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First record of *Dolichospermum mucosum* (Nostocales, Cyanobacteria) in Korea: A study from the Han River

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The heterocytous cyanobacterium *Dolichospermum mucosum* was observed in Cheongpyeong Lake, located in the mid-to-downstream region of the North Han River. This species is morphologically characterized by irregularly and circularly coiled trichomes, which are consistently surrounded by a transparent mucilaginous envelope at a distance of 19.8 ± 11.3 μm. Akinetes were solitary, nearly spherical, and positioned at a distance from the heterocysts, with a width-to-length ratio of 1.1 ± 0.2. In phylogenetic analyses based on the 16S rRNA and *rbcLX* genes, this strain clustered within the same clade as previously reported *D. mucosum*. However, in the phylogenetic analysis, it grouped mostly closely with *D. smithii*, which exhibited the highest nucleotide sequence similarity. Structural variations in Box-B and V3 helices enabled clear differentiation between *D. mucosum* and *D. smithii*. Based on these morphological and phylogenetic characteristics, the *Dolichospermum* strain was identified as *D. mucosum*, marking its first recorded occurrence in the Republic of Korea.

Keywords: *Dolichospermum*, heterocytous cyanobacteria, morphological and phylogenetic analysis, secondary structure, unrecorded species

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Introduction

Cyanobacteria are primary producers in freshwater ecosystems and play vital roles in ecosystem dynamics (Tomitani *et al*., 2006; Bae *et al*., 2020). Heterocytous cyanobacteria such as the genus *Dolichospermum* contribute to the nitrogen cycle by fixing atmospheric nitrogen within specialized cells called heterocysts (Paerl *et al*., 2001; Deng *et al*., 2024). Species of the genus *Dolichospermum* inhabit lakes and rivers and are among the key cyanobacterial taxa responsible for harmful algal blooms (HABs) (Chia *et al*., 2018; Song *et al*., 2022). Depending on their ability to produce cyanotoxins, *Dolichospermum* species can be either toxic or non-toxic (Li *et al*., 2016; Zhang *et al*., 2023). Therefore, investigating species diversity within this genus is essential for monitoring algal blooms and managing water quality in freshwater ecosystems.

This genus was historically known as *Anabaena* (Bornet and Flahault, 1888). However, a molecular phylogenetic study in 2009 revealed that benthic and planktonic species formed distinct clades in the 16S rRNA phylogenetic tree owing to genetic divergence. Consequently, planktonic species possessing gas vesicles were reclassified into the newly designated genus *Dolichospermum* (Wacklin *et al*., 2009).

Traditionally, *Dolichospermum* species have been identi-

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fied based on their morphological characteristics and phylogenetic analyses. However, relying solely on morphology can lead to misidentification owing to several factors. First, morphological variations may arise owing to long-term cultivation or environmental changes (Rippka *et al*., 1979; Watanabe *et al*., 2004; Wang *et al*., 2024). Second, the formation rate of the akinetes was low when the samples were collected from the field. Therefore, isolating and culturing a single strain for a certain period is essential for accurate species identification. However, if the akinetes remain immature after prolonged incubation, the morphological characteristics necessary for species identification may not be fully understood (Choi *et al*., 2018).

Therefore, to overcome the limitations of species identification based solely on morphological characteristics, molecular markers, such as the housekeeping gene 16S rRNA (Zapomelova *et al*., 2008), the *rbcLX* gene associated with photosynthetic function (Tuji and Niiyama, 2010; Singh *et al*., 2015), and the Internal Transcribed Spacer (ITS) region, which exhibits high intraspecific variation, are widely utilized for cyanobacterial identification s (Li and Brand, 2007).

Currently, 61 *Dolichospermum* species are registered in the international algal database AlgaeBase (https://www.algaebase.org). The Korean algal database maintained by the National Institute of Biological Resources (https://species.nibr.go.kr) records 12 species (*Dolichospermum affine*, *D. circinale*, *D. crassum*, *D. flos-aquae*, *D. hangangense*, *D. helicoideum*, *D. mendotae*, *D. planctonicum*, *D. sigmoideum*, *D. smithii*, *D. spiroides*, and *D. viguieri*), representing 19.7% of the globally documented species.

In this study, we aimed to identify and characterize a *Dolichospermum* strain newly discovered in the Han River system using morphological observations and molecular phylogenetic analyses.

Materials and Methods

Sampling site and sampling method

Water samples were collected from Cheongpyeong Lake (37°42′31.42″N, 127°27′07.30″E) in the downstream of the North Han River in July 2013. Plankton nets with mesh diameters of 20 μm were used to collect phytoplankton (Wehr and Descy, 1998). The collected samples were sealed in the field and transported immediately to the laboratory.

Cultivation

Single trichomes were isolated from the samples using a Pasteur pipette under an IX73 inverted microscope (Olympus, Tokyo, Japan), and the isolated cells were grown in 1 mL of CB medium (pH 9.0) (Ichimura and Itoh, 1977) and incubated in 48-well plates. After culturing for 2–3 weeks, the cell density increased, and it was confirmed under a microscope that there was no contamination and only a single type of cell existed. The culture was then transferred to a filter-cap type 25 cm^2 cell culture flask (SPL, cat. no. 70025). The culture was maintained under constant culture conditions (CB medium, 20°C, 45 μmol photons m^{-2} s^{-1} light intensity, 12 : 12h light : dark cycle). The strain has been maintained in culture for 12 years through monthly subculturing. The single culture obtained was deposited in the Culture Collection of the Korea Research Institute of Bioscience and Biotechnology (KRIBB). The culture was assigned the resource number (KCTC-AG60755).

Morphological characteristics

Morphological characteristics of *D. mucosum* were evaluated using a fresh strain. For morphological observation of this species, a BX51 upright microscope (Olympus, Tokyo, Japan) was used. For species identification based on morphological characteristics, *The Subwasserflora Von Mitteleuropa, Bd. 19/3, Cyanoprokaryota - 3. Teil /3rd Part: Heterocytous Genera* (J. Komárek, Springer) was used as a reference. Cell dimensions were measured using the Cell sens program v.1.8 (Olympus, Tokyo, Japan). The following morphological characteristics were determined for at least 30 cells: long and wide vegetative cells, heterocysts, akinetes, and distance between trichomes and the mucilaginous sheath.

DNA extraction

The biomass of the strain was harvested during the exponential growth stage. Genomic DNA was extracted from the samples using the Chargeswitch™ Forensic DNA Purification Kit (Invitrogen, Thermo Fisher Scientific).

PCR and sequencing

The polymerase chain reaction (PCR) mixture (total volume 20 μL) consisted of 11.9 μL sterile water, 2 μL 10× Taq buffer, 2 μL dNTP, 0.1 μL Ex Taq of the Takara Ex Taq PCR kit (Takara Shuzo, Osaka, Japan), 1 μL of each primer (0.01 mM), and 2 μL template DNA. PCR was performed on an i-cycler (Bio-Rad, Hercules, CA, USA) using 16S rRNA primers CYA108F (5′-ACG GGT GAG TAA CRC GTR A-3′) (Nübel *et al*., 1997) and 16S CYR (5′-CTT CAY GYA GGC GAG TTG CAG C-3′) (Urbach *et al*., 1992) and amplified using the following steps: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, and a final extension step of 72°C for 5 min. PCR amplification of *rbcLX* was performed with primers CW (5′-CGT AHC TTC CGG TGG TAT CCA CG T-3′) and CX (5′-GGG GCA GGT AAG AAA GGG TTT CGT A-3′) (Rudi *et al*.,

1998). In addition, amplification of *rbcLX* was performed in two steps as follows: initial denaturation of the first step at 94°C for 4 min, two cycles of 94°C for 30 s, 40°C for 30 s, 72°C for 2 min, followed by initial denaturation of the second step at 94°C for 30 s, followed by 38 cycles of 55°C for 30 s, 72°C for 2 min, and a final extension reaction at 72°C for 7 min. Additionally, PCR amplification of 16S-23S ITS was performed with primers 322 (5′-TGT ACA CAC CGC CCG TC-3′) and 340 (5′-CTC TGT GTG CCT AGG TAT CC-3′) (Laloui *et al*., 2002). Amplification of 16S-23S ITS was performed in two steps as follows: initial denaturation of the first step at 94°C for 10 min, 38 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension reaction at 72°C for 7 min.

Phylogenetic and secondary structures analysis

Phylogenetic analyses were performed using the sequences of our strain and other sequences of the same genus retrieved from a public database. They were aligned using the BioEdit program v.7.1.3 (Hall, 1999). The alignment was edited manually. We determined the partial sequences of the 16S rRNA (1,250 bp) and *rbcLX* (747 bp). The sequences were deposited in GenBank (http://www. ncbi.nlm.nih.gov) with the accession numbers (16S rRNA: MN207474, *rbcLX*: MN205557). The topology of the phylogenetic tree was derived using the maximum-likelihood (ML) method. The GTR + G + I (the General Time Reversible model incorporating variant sites and a gamma distribution) model was selected for ML analyses as determined by ModelTest in MEGA 12.0 (Kumar *et al*., 2024). MEGA software (version 12.0) was used for ML and neighbor-joining phylogenetic analyses using *p*-distance models. All analyses were performed using bootstrapping with 1,000 replicates. Box-B and V3 helices of the ITS were edited using the BioEdit program v.7.1.3 (Hall, 1999). The secondary structures were constructed using Mfold 3.1 (Zuker, 2003).

Results and Discussion

Morphological characterization of *D. mucosum* KCTC-AG60755

Family: Aphanizomenonaceae
Genus: *Dolichospermum*
Species: *Dolichospermum mucosum*

Basionym: *Anabaena mucosa* Komárková-Legnerová & Eloranta.

Synonyms: *Anabaena spiroides* f. *ukrainica* (Schkorbatow) Elenkin 1938; *Anabaena ucrainica* (Schkorbatow) M.Watanabe 1996; *Dolichospermum ucrainicum* (Schkorbatow) Tuji & Niiyama 2012.

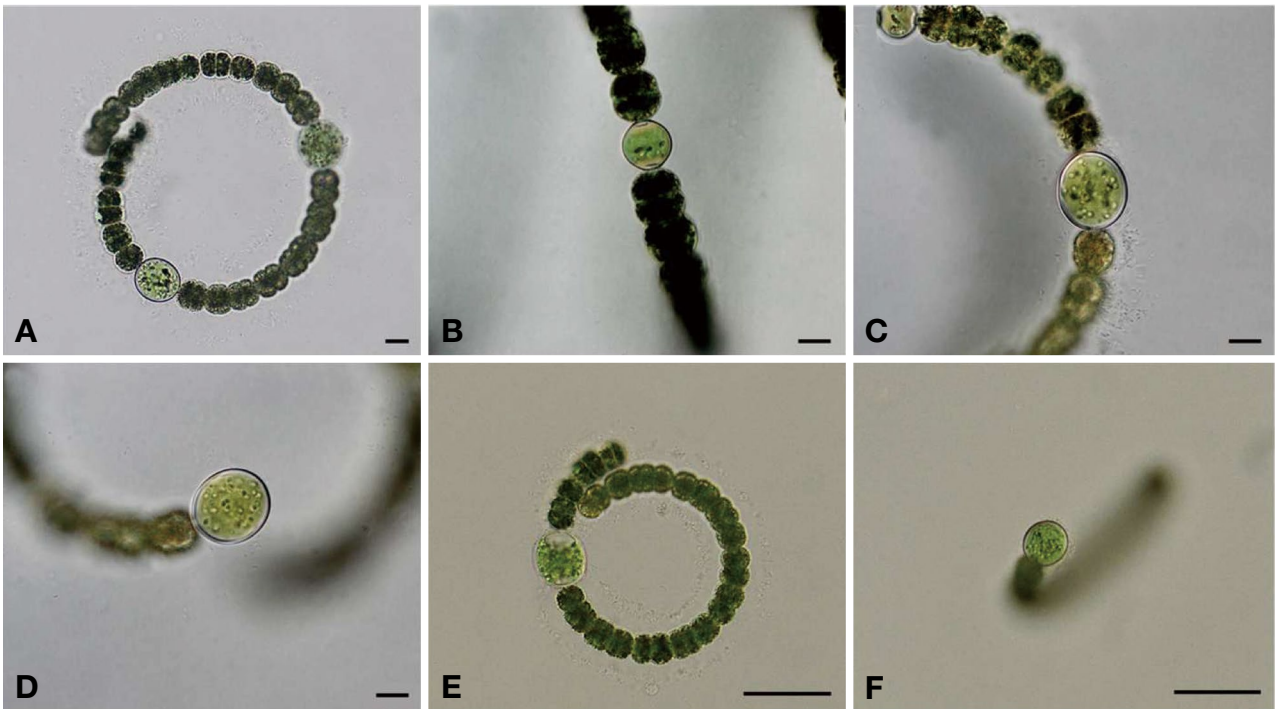


Fig. 1. Morphological characteristics of *Dolichospermum mucosum* (KCTCAG60755) isolated from the Han River. (A) Trichome. Transparent mucilaginous sheath was formed surrounding trichome. (B) Heterocyst. (C) Ripen akinete. (D) Akinete formed at the end of the trichome. (E) Trichome morphology of *D. mucosum* photographed in 2013. (F) Akinete formed at the end of the trichome, photographed in 2013. Scale bars = 10 μm.

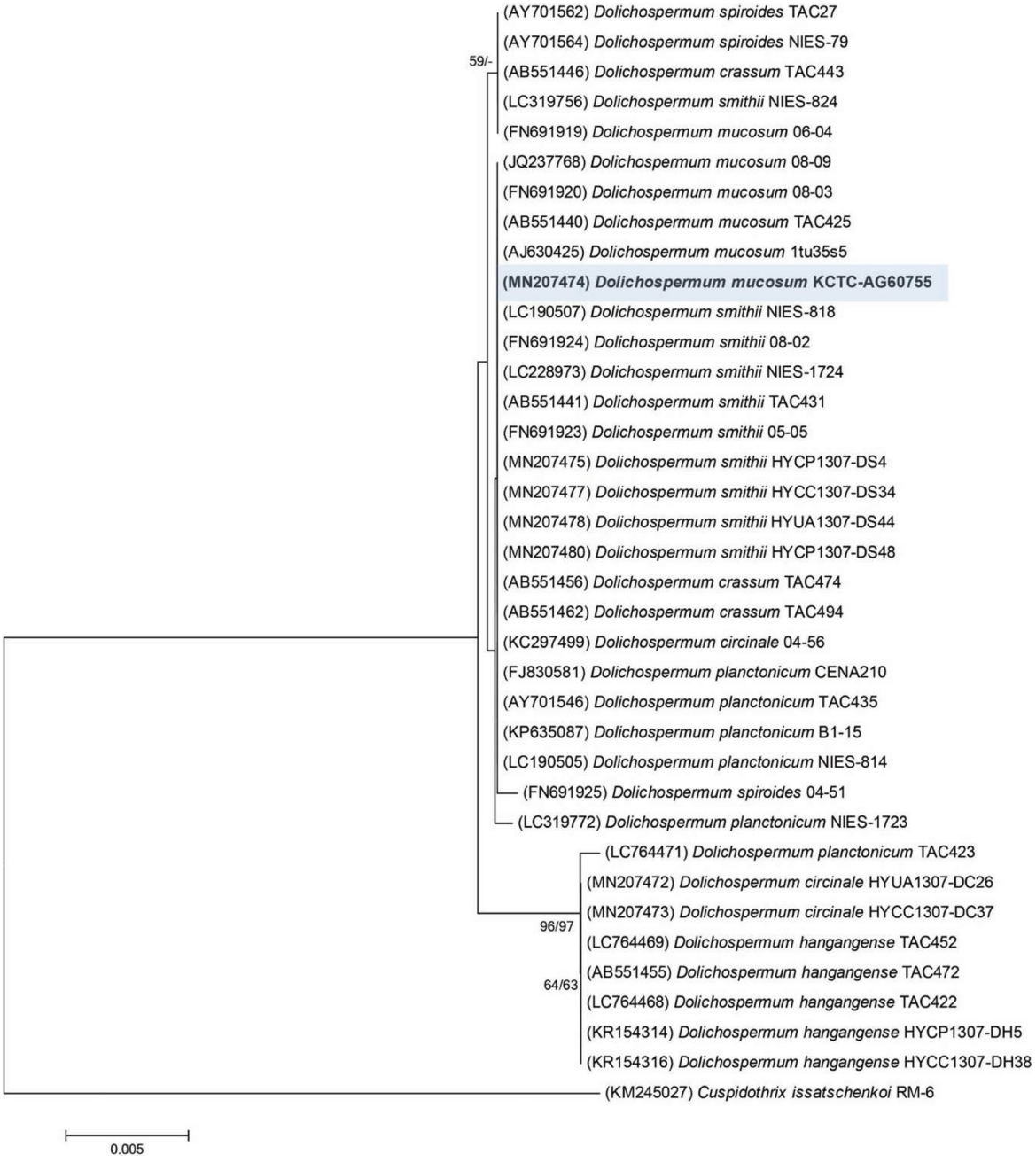


Fig. 2. Neighbor joining based on 16S rRNA (1,250 bp) sequences showing the clustering of cultured *Dolichospermum* spp. strains. Numbers above branches indicate bootstrap support (>50%) from neighbor joining/maximum likelihood. *D. mucosum* has skyblue background. *Cuspidothrix issatchenkoi* RM-6 was used as the out group.

Trichomes were free-floating and solitary, exhibiting an irregular, circularly coiled form that occasionally appears loosely coiled. A transparent mucilaginous envelope consistently surrounded and maintained a uniform distance from the trichome. The distance between the trichome and the mucilaginous sheath ranges from 9.9 to 25.9 μm (mean ± SD: 19.8 ± 11.3 μm). The trichomes were not tapered at their ends. Vegetative cells were spherical to barrel-shaped, dark blue-green, and contained numerous aerotopes, measuring 9.5–17.3 μm in length (12.5 ± 5.5 μm) and 4.3–11.1 μm in width (7.2 ± 4.8 μm) (Fig. 1A). Heterocysts were solitary, spherical, and exhibited a greenish hue, resembling hollow beads. They measure 9.6–13.7 μm in length (11.7 ± 2.9 μm) and 9.4–14.2 μm in width (11.4 ± 3.4 μm) (Fig. 1B). Akinetes were solitary, intercalary, and positioned at a distance from heterocysts. They

Table 1. Sequence similarity and *p*-distance between 5 closely related species and *D. mucosum* of the partial 16S rRNA gene sequences.

Species names of relatives	Strain names	Accession no.	Sequence similarity (%)	*p*-distance (%)
Dolichospermum smithii	HYCC1307-DS34	MN207477	100	0.0000
	HYUA1307-DS44	MN207478	100	0.0000
	HYCP1307-DS48	MN207480	100	0.0000
	HYCP1307-DS4	MN207475	99.92	0.0008
	05-05	FN691923	99.04	0.0096
	08-02	FN691924	98.96	0.0104
	TAC431/NIES-820	AB551441	98.96	0.0104
	NIES-818	LC190507	98.96	0.0104
	NIES-1724	LC228973	98.96	0.0104
Dolichospermum crassum	TAC474/NIES-1657	AB551456	99.04	0.0096
	TAC494/NIES-1665	AB551462	99.04	0.0096
Dolichospermum mucosum	08-03	FN691920	98.96	0.0104
	08-09	JQ237768	98.96	0.0104
	TAC425/NIES-1677	AB551440	98.96	0.0104
	1tu35s5	AJ630425	98.96	0.0104
Dolichospermum circinale	04-56	KC297499	98.96	0.0104
Dolichospermum planctonicum	NIES-814	LC190505	99.04	0.0096
	CENA210	FJ830581	98.96	0.0104
	B1-15	KP635087	98.88	0.0112

were nearly spherical, measuring 18.2−26.3 μm in length (21.5 ± 5.7 μm) and 19.5−30.4 μm in width (23.2 ± 7.7 μm). The width-to-length ratio of akinetes ranged from 1.0 to 1.2 (1.1 ± 0.2) (Fig. 1C). Akinetes were frequently formed at the ends of trichomes (Fig. 1D).

A distinguishing morphological characteristic of *D. mucosum* is the presence of spherical akinetes and a transparent mucilaginous sheath surrounding the trichome. However, this study identified a previously unreported feature: the mucilaginous sheath maintains a consistent distance from the trichomes. To quantify this trait, the distance between the trichome and the mucilaginous sheath was measured in more than 30 trichomes, and the range, mean value, and standard deviation were recorded. This characteristic is also depicted in an illustration of *D. mucosum* on page 712 of *Süßwasserflora von Mitteleuropa, Bd. 19/3: Cyanoprokaryota* (2013). Therefore, the distance between the trichome and the mucilaginous sheath can be considered a species-specific morphological feature of *D. mucosum*.

Long-term cultivation had no effect on the morphological characteristics of *D. mucosum*, including the coiled and circular trichome structure, wide gap between the trichome and mucilaginous sheath, spherical akinetes, and occasional formation of akinetes at the ends of the trichome (Fig. 1E, F). This finding indicated that the key morphological traits of *D. mucosum* were stable over time.

Molecular phylogeny of *D. mucosum*

The cultured strain of the cyanobacterium *D. mucosum* isolated in this study was registered in the NCBI database with the nucleotide sequences of the 16S rRNA and *rbcLX* regions (accession numbers: 16S rRNA, MN207474; *rbcLX*, MN205557). Phylogenetic analysis of the 16S rRNA gene showed that *D. mucosum* (KCTC-AG60755) formed a clade with four *D. mucosum*, nine *D. smithii*, two *D. crassum*, one *D. circinale*, and four *D. planctonicum* strains from the Korean and international culture collections (Fig. 2).

The nucleotide sequence similarity between *D. mucosum* and its closely related species ranged from 98.88% to 100%, with a *p*-distance of 0−0.0112%. These five species shared a minimum sequence similarity of 98.88%, making species-level identification challenging (Table 1). Thus, the 16S rRNA region is known to have low resolution for distinguishing species, particularly in heterocytous cyanobacteria (Wacklin *et al*., 2009; Zapomelova *et al*., 2009; Ki, 2010; Lee *et al*., 2020).

To address this limitation, a phylogenetic analysis of the *rbcLX* gene was conducted. *Dolichospermum mucosum* (KCTC-AG60755) formed a clade with one *D. mucosum* strain and four Korean *D. smithii* strains. Although phylogenetic analysis of the *rbcLX* region provided a higher resolution for species identification than that of the 16S

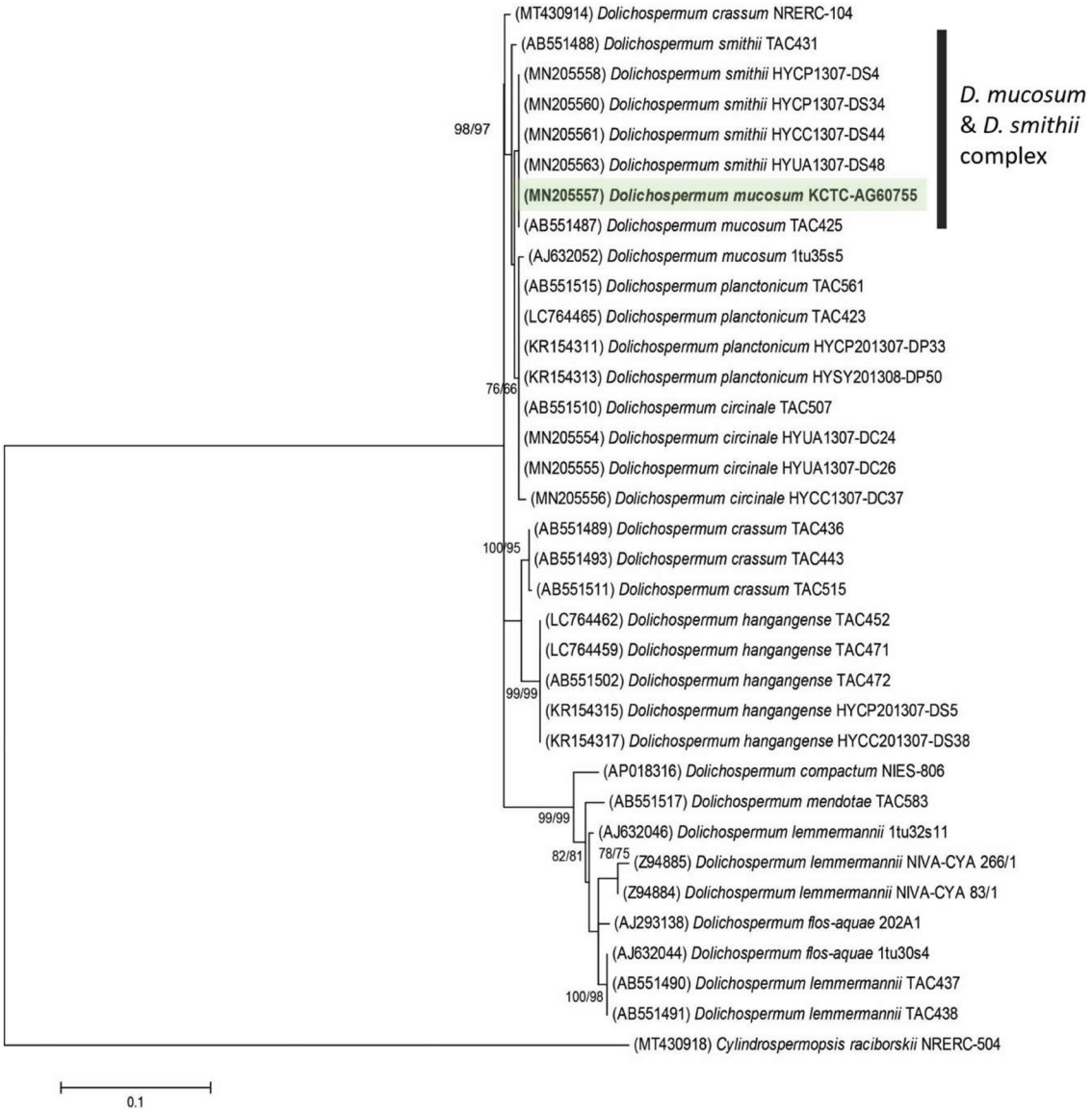


Fig. 3. Neighbor joining based on *rbcLX* (747 bp) sequence showing the clustering of cultured *Dolichospermum* spp. strains. Numbers above branches indicate bootstrap support (>50%) from neighbor joining/maximum likelihood. *D. mucosum* has green background. *Cylindrospermopsis raciborskii* NRERC-504 was used as the out group.

rRNA region, it still exhibited limitations in differentiating *D. mucosum* from *D. smithii* (Fig. 3).

The *rbcLX* gene encodes ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), a key enzyme involved in carbon fixation that plays a crucial role not only in cyanobacteria but also in various photosynthetic organisms. This gene exhibits high resolution for detecting subtle differences at the species level and provides reliable phylogenetic insights, particularly for heterocytous cyanobacteria (Gugger *et al*., 2002; Tuji *et al*., 2023).

Secondary structure

To differentiate *D. mucosum* from the closely related *D. smithii*, which could not be resolved through phylogenetic analysis, secondary structure analysis was performed on the Box-B and V3 helix regions within the 16S-23S ITS gene.

Both *D. smithii* and *D. mucosum* possessed three loops and a nucleotide sequence consisting of 48 bases in the Box-B helix. However, differences in loop size and nucleotide composition distinguish these species. The nucleotide sequence of the first loop differed between *D. mucosum* and *D. smithii* (AUAAAAU vs. UAUGC), whereas that of the second loop varied (CGACUAC vs. GUUAACGCA CCU) (Fig. 4A).

In the V3 region, the secondary structure of *D. smithii*

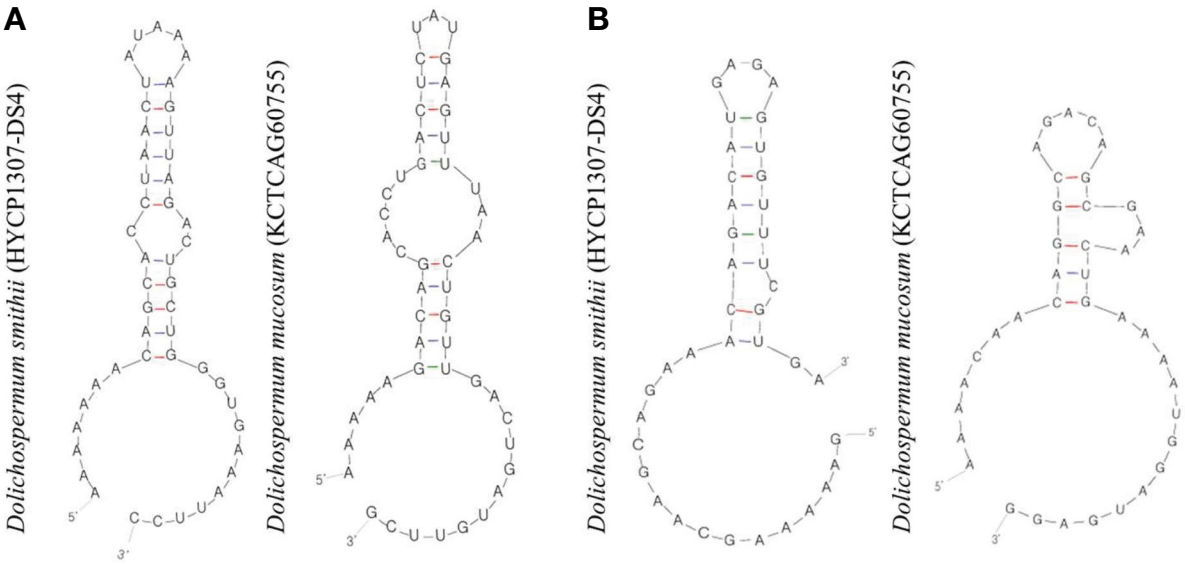


Fig. 4. Comparison of *Dolichospermum mucosum* and its closely related species *D. smithii* based on the secondary structures of (A) the Box-B helix and (B) the V3 helix within the 16S-23S ITS gene.

consisted of three loops with 39 bases. Similarly, *D. mucosum* possessed three loops; however, its total nucleotide count was 38 bases. Additionally, the size and shape of the loops differed significantly, allowing for a clear differentiation between the two species (Fig. 4B). The 16S-23S ITS gene is a non-coding region with high genetic variability among species, making it a valuable marker for species-level classification and for distinguishing closely related taxa (Johansen *et al*., 2011; Vaccarino and Johansen, 2012). Its secondary structure, which is composed of helices, loops, and stems, is species-specific and evolutionarily conserved, enhancing the reliability of species identification (Iteman *et al*., 2000; Kim *et al*., 2021). In addition, the Box-B and V3 helices are the most variable regions within heterocytous cyanobacteria and are widely used as molecular markers for species- and intraspecies-level identification (Li and Brand, 2007; Ni *et al*., 2008; Stanojkovic *et al*., 2022).

This is the first report of a previously unrecorded species, *D. mucosum* in Korea, with species identification based on an integrative approach combining morphological and molecular phylogenetic analyses. The findings of this study are expected to serve as valuable reference data for future studies on the identification and classification of heterocytous cyanobacteria.

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Conflicts of Interest

The authors of this paper have no affiliation with any interests and are solely responsible for the paper.

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