives were selected based on the a previous report (Vaz *et al*., 2015) and our preliminary analysis via BLAST. These included *Chroakolemma* Becerra-Absalón and Johansen 2018, *Euryhalinema* Chakraborty et Mukherjee 2019, *Leptodesmis* Raabová, Kovacik and Strunecký 2019, *Leptolyngbya*, *Leptothoe* Konstantinou and Gkelis 2019, *Onodrimia*, *Pinocchia* Dvořák, Jahodářová et Hašler 2015, *Planktolyngbya* Anagnostidis and Komárek 1988, *Plectolyngbya*, *Rhodoploca* Konstantinou and Gkelis 2021, *Scytolyngbya*, *Stenomitos*, *Pseudanabaena* Lauterborn 1915.

We used three methods to infer the phylogenetic relationships between taxa: General Time Reversible (GTR) maximum likelihood model using RAxML ver. 7.0.3 (Stamatakis, 2006), and neighbor-joining approach adopted the Kimura 2-parameter model using the MEGA 7.0 (Kumar *et al*., 2016), and Bayesian inference with the K2 + G + I model using MrBayes ver. 3.1.2. We used TreeView ver. 1.6.6 (Page, 1996) to visualize the trees.

Structure analyses of 16S–23S internal transcribed spacer (ITS)

The Mfold (accessed 1 August 2021) was adopted to figure out the secondary structure on the D1–D1′, Box-B, and V3 helices of the 16S–23S ITS whose drawing was

based upon the PseudoViewer3 (http://pseudoviewer.inha.ac.kr/) (Zuker, 2003).

Results

The *Pantanalinema rosaneae* and *Alkalinema pantanalense* belonging to Leptolyngbyaceae and Oscillatoriales were identified through their morphological traits and the phylogenetic analysis using and 16S−23S rRNA sequences, both of which are unreported genera and species in Korea.

Order Synechococcales Hoffmann, Komárek and Kastovsky 2005
Family Leptolyngbyaceae (Anagnostidis and Komárek) Komárek, Kastovsky, Mareš and Johansen 2014
Genus *Pantanalinema* Vaz *et al*. 2015

Pantanalinema rosaneae Vaz *et al*. 2015 (Fig. 2)

Morphology and description

The filaments are entangled and flexuous. The cells are slightly constricted, and the cross wall is translucent. The sheath is hyaline, attached to the trichome and always present. Cells are isodiametric or wider than they are long, 1.5−3.2 μm long by 1.8.−2.5 μm wide. The apical cell is cylindrical with a rounded apex. Cell content is homogeneous and brownish green. As above, morphological characteristics were consistent with the previously reported description of this species (Vaz *et al*., 2015).

Ecology: This species was isolated in water samples collected from Salina Verde in the Pantanal wetlands, Mato Grosso do Sul State, Brazil (Vaz *et al*., 2015). In this study, this species was isolated from the soil of a stone wall, Seoul of Korea.
Site of collection: Chungjeong-ro 3-ga, Seodaemun-gu, Seoul (37°33′51.1″N / 126°57′38.6″E).
Date of collection: July 24, 2020.
Specimen Locality: FBCC-A1470, FBCC-A1471 in the Nakdonggang National Institute of Biological Resources (NNIBR).
Gene sequences: The 16S rRNA to 23S rRNA gene sequences: GenBank accession Nos. MZ567050, MZ567052.

16S rRNA gene and phylogenetic affiliation of *Pantanalinema rosaneae*

The region from 16S rDNA to 23S rDNA were sequenced for two strains (MZ567050, MZ567052) of *Pantanalinema rosaneae*. Upon DNA analysis, 16S rDNA were found to be nearly identical to that of the type strain (DNA similarity 99.9%). Phylogenetic relationships of the genus *Pantanalinema* and other relatives were investigated by using a ML tree constructed from their partial 16S rRNA gene sequences. The additional Bayesian tree turned out to be quite close to the ML tree (Fig. 4). The strains of *P. rosaneae* (FBCC-A1470, FBCC-A1471) clustered with the type strain *P. rosaneae* (CENA 516), inferring that the strains of *P. rosaneae* (FBCC-A1470, FBCC-A1471) and *P. rosaneae* (CENA 516) were the same species.

Pantanalinema rosaneae FBCC-A1470 showed a 100% DNA similarity to *P. rosaneae* FBCC-A1471 and 0.00% *p*-distance between them. On the other hand, the strains of *P. rosaneae* (FBCC-A1470, FBCC-A1471) showed a 99.5 to 99.9% DNA similarity to *P. rosaneae* (CENA 516, 517, 521, 537) and 0.00% *p*-distance between them (Table 1).

Characterization of 16S−23S ITS regions of *Pantanalinema rosaneae*

This study revealed secondary structures of D1−D1′, Box-B, and V3 helices on the *P. rosaneae* (FBCC-A1471) using the Mfold server (Fig. 5, A). A total of four secondary D1−D1′ helical structures of the *P. rosaneae* have been reported so far. *P. rosaneae* (FBCC-A1471), one of the four reported D1−D1′ helical secondary structures, composed of the same six loops and 81 bases as *P. rosaneae* (CENA 516, 517). One of the three reported Box-B helical structures, *P. rosaneae* (FBCC-A1471) composed of the same four loops and 44 bases as *P. rosaneae* (CENA 516, 521, 539, CClbt1046). *Pantanalinema rosaneae* (FBCC-A1471) has the same 44- bases as *P. rosaneae* (CENA 517) but ta different stem. The V3 helix of *P. rosaneae* (FBCC-A1471) formed the same four loops and 57 bases as *P. rosaneae* (CENA 537, 539, CClbt1046). Nevertheless, both *P. rosaneae* (FBCC-A1471) and *P. rosaneae* (CENA 537, 539, CClbt1046) each showed a novel structure of different loop sizes.

Besides, the strains of *P. rosaneae* (FBCC-A1470, FBCC-A1471) illustrated a similar ring form to the ITS secondary structure of conventional *P. rosaneae* (CENA 516, 517, 521, 537) (Fig. 5, A).

Order Synechococcales Hoffmann, Komárek and Kastovsky 2005
Family Leptolyngbyaceae (Anagnostidis and Komárek) Komárek, Kastovsky, Mareš and Johansen 2014
Genus *Alkalinema* Vaz *et al*. 2015

Alkalinema pantanalense Vaz *et al*. 2015 (Fig. 3)

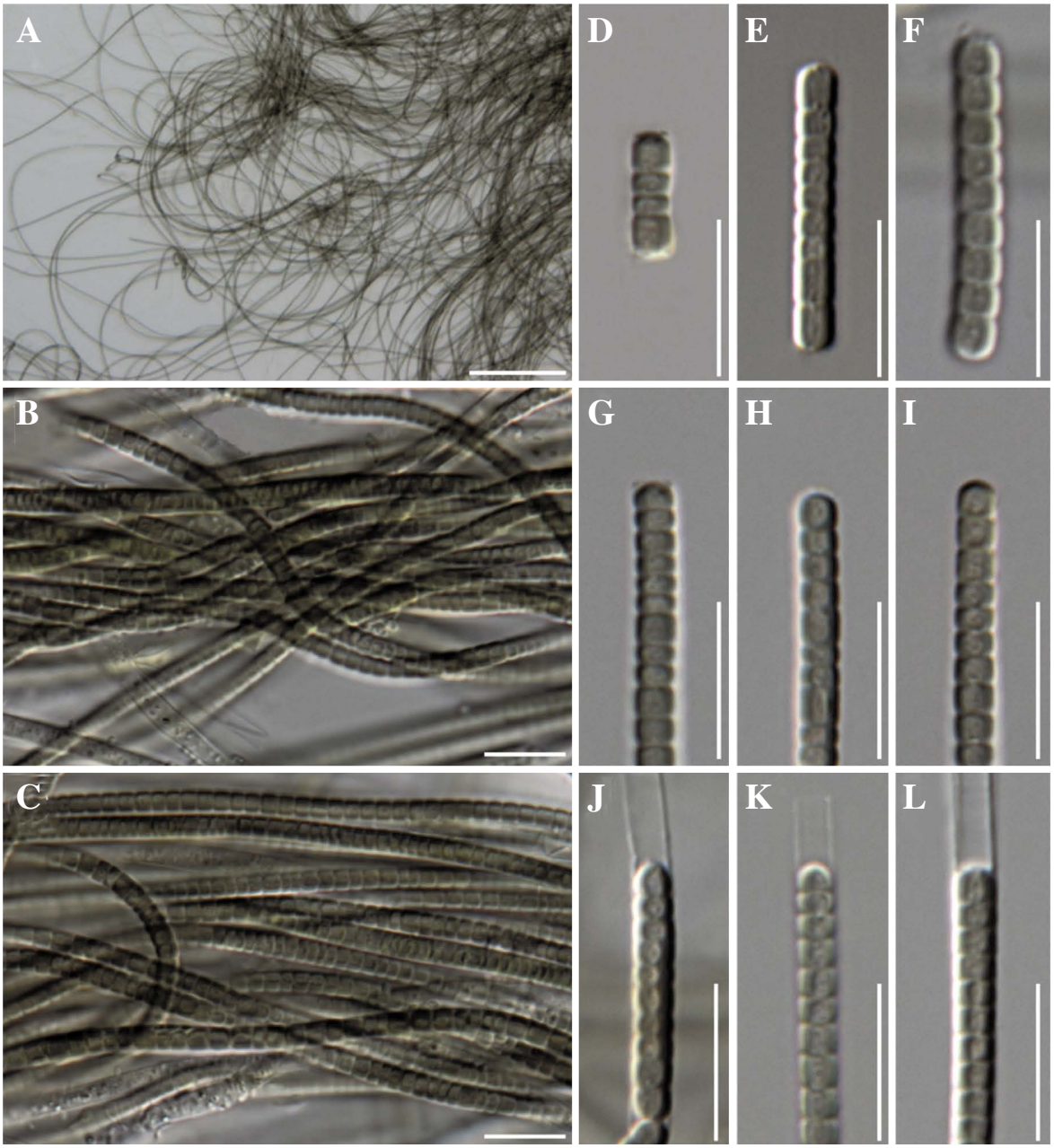

Fig. 2. Microphotographs of *Pantanalinema rosaneae* from the strain FBCC-A1471. (A−C) Colonies (mats) formed in culture, (D−F) Hormogonia, (G−I) Morphologies of apical cells, (J−L) Firm sheathes of trichomes in culture. Scale bars = (A) 100 μm, (B−L) 10 μm.

Morphology and description

The trichomes do not have sheaths but do have a diffluent mucilage. Colonies are grown on interwoven mats. Cells are longer than they are wide, 2.2−3.1 μm long by 2.8−3.2 μm wide, with a narrowed apical cell. As above, morphological features of this strain were identical to the original description of the type species (Vaz *et al*., 2015).

Ecology: The species was first collected in the saline-alkaline lake of wetland in Pantanal, Brazil and named as *Alkalinema pantanalense*. *Alkalinema pantanalense* (CENA 528) was reported to survive in the range of pH 8.4 to 9.9 for growth experiment but brought about a change to 8.4 to 9.9 pH in the cultured medium (Vaz *et al*., 2015). In this study, it turned out to be an epilithic species growing on gravels in the freshwater, implying that they can be distributed in an extensive water environment.

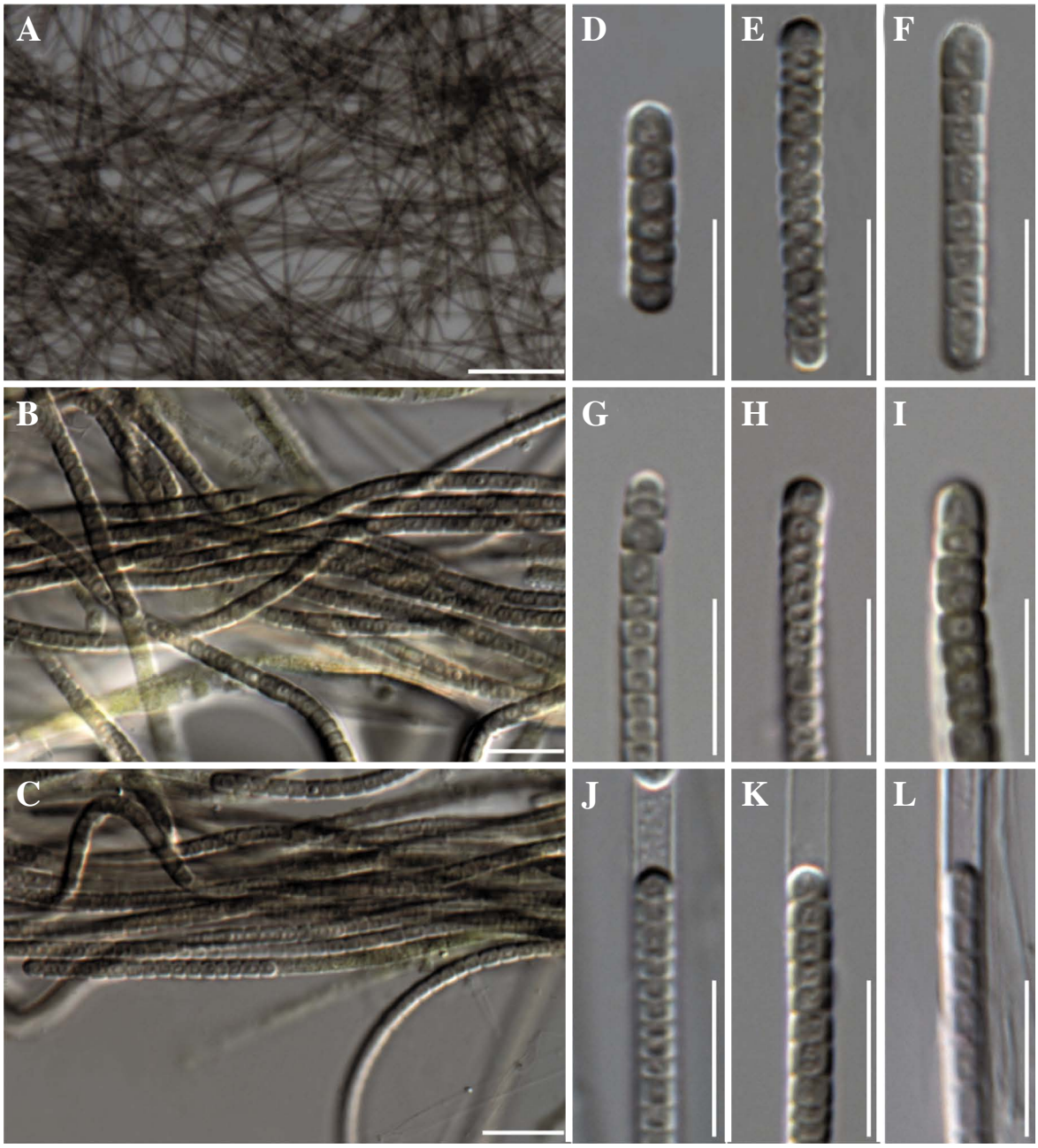

Fig. 3. Microphotographs of *Alkalinema pantanalense* from the strain FBCC-A1469. (A–C) Colonies (mats) formed in culture, (D–F) Hormogonia, (G–I) Morphologies of apical cells, (J–L) Firm sheathes of trichomes in culture. Scale bars = (A) 100 μm, (B–L) 10 μm.

Site of collection: Anseongcheon, Anseong-si, Gyeonggi Province (37°00′04.2″N / 127°16′13.5″E).
Date of collection: September 20, 2020.
Specimen Locality: FBCC-A1468, FBCC-A1469 in the Nakdonggang National Institute of Biological Resources (NNIBR).
Gene sequences: The 16S rRNA to 23S rRNA gene sequences: GenBank accession Nos. MZ567048, MZ567049.

16S rRNA gene and phylogenetic affiliation of *Alkalinema pantanalense*

The region from 16S rDNA to 23S rDNA were sequenced for two strains (MZ567048, MZ567049) of *Alkalinema pantanalense*. DNA analysis showed that the two strains had completely identical 16S rRNA gene sequences (DNA similarity 99.7%). The phylogenetic relationships of the genus *Alkalinema* and other relatives were investigated by using a ML tree constructed from their

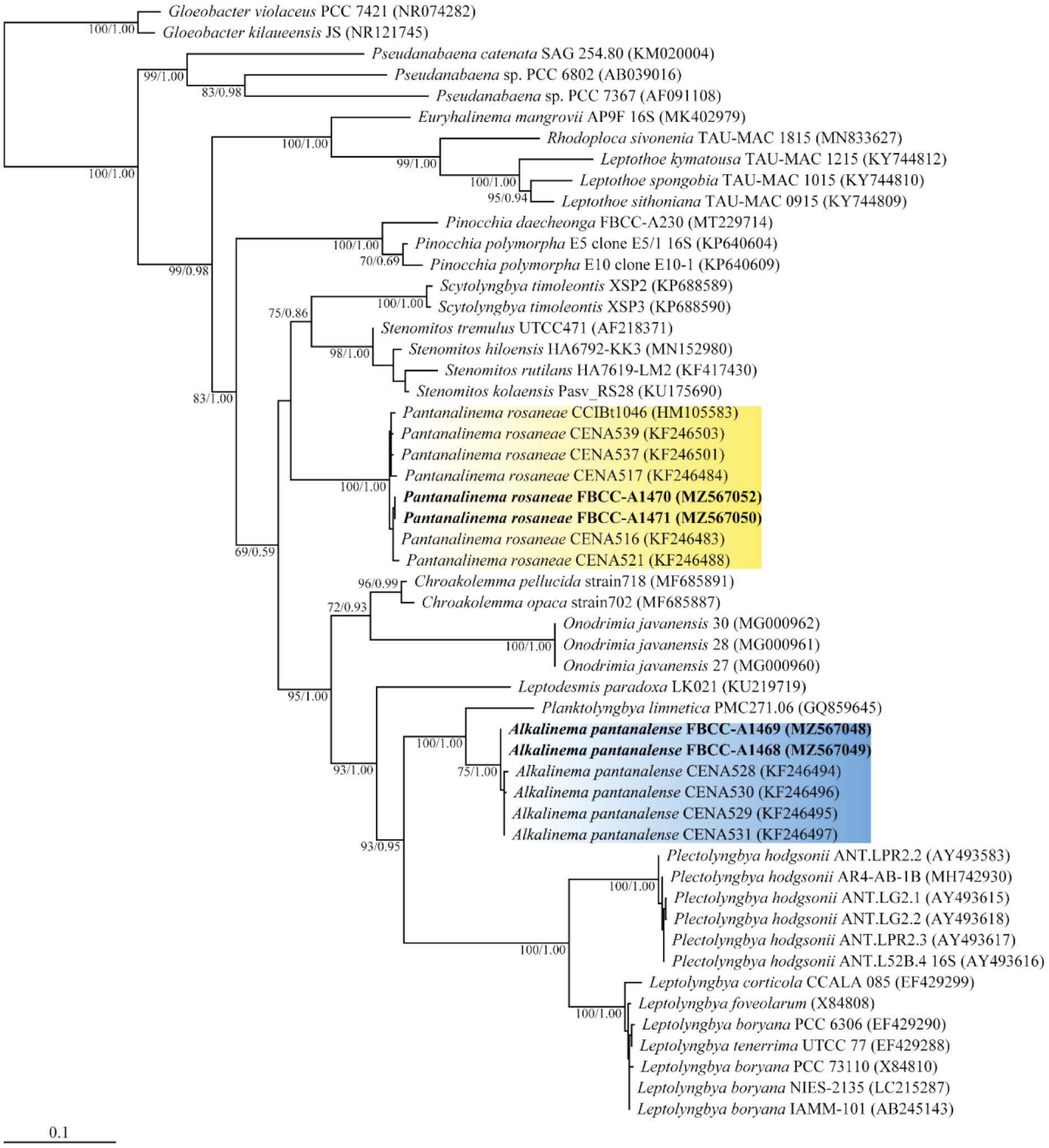

Fig. 4. Phylogenetic position of *Pantanalinema* and *Alkalinema* within the family Leptolyngbyaceae inferred from 16S rRNA sequences with the maximum-likelihood (ML) algorithm. 16S rRNA sequences of genera (*Gloeobacter violaceus*, Accession No. NR074282/ *Gloeobacter kilaueenis*, Accession No. NR121745) were included as the outgroup. Additionally, the probability of Bayesian analysis was incorporated into the ML tree to support the strength of each branch. The first and second numbers at the nodes display the bootstrap proportions (BP) (>50%) in ML and posterior probabilities (PP; >0.50) in Bayesian analysis, respectively. The branch lengths are proportional to the scale given.

partial 16S rRNA gene sequences. The additional Bayesian tree turned out to be quite close to the ML tree (Fig. 4). The strains of *A. pantanalense* (FBCC-A1468, FBCC-A1469) clustered with the type species *A. pantanalense* (CENA 528) and another strains of *A. pantanalense* (CENA 529, 530, 531), and are clearly identified to be the same species in the ML tree.

The strain of *A. pantanalense* FBCC-A1468 showed a 100% DNA similarity to FBCC-A1469 and 0.00% *p*-distance between them. On the other hand, the strains of *A.*

Table 1. Similarity scores (above diagonal) and % genetic *p*-distance (below diagonal) estimated by the Kimura 2-parameter model between 16 pairs of the aligned sequence data of the partial 16S rRNA gene from *Alkalinema*, *Pantanalinema*, *Plectolyngbya* and *Scytolyngbya*.

No.	Species and strain	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]
		DNA similarity (%)															
[1]	*P. rosaneae* FBCC-A1471 (MZ567050)[#]		**100**	**99.5**	**99.9**	**99.5**	**99.7**	**99.6**	**99.6**	91.3	91.3	91.2	91.3	91.3	91.3	89.6	92.8
[2]	*P. rosaneae* FBCC-A1470 (MZ567052)[#]	**0.00**		**99.5**	**99.9**	**99.5**	**99.7**	**99.6**	**99.6**	91.3	91.3	91.2	91.3	91.3	91.3	89.6	92.8
[3]	*P. rosaneae* CCIBt1046 (HM105583)	**0.00**	**0.00**		99.6	99.6	99.4	99.7	99.7	91.3	91.3	91.1	91.2	91.1	91.2	89.6	92.7
[4]	*P. rosaneae* CENA516 (KF246483)	**0.00**	**0.00**	0.00		99.5	99.7	99.7	99.7	91.2	91.2	91.1	91.3	91.2	91.3	89.6	92.8
[5]	*P. rosaneae* CENA517 (KF246484)	**0.01**	**0.01**	0.00	0.00		99.3	99.7	99.7	91.2	91.2	91	91.1	91.1	91.1	89.6	92.6
[6]	*P. rosaneae* CENA521 (KF246488)	**0.00**	**0.00**	0.01	0.00	0.01		99.5	99.5	91	91	90.9	91.1	91	91.1	89.5	92.6
[7]	*P. rosaneae* CENA537 (KF246501)	**0.00**	**0.00**	0.00	0.00	0.00	0.01		99.8	91.3	91.3	91.1	91.3	91.2	91.3	89.7	92.9
[8]	*P. rosaneae* CENA539 (KF246503)	**0.00**	**0.00**	0.00	0.00	0.00	0.01	0.00		91.3	91.3	91.1	91.3	91.2	91.3	89.7	92.8
[9]	*A. pantanalense* FBCC-A1469 (MZ567048)[#]	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09		**100**	**99.7**	**99.8**	**99.7**	**99.8**	90	90.9
[10]	*A. pantanalense* FBCC-A1468 (MZ567049)[#]	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	**0.00**		**99.7**	**99.8**	**99.7**	**99.8**	90	90.9
[11]	*A. pantanalense* CENA528 (KF246494)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	**0.00**	**0.00**		99.8	99.7	99.8	89.8	90.7
[12]	*A. pantanalense* CENA529 (KF246495)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	**0.00**	**0.00**	0.00		99.9	100	90	90.8
[13]	*A. pantanalense* CENA530 (KF246496)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	**0.00**	**0.00**	0.00	0.00		99.9	89.9	90.8
[14]	*A. pantanalense* CENA531 (KF246497)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	**0.00**	**0.00**	0.00	0.00	0.00		90	90.8
[15]	*Pl. hodgsonii* ANT.LPR2.2 (AY493583)	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.10	0.10	0.11	0.10	0.11	0.10		89.6
[16]	*S. timoleontis* XSP1 (KP688588)	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.09	0.09	0.10	0.09	0.10	0.09	0.11	
		p-distance (%)															

[#]Sequences determined in this study. Bolds represent more than 99.5−100% similarity and less than 0.00% of genetic distance.

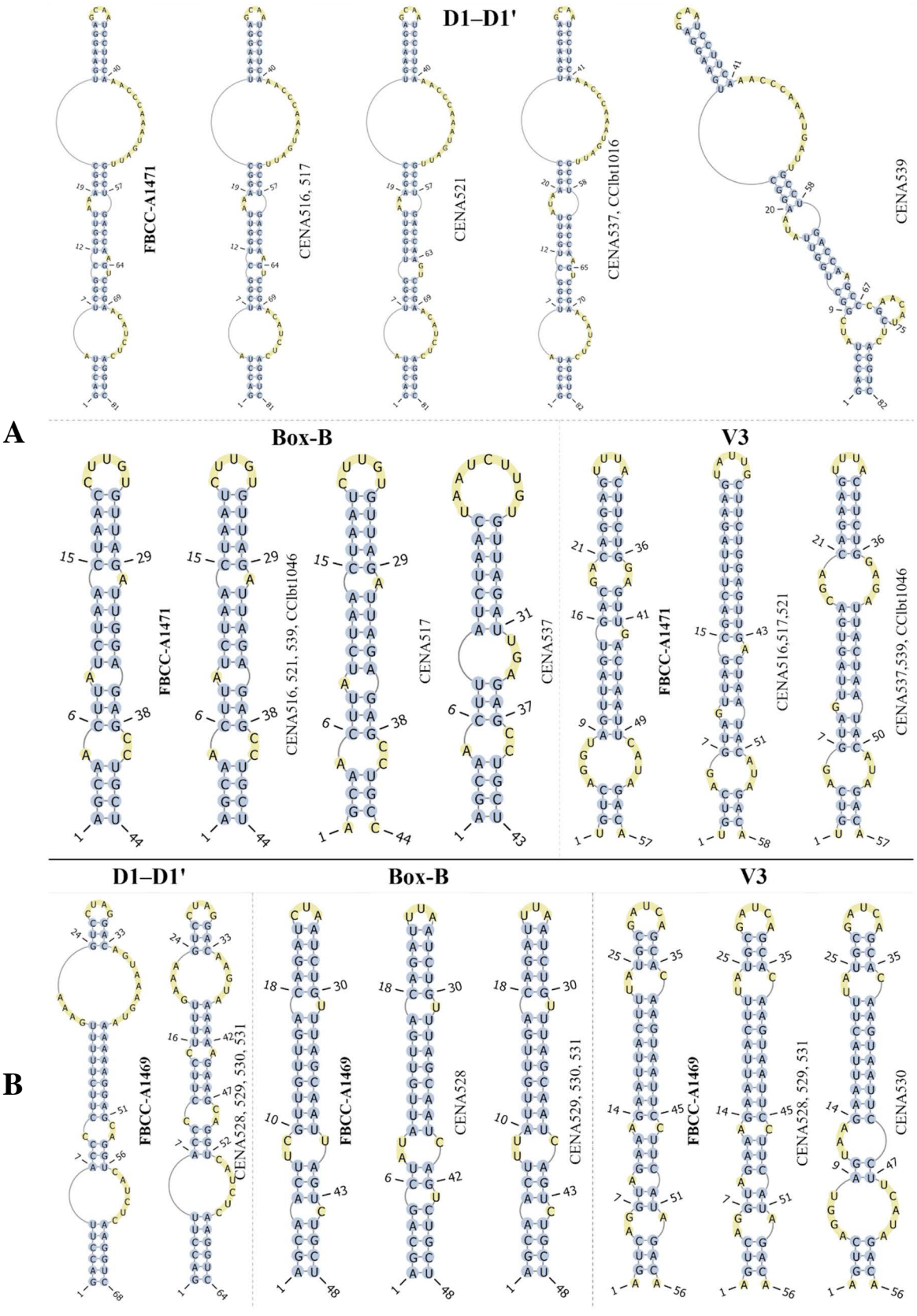

Fig. 5. Folded 16S−23S rRNA ITS secondary structures of strains of (A) *Pantanalinema rosaneae* and (B) *Alkalinema pantanalense*.

pantanalense (FBCC-A1468, FBCC-A1469) showed a 99.5 to 99.9% DNA similarity to type species *A. pantanalense* (CENA 528) and 0.00% *p*-distance between them (Table 1).

Characterization of 16S–23S ITS regions of *Alkalinema pantanalense*

The secondary structures were predicted with the Mfold server for D1–D1′ and Box-B helices (Fig. 5, B). A single secondary D1–D1′ helical structure of the *Alkalinema pantanalense* was reported. The secondary D1–D1′ helical structure of the strain of *A. pantanalense* (FBCC-A1469) composed of four loops and 68 bases. On the other hand, already-reported secondary D1–D1′ helical structure of the *A. pantanalense* (CENA 528–531) five loops and 64 bases.

In comparison with the Box-B helical structure of *A. pantanalense*, the strain of *A. pantanalense* (FBCC-A1469) has the same four loops and 48 bases but showed a slight difference in the DNA sequence inside the loop. The strain of the *A. pantanalense* (FBCC-A1469) formed the same five loops and a composition of 56 bases as *A. pantanalense* (CENA 528, 529, 531) which was reported in the V3 helix. However, the strain of *A. pantanalense* (FBCC-A1469) revealed a different structure in the D1–D1′ helix and the same secondary structure in the Box-B helix from/as what had already been reported. In spite of the difference in interior DNA sequences, the strain of *A. pantanalense* (FBCC-A1469) showed the same structure in the previously reported V3 helix.

Besides, the strains of *A. pantanalense* (FBCC-A1468, FBCC-A1469) illustrated a similar ring form to the ITS secondary structure of *A. pantanalense* (CENA 528–531) (Fig. 5, B).

Discussion

Because the genera *Pantanalinema* and *Alkalinema* were *Leptolyngbya* morphotypes, they were identified based upon the ecological and genetic traits rather than the morphological one (Vaz *et al*., 2015). What's more, it has been known that the genus *Leptolyngbya* is polyphyletic, and that the taxonomic re-evaluation should be performed (Casamatta *et al*., 2005; Zammit *et al*., 2012).

The genera *Pantanalinema* and *Alkalinema* are morphologically similar to each other; however, trichomes of the genus *Alkalinema* are interwoven, making the arrangement pattern different (Vaz *et al*., 2015). The genus *Pantanalinema* was isolated from the saline-alkaline wetland lake in Pantanal, Brazil. On the other hand, the genus *Alkalinema* was obtained from alkaline soil in Mexico (Vaz *et al*., 2015). The *Pantanalinema* strains collected from soil in Korea was subaerial. The *Alkalinema* strains were epilithic, living attached to the gravel of a freshwater stream in Korea. Hence, these two genera revealed a remarkable difference from the initially reported habitats. Cyanobacteria generally are alkaliphilic and dominant in the alkaline lake (Dadheech *et al*., 2012); however, they can survive in the acidic condition as well (Steinberg *et al*., 1998). Therefore, ecological features, like morphological traits, do not significantly contribute to distinguishing these two species. Thus, *P. rosaneae* and *A. pantanalense* are cosmopolitan species distributed in the freshwater and in the aerial condition of the temperate climate, also surviving in the saline-alkaline water of the tropical rainforest.

The 16S rRNA gene is mostly selected as a taxonomic marker, and genetic similarities of the genes are an important criterion for differentiating species. In the phylogenetic analysis using the 16S rRNA gene sequences, the strains of *Pantanalinema* and *Alkalinema* in this study are separated from *Leptolyngbya sensu stricto*, forming an independent cluster along with previously reported *Pantanalinema* and *Alkalinema* strains (Fig. 4). In this study, the DNA similarity of the *Pantanalinema* (FBCC-A1471) showed 99.5–99.9% to the genus *Pantanalinema* (CENA 516, 517, 521, 537, 539); likewise, the genus *Alkalinema* (FBCC-A1469) showed 99.7–99.8% to genus *Alkalinema* (CENA 528–531) (Table 1). According to Kim *et al*. (2014), a similarity of more than 98.5% might be considered a same species when compared to already-known species. Present study revealed that the 16S rRNA of *P. rosaneae* (FBCC-A1471) and *A. pantanalense* (FBCC-A1469) were similar with the type strains of two species, of which scores were calculated to 99.9% and 99.7% similarity in the type species respectively (Table 1). In the comparison of the secondary structure of the ITS region (D1–D1′ helix, Box B), *P. rosaneae* FBCC-A1471 showed genetic similarity to the type species of *P. rosaneae* (CENA 516). However, V3 helix comparison showed more structural diversity among *P. rosaneae* strains. In *A. pantanalense*, FBCC-A1469 and a type species CENA 528, were similar in Box-B and V3, but showed more diversity in D1–D1′ helix. As above, the diversity of ITS secondary structure between strains within the same species was also found in *Pantanalinema* and *Alkalinema* strains (Vaz *et al*., 2015), *Wilmottia murrayi* strains (Lee *et al*., 2020), and *Cephalothrix komarekiana* strains (Malone *et al*., 2015) as well. This means that ITS sequence can be used as an auxiliary tool in species-level studies.

Therefore, our phylogenetic results confirmed that Korean strains of this study belonged to *P. rosaneae* and *A. pantanalense* in the family Leptolyngbyaceae within the order Synechococcales (Fig. 4).

From the above results, two species were added to the cyanobacteria flora and it is expected that more species

can be revealed by isolating cyanobacteria from more habitats in the future.

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