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New records of the genus *Cyanobium* and *Cyanobium gracile* (Synechococcales, Cyanophyceae) in Korean freshwater

Dae Ryul Kwon†, Bok Yeon Jo†, Seok Won Jang, Chang Soo Lee and Seung Won Nam*

Nakdonggang National Institute of Biological Resources, Sangju 37242, Republic of Korea

***Corresponding author**
Seung Won Nam
Tel. 054-530-0843
E-mail. seungwon1007@gmail.com

†These authors contributed equally to this work

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Abstract: *Cyanobium* is a genus of picoprokaryotic cyanophytes, which includes species worldwide. The present study investigated the morphology, ultrastructure, and molecular phylogeny of the unrecorded genus *Cyanobium* Rippka & Cohen-Bazire 1983 and species *Cyanobium gracile* Rippka & Cohen-Bazire 1983. A *C. gracile* culture from a freshwater sample collected from the Adongji pond was established by single-cell isolation. Morphological data were analyzed using light and transmission electron microscopy. *C. gracile* lives as solitary cells without gelatinous envelopes and is ovate, oval, or shortly rod-shaped. Thylakoids are laid along the cell walls, with three thylakoid membranes parallel to each other. Nucleoplasm was observed in the center of the cell. Molecular phylogeny performed with data from 16S small subunit ribosomal DNA gene (SSU rDNA) sequences showed that the three strains of *C. gracile*, including the type strain (PCC6307) and a newly recorded strain (Adong101619), formed a distinct clade with a high supporting value (maximum-likelihood = 100, pp = 1.00). Based on morphology and molecular data, we report the newly recorded *C. gracile* in Korea.

Keywords: cyanobacteria, *Cyanobium*, phylogeny, ultrastructure, unrecorded

INTRODUCTION

Cyanobacteria are widely distributed worldwide and a major causative species of algae bloom in freshwater ecosystems (Robarts and Zohary 1987; Yunes *et al.* 2003; Havens 2008; You *et al.* 2013). This algae blooming caused by cyanobacteria induces visual disturbance (Barnett 1984). Some species produce off-flavor compounds, such as geosmin and 2-MIB (Kim *et al.* 2015), and liver or neurotoxin substances, such as microcystin and anatoxin-a (Suffet *et al.* 1995; Zander and Pingert 1997). Taxonomic studies on cyanobacteria have emerged due to the harmful effects of these cyanobacteria on the environment (Komárek 2006; Ryu *et al.* 2018). Taxonomic studies on cyanobacteria have been mainly based on morphological characteristics (Pfannkuche and Lochte 1993; Choi *et al.* 1998). However, these methods still suffer from light microscopy given their morphological changes in response to various environmental conditions and insufficient taxonomic characteristics (Lehtimäki *et al.* 2000; Gugger *et al.* 2002; Willame *et al.* 2006). Recently, molecular phylogenetic data and ultrastructural and morphological characteristics have been used together to describe cyanobacterial species (Komárek and Anagnostidis 2005; Komárek 2016).

In Korea, studies on cyanobacteria focus on taxa with high environmental impacts, such as the genus *Microcystis*, which produces toxins, and the genus *Anabaena*, which generates odorants and neurotoxins. Additionally, only 377 cyanobacterial species have been reported (NIBR 2019), which is less than 10% of the number of cyanobacteria reported worldwide (4,707 species, Guiry and Guiry 2020). Thus, studies on the diversity of cyanobacteria species in

Korea are quite insufficient (Ryu *et al.* 2018).

The genus *Cyanobium* is an oval-shaped or short rod-shaped unicellular cyanobacteria (Rippka and Cohen-Bazire 1983; Komárek *et al.* 1999). This genus is often considered morphologically similar to the genus *Synechococcus* (Nägeli 1849; Padisák *et al.* 1997). However, a difference is noted between the two genera in terms of DNA base composition. The average GC content of *Cyanobium* is 66–71 moles%, whereas that of *Synechococcus* is 48–56 moles% (Rippka and Cohen-Bazire 1983).

The genus *Cyanobium* is an important primary producer in oligotrophic and mesotrophic environments (Jezberová and Komárková 2007). However, only 14 species have been reported worldwide (Guiry and Guiry 2020) due to the absence of distinct morphological characteristics and the low population density (Komárek *et al.* 1999). Additionally, this genus and species have not yet been reported in Korea.

In this study, morphological and ultrastructural studies and phylogenetic analysis were performed to report the unrecorded genus *Cyanobium* and the unrecorded species *Cyanobium gracile* in Korea.

MATERIALS AND METHODS

1. Sampling and clonal culture of *Cyanobium gracile*

Cultures of *C. gracile* were established by single-cell isolation from freshwater samples collected at Adongji pond, Korea (35°58′47.4″N, 126°46′14.6″E) in October 2019. The cultures were grown in BG11 medium at 25°C under a 14 : 10 light : dark cycle and a light intensity of 4,000 lux provided by cool-white fluorescent lamps.

2. Light microscopy

Living *C. gracile* cells were studied using a Nikon ECLIPSE Ni-U (Nikon, Japan) equipped with differential interference contrast optics. Images were captured using a digital camera (DS-Ri2, Nikon).

3. Transmission electron microscopy

For transmission electron microscopy, the cells were prefixed in a 1 : 1 mixture of 5% (V/V) glutaraldehyde and BG11 culture media for 1 h at 4°C. The glutaraldehyde-fixed cells were washed 3 times in BG11 culture media and postfixed in 1% (W/V) OsO_4 for 1 h at 4°C. The fixed cells were rinsed three times with distilled water. Dehydration was carried out at 4°C using a graded ethanol series of 50, 60, 70, 80, and 90% for 10 min each and three 10 min changes of pure ethanol. Pellets were then brought to room temperature and transferred through propylene oxide two times for 20 min each with 50% and 75% Spurr's embedding resin (Spurr 1969) in propylene oxide for 1 h each and 100% overnight. On the following day, pellets were moved to new pure resin and polymerized at 70°C. Blocks were thin-sectioned on a PT-X ultramicrotome (RMC Products, Boeckeler Instruments, Tucson, AZ). Sections of 70 nm thickness were collected on slot copper grids, stained with 3% (w/v) uranyl acetate and Reynold's lead citrate (Reynolds 1963), and observed using a JEM-1400 Plus at Korea Basic Science Institute (KBSI) operated at 120 kV (JEOL, Tokyo, Japan).

4. DNA extraction, amplification, and sequencing

Approximately 10 mL aliquots of culture media were obtained in the exponential growth phase. Cells were harvested by centrifugation (1,330 × g, model 5415; Eppendorf, Hamburg, Germany) for 1 min at room temperature followed by washing three times with sterilized distilled water. According to the manufacturer's protocol, total genomic DNA was extracted from the pellet using InstaGenetm Matrix (BIO-RAD, CA, USA). Polymerase chain reaction (PCR) was performed using 27F/1492R universal primers to amplify 16S SSU rDNA (Edwards *et al.* 1989). PCR amplification was performed with a total volume of 30 μL containing EF-Taq (SolGent, Daejeon, Korea), each dNTP, 10·Ex Taq Buffer, each primer, and 20 ng of template DNA. The 16S SSU rDNA gene was amplified using a DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD, CA, USA) with the following conditions: initial denaturation at 95°C for 2 min; 35 cycles each of 95°C for 2 min, 55°C for 1 min, and 72°C for 1 min; final extension at 72°C for 10 min; and holding at 4°C. According to the manufacturer's protocol, the PCR products were purified using a Multiscreen filter plate (Millipore Corp., MA, USA). The purified template was sequenced with PRISM BigDyeTM Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). The 16S SSU rDNA gene sequence alignment was edited using the Genetic Data Environment (GDE 2.2) program (Smith *et al.* 1994), and the aligned sequence was registered in GenBank (Accession Number MT644519).

5. Phylogenetic analyses

Sequence data of 14 strains (Table 1) were used for the analysis of MODELTEST v.3.7 (Posada and Cradall 1998), and maximum likelihood (ML). ML analysis was performed with RAxML v8.2.4 (Stamatakis 2014) using the general time reversible plus gamma (GTR+G) model with random sequence addition 1,000 times followed by a heuristic search using tree-bisection reconnection (TRB) branch swapping. Bayesian analysis was performed using MrBayes v3.2 (Ronquist *et al.* 2012) to construct random inference trees with the GTR+G+I model 2,000,000 times. The phylogenetic tree was constructed every 1,000 cycles, and the burn-in point was graphically identified based on the likelihood score in the phylogenetic tree (Tracer v1.5; http://tree.bio.ed.ac.uk/software/tracer/).

RESULTS AND DISCUSSION

1. Taxonomic summary

Phylum Cyanobacteria Stanier ex Cavalier-Smith, 2002
Class Cyanophyceae Schaffner, 1909
Order Synechococcales Hoffmann, Komárek & Kastovsky, 2005

Table 1. List of strains used in the molecular study and GenBank accession number

Species	Strains	Accession no.	References
Cyanobium gracile	**Adong191016**	**MT644519**	**This study**
Cyanobium gracile	PCC6307	CP003495	Shih *et al.* 2013
Cyanobium gracile	PCC9604	AF216944	Robertson *et al.* 2001
Prochlorococcus marinus	AS9601	NC_008816	Kettler *et al.* 2007
Prochlorococcus marinus	MIT9312	CP000111	Coleman *et al.* 2006
Prochlorococcus marinus	MIT9515	CP000552	Kettler *et al.* 2007
Prochlorococcus marinus	CCMP1375	AE017126	Dufresne *et al.* 2003
Synechococcus elongatus	PCC6301	AP008231	Sugita *et al.* 2007
Synechococcus elongatus	PCC7942	CP000100	Holtman *et al.* 2005
Synechococcus spongiarum	15L	JYFQ01000001	Burgsdorf *et al.* 2015
Synechococcus spongiarum	142	JXUO01000104	Burgsdorf *et al.* 2015
Synechococcus spongiarum	SH4	JENA01000091	Gao *et al.* 2014
Synechococcus sp.	WH7803	CT971583	Doron *et al.* 2016
Synechococcus sp.	RS9917	NZ_CH724158	Dufresne *et al.* 2008

New sequences are indicated in bold type.

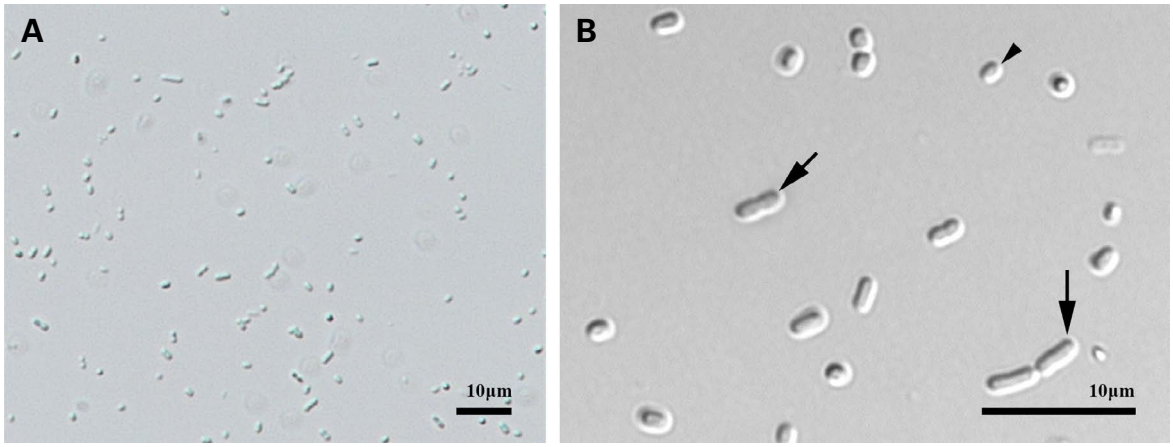

Fig. 1. Light micrographs of *Cyanobium gracile* Adong101619 showing symmetrical oval, ellipsoid (arrowhead), and dividing (arrow) cells. A. ×400. B. ×1000.

Family Synechococcaceae Komárek & Anagnostidis, 1995
Genus *Cyanobium* Rippka & Cohen-Bazire, 1983

***Cyanobium gracile* Rippka & Cohen-Bazire 1983**

Holotype. Type strain deposited at Pasteur Culture Collection (PCC), PCC6307.
Material examined. Freshwater was collected from the Adongji pond, Adong-ri, Gaejeong-myeon, Gunsan-si, Jeollabuk-do, Republic of Korea (35°58′47.4″N, 126°46′14.6″E) on October 16, 2019.
Diagnosis. Cells are a pale blue-green single cell. The cell shape is ovate, oval, or short rod-shaped without gelatinous envelopes. Cells solitary or in twos after division, not in colonies. 1.08−3.87 μm long and 0.75−1.51 μm wide. The thylakoid membranes are stacked in three and arranged along the cell walls. Obligatory photoautotroph.
Distribution. North America (Smith 2010) and the Republic of Korea.
Voucher slide. Two slides of gelatin-embedded specimens were deposited at Nakdonggang National Institute of Biological Resources, Korea (NNIBRCY894 and NNIBRCY 895).

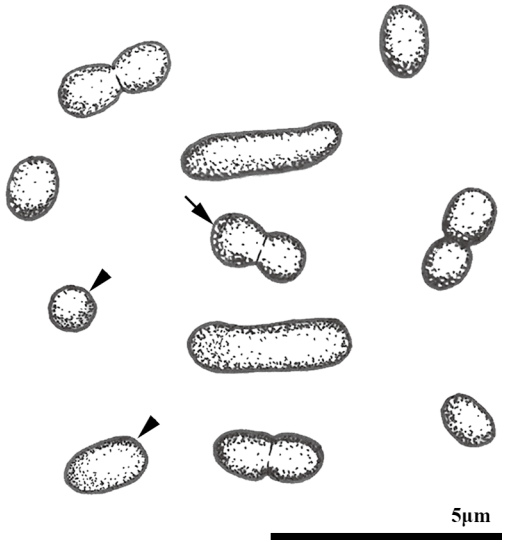

Fig. 2. Schematic drawing showing symmetrical oval, ellipsoid (arrowhead) to shortly rod-shaped and dividing (arrow) cells.

2. Morphology and ultrastructure

Cyanobium gracile was pale blue-green and ovate-, oval-, or rod-shaped (Figs. 1, 2). *Synechococcus* species, similar to *Cyanobium* species in morphology, generally have a long cylindrical shape and are occasionally asymmetrical (Komárek *et al.* 1999), but *C. gracile* cells were observed to be symmetrical (Figs. 1, 2). When the cell divided by simple dichotomy, the cell was elongated rod-shaped or eight-shaped (Figs. 1B, 2). *C. gracile* cells were 1.08−3.87 μm ($n=75$, mean = 1.84 ± 0.43 μm) long and 0.75−1.51 μm ($n=75$, mean = 1.08 ± 0.18 μm) wide. Cells were larger than that of the type strain PCC6307 (0.4−2.4 × 0.25−0.4 μm, Komárek *et al.* 1999). The nucleolus was observed in the center of the cell of *C. gracile*. Peripheral thylakoid membranes were stacked in three, and each of the thylakoid membranes was arranged in parallel (Fig. 3). This thylakoid architecture is a typical characteristic of the genus *Cyanobium* (Gantt and Conti 1969; Komárek and Cepak 1998). Additionally, an electron opaque material, which was presumed to be polyphosphate granules, was observed in the cytoplasm. These granules were distributed between the cell wall and the outer thylakoid membrane (Fig. 3).

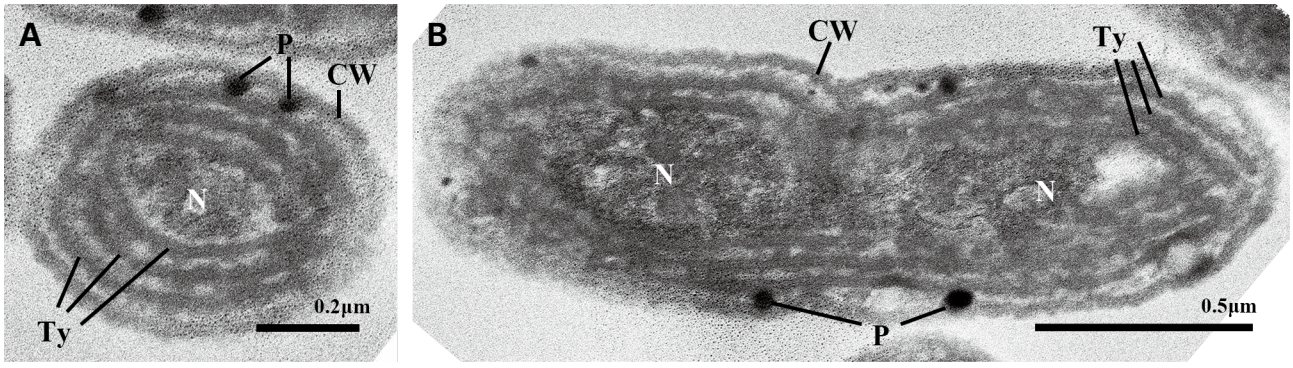

Fig. 3. Transmission electron micrographs of *Cyanobium gracile* Adong101619 showing thylakoids arranged parallel to the cell walls. A. Cross-sectional image. B. Longitudinal section image. CW, cell wall; N, nucleoplasm; P, polyphosphate granule; Ty, thylakoid membrane.

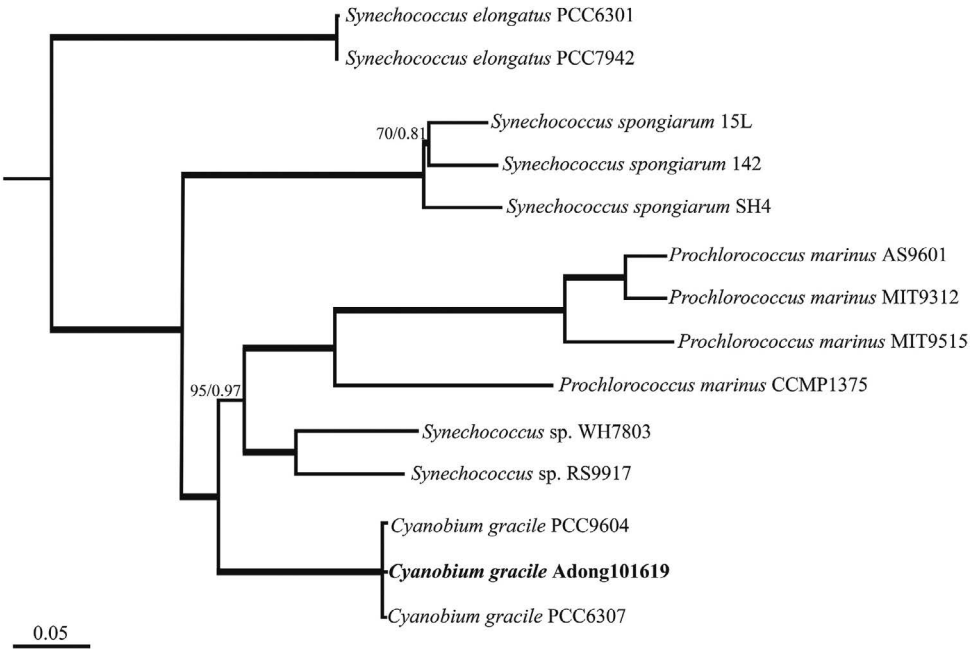

Fig. 4. Maximum-likelihood (ML) tree of the genus *Cyanobium* based on 16S SSU rDNA sequence data. Bayesian posterior probability (pp) and maximum-likelihood (ML) bootstrap values are shown above the branches. The bold branches indicate strongly supported values (ML = 100, pp = 1.00). Scale bar = 0.05 substitutions/site.

3. Phylogeny

BLAST analysis indicated that the 16S SSU rDNA sequence of *C. gracile* showed 100% similarity to the reference sequences of CP003495, NR_102447, AF216944, MT488300, DQ275599, and NR_114406. Bayesian and maximum likelihood (ML) analyses were performed with the 16S SSU rDNA sequence of *C. gracile* and 13 references (Fig. 4). In the phylogenetic tree, *C. gracile* formed a monophyletic clade with *C. gracile* PCC9604 and PCC6307 (ML = 100, pp = 1.00). In addition, *C. gracile* formed a sister group with four strains of *Prochlorococcus marinus* and two strains of *Synechococcus* sp. (ML = 100, pp = 1.00).

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