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New report on cyanophyte in Korea, *Microseira wollei* (Farlow ex Gomont) G.B.McGregor and Sendall ex Kennis (Oscillatoriaceae)

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Microseira wollei (Farlow ex Gomont) G.B.McGregor and Sendall ex Kennis, a mat-forming filamentous harmful cyanobacterium, has historically been found in the United States. *Microseira wollei* produces neurotoxins and hepatotoxins which affect declining water quality. In the present research, we report of unrecorded *M. wollei* with morphology, TEM anatomy, molecular phylogeny on the Korean population. Based on 16S rRNA gene sequences, Korean population were different by 0.02% (2 bp) to the Japanese population, 1.2−1.3% to the Australian population, and 2.5−3.7% to the United States populations. *nifH* gene sequences were 8.4−8.7% different to Australian ones and 3.5−3.8% to other population, however molecular phylogenetic analysis of *M. wollei* living in Korea revealed monophyly with the geographical populations of U.S.A., Australia, and other geographical populations. Since the mat of *M. wollei* has been reported to be maintained for several years in other countries, it is necessary further investigate the seasonal and regional distribution of this species in Korea.

Keywords: 16S rRNA, *Microseira wollei*, morphology, unrecorded

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Introduction

Cyanobacteria (also known as blue-green algae), which played an important role in increasing oxygen in the atmosphere in the Precambrian era, are photosynthetic prokaryotes. They are considered important primary producers in aquatic ecosystems, such as surrounding diatoms. Most cyanobacteria have the capacity to form sheaths, capsules, and akinetes, and some of these characters provide the ability to exist in various terrestrial and aquatic environmental conditions and survive in extreme environments, polar regions, hypersaline seawater, and deserts.

Cyanobacteria possess a diversity morphological features such as unicellular, multicellular, and filamentous forms. Using morphological features of cyanobacteria, 1,500 species and 150 genera were identified by Geitler (Mishra, 2002). The traditional taxonomy based on morphologic traits does not reflect the results of phylogenetic analyses (Gugger and Hoffmann, 2004). The predominance of morphology polyphyletic species, genera and higher taxonomic categories based on morphology indicate the need for taxonomic revision (Komárek *et al*., 2014).

Only 300 taxa of cyanobacteria, however, have been reported so far in Korea using morphological features (Park, 2012; NIBR, 2015). The controversies in traditional and modern approaches to cyanobacterial taxonomy persist, several studies have been reported with molecular method since 2000's in Korea (Li *et al*., 2013).

The genus *Microseira* was established as a new genus, based on the type species *M. wollei* (McGregor and Sendall, 2014). *Microseira* was distinct from other Oscillatorialeans based on large cells, molecular phylogeny of 16S rRNA gene, *nifH* gene, and toxin coding genes. *Microseira wollei* was validated by Kenins (2017) due to the invalid citation of basionym as *Lyngbya wollei* (Farlow ex Gomont) Speziale and Dyck, not as *Plectonema wollei* Farlow ex Gomont. *Plectonema wollei* had been transferred to genus *Lyngbya* (Speziale and Dyck, 1992) with emphasis of the 'false branching' characteristic of *P. wollei* in the original description (Gomont, 1892) was undue in spite of variability according to season and environment.

Microseira wollei have had recurrent blooms over the

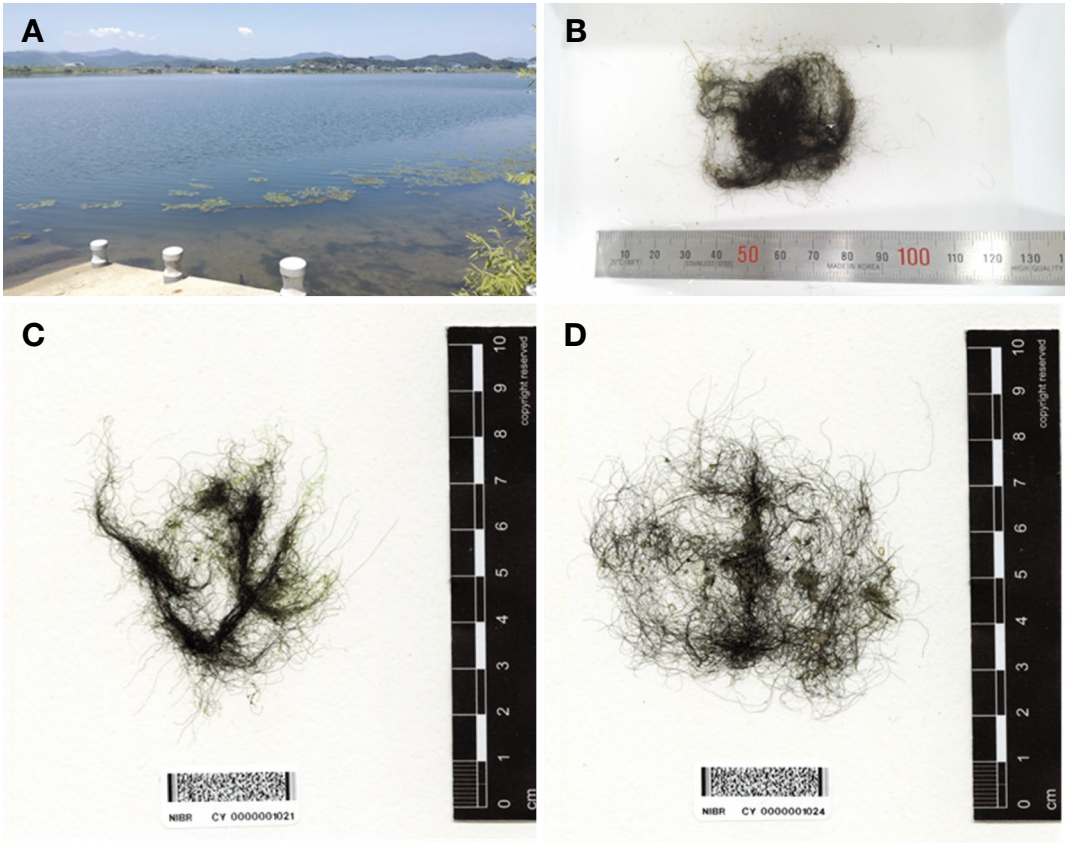

Fig. 1. Collection site and specimen information of *Microseira wollei*. A. Collection site near Gangjeong-Goryung weir in the Nakdong river. B. Natural form of *Microseira wollei*. C. Dried specimens deposited in KB, NIBRCY0000001021 (13 May 2020). D. NIBRCY0000001024 (16 Jun 2020).

past 100 years in the United States. Besides, it has been known as one of the toxin producers of the potent neurotoxic saxitoxins (Carmichael *et al*., 1997) or paralytic shellfish toxins (Foss *et al*., 2012). McGregor and Sendall (2014) investigated phylogeny of toxin gene, cylindrospermopsin (CYN) and deoxy-cylindrospermopsin (deoxy-CYN) of Australian population. In Korea, *M. wollei*-like species were recognized in the Nakdong River, which is one of the important water sources for citizens of southwestern South Korea (Lee *et al*., 2012).

In this study, we report the presence of the cyanophytic species *M. wollei* on the Korean population for the first time, based on morphology, TEM anatomy, and molecular phylogeny. In addition, presence of toxin biosynthesis genes was investigated by PCR method.

Materials and Methods

Collection

The field collections were conducted near Gangjeong-Goryeong weir of the Nakdong River on 13 May and 16 June in 2020. The collected samples were immediately moved to the laboratory in a cooled ice box. Standardized specimen preservation was used, according to the Institution's manual (National Institution of Biological Resources, 2009). Specimen were registered at the NIBR Biological Resources Management System by serial number. The specimens were stored at the herbarium of plant storage center of the NIBR International Standards Herbarium (KB).

Morphology

Gross morphology and anatomical features were observed with fresh trichomes. Observations were aided with light microscope (Olympus BX51) with a camera system (Olympus DP72). For transmission electron microscope (TEM) observation, cells were fixed in 0.1 M sodium cacodylate containing 2% glutaraldehyde, 2% paraformaldehyde for 2 h at room temperature. After washing three times with 0.1 M sodium cacodylate, cells were dehydrated through a gradient series of ethanol, 20 min each step, starting from 50% ethanol and ending with 100% ethanol. Afterwards, cells were incubated with progressively concentrated propylene oxide dissolved in ethanol

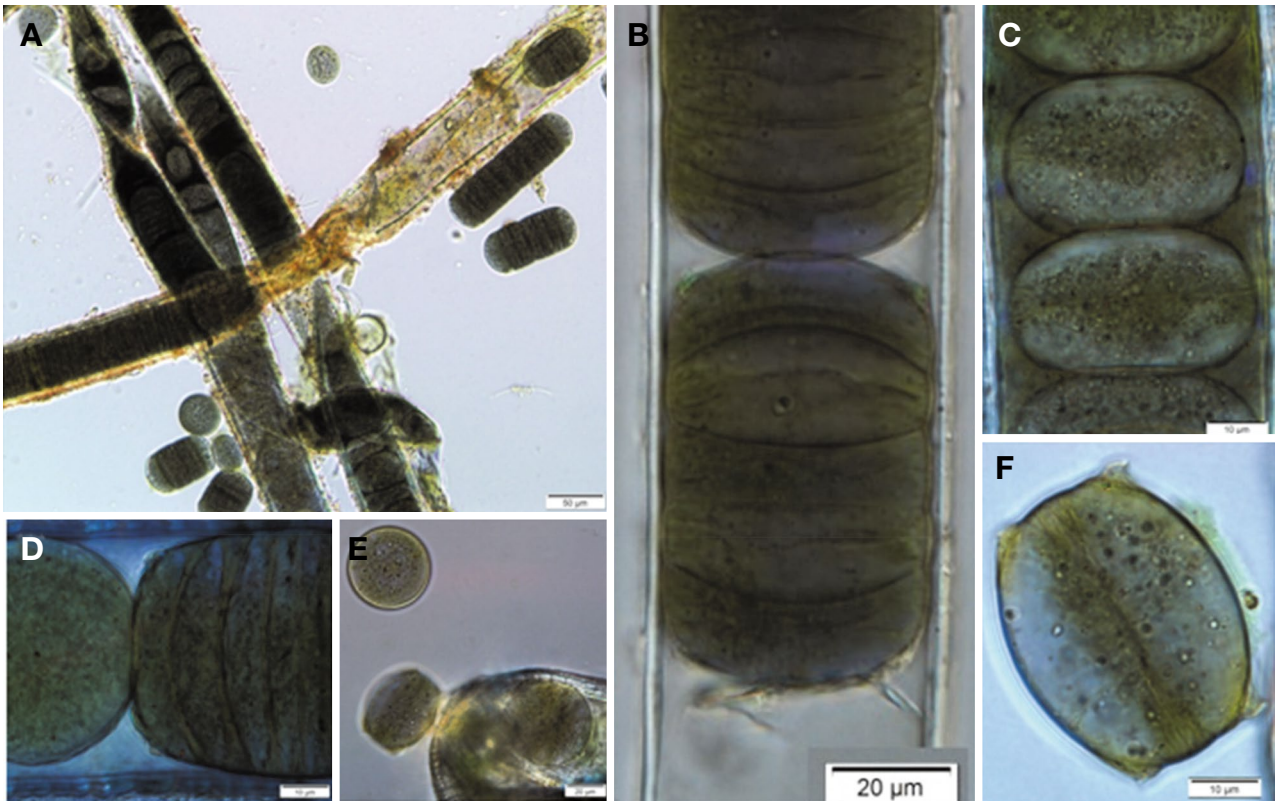

Fig. 2. Light micrographs of *Microseira wollei* collected in Gangjeong-Goryeong weir. A–D. Unbranched filament, showing thick sheath and trichomes. E, F. Discoid single cell released from filament.

then infiltrated with increasing concentration of Eponate 812 resin. Samples were baked in a 65°C oven overnight then sectioned using an ultramicrotome. Sections were viewed with an energy filtering TEM unit (JEM-2100F, Japan) at the Korea Basic Science Institute, South Korea.

DNA extraction, PCR, and Sequencing

Materials for DNA extraction were picked by isolation under light microscope as usual for indoor culture. Isolated single disk or filament was washed with distilled water. Sample was moved into 1.5 mL microtube with 100 μL of 10% Chelex 100 DW solution (Walsh *et al.*, 1991). Samples in microtubes were vortexed for 10 sec and then incubated at 99°C for 30 min. After boiling, microtubes were on ice for 5 min. Finally, Chelex 100 and samples were centrifuged at maximum rpm for 5 min. The supernatant was moved to a new microtube by micropipette avoiding the Chelex 100 pellet.

The 16S rDNA region was amplified and sequenced with primers, 27F1 (UFP)/1494Rc (URP) (Nelian *et al.*, 1997) or CYA106F (Nübel *et al.*, 1997)/ FDSKS_CyaF1, FDSKS_CyaF2, FDSKS_CyaF3, FDSKS_CyaR1 (Shiels *et al.*, 2019). The nitrogenase reductase gene was amplified with the primers (nifH) nifH seqF/nifH seqR (McGregor and Sendall, 2015). The toxin biosynthase gene was amplified with primer set LWpks-F1/LWpks-R2 for *CyrC* gene (McGregor and Sendall, 2015), and primers AMT1/AMT2 for *CyrJ* gene (Campbell 2009 in McGregor and Sendall, 2015). The reaction solution contained 1 μM primers, dNTPs of 2.5 mM each, 25 mM $MgCl_2$, Takara Ex Taq^{TM}, 10X Ex Taq^{TM} buffer (Mg^{2+} free), and template DNA. The mixture was used for polymerase chain reaction (PCR) using a 2720 thermal cycler (The Applied Biosystems) with the following procedures: 5 min at 94°C for initial denaturation, 30 sec at 94°C for denaturation, 30 sec at 55°C for primers annealing according to the samples, 1 min at 72°C for polymerization extension for 35 cycles, and a final 10 min extension at 72°C. To visualize the amplified product, it was loaded with size marker and run on 1% agarose gel, containing dye, and illuminated in UV light.

PCR products were submitted to Xenotech corp. (Daejeon, Korea) for sequencing. Forward and reverse sequences were assembled and edited by eye using the programs Chromas and BioEdit.

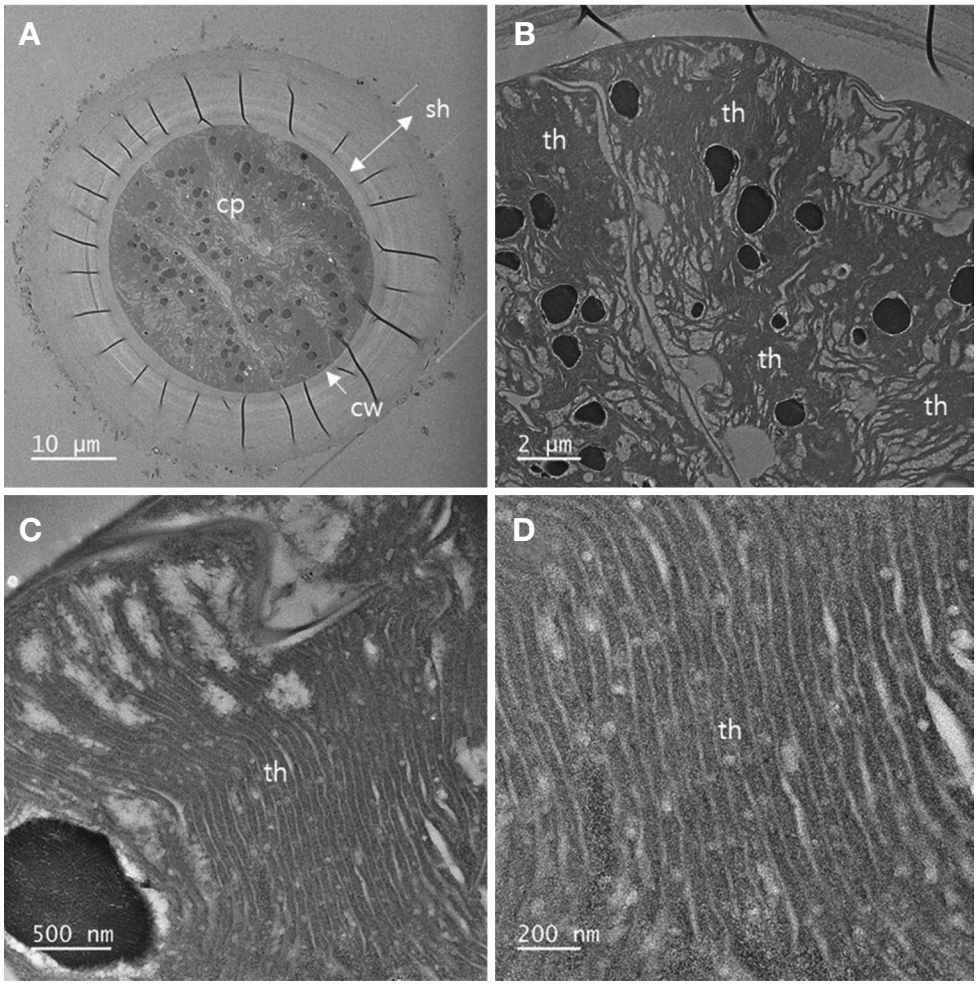

Fig. 3. Ultrastructures of *Microseira wollei* by transmission electron microscopy (TEM). A. Cross-section of filament surrounded by cell wall and thickened sheath. B. Cytoplasm filled with numerous thylakoids. C. Densely stacked thylakoids. D. Magnified thylakoids. cp (cytoplasm), cw (cell wall), sh (sheath), th (thylakoid).

Phylogenetic analysis

Sequences were aligned using Clustal W in MEGA-X Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al*., 2018). Total eight 16S rRNA gene sequence samples of Korean *M. wollei* were compared with one from Japan, six from Australia, and six from USA from the NCBI database. The outgroup was hypothesized as *Nostoc commune* in Nostocales. In the case of *nifH* gene, eight Korean samples were compared with six sequences from NCBI. The aligned sequences were loaded to MEGA-X for the phylogenetic analysis. Pairwise distances were calculated by maximum composite likelihood model. Datasets were analyzed using maximum parsimony (MP), maximum likelihood (ML), and neighbor joining (NJ) analysis of MEGA-X. Each generated tree by model was run with 1,000 bootstrap replicates for testing robustness of each clade.

Results

Description

Order Oscillatoriales Schaffner 1922
Family Oscillatoriaceae Engler 1898
Genus *Microseira* G.B.McGregor and Sendall 2014
Microseira wollei (Farlow ex Gomont) G.B.McGregor and Sendall ex Kennis 2017

Basionym: *Plectonema wollei* Farlow ex Gomont 1892
Homotypic synonym: *Lyngbya wollei* (Farlow ex Gomont) Speziale and Dyck 1992
Type specimen: 945406 (syntype, NY)
Habitat: The filaments of *Microseira wollei* were found in sublittoral habitat of a freshwater river entwined in the basal part of aquatic macrophytes or plants.
Collection site: The riverside of 7 km upper stream from

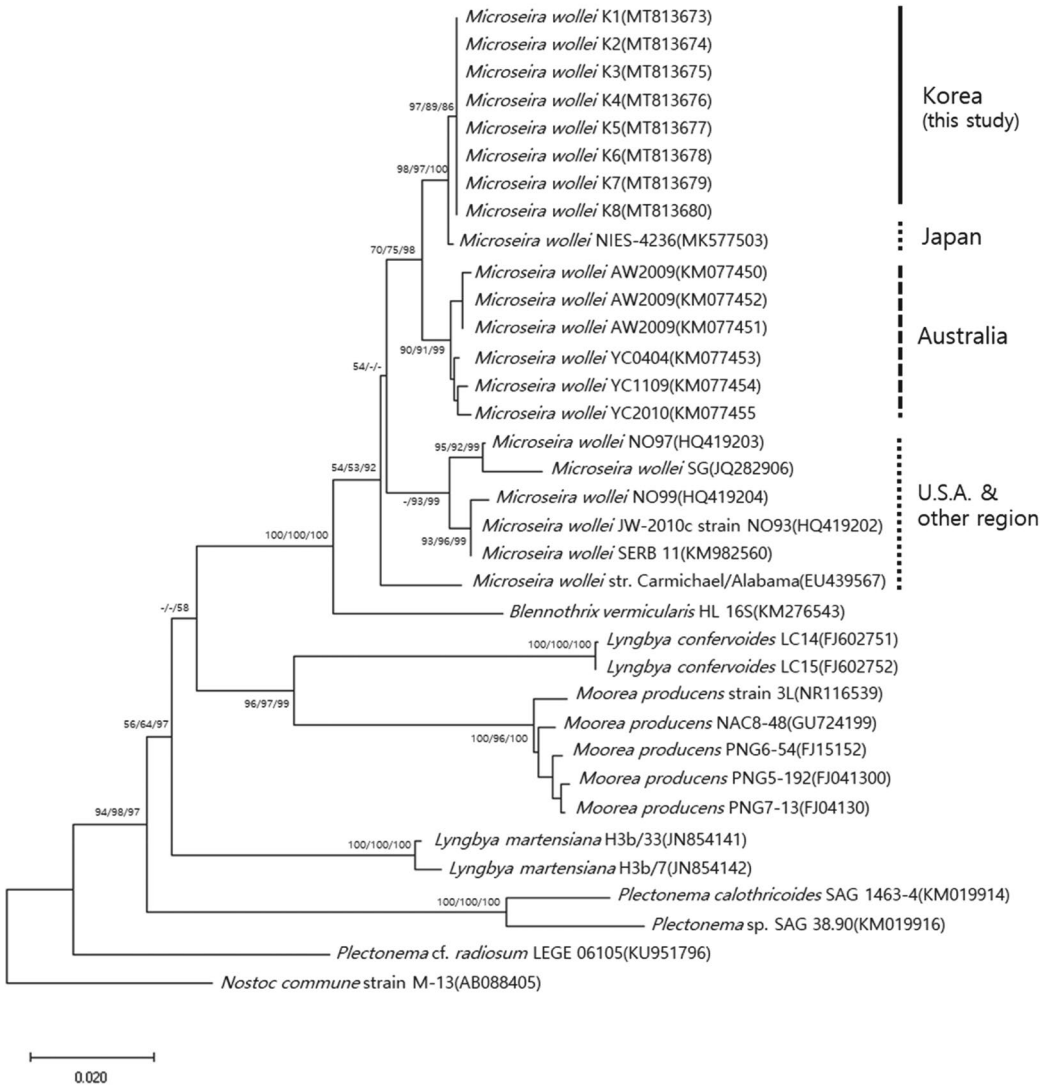

Fig. 4. Phylogenetic tree based on the 16S rRNA gene partial sequences aligned 1,267 bp showing the genetic position of *Microseira wollei* of Korea along with other geographical populations and related taxa with some taxonomic treatment of *M. wollei*. Numbers indicate bootstrap value (>50%) from 1,000 replicates maximum parsimony (MP), maximum likelihood (ML), neighbor-joining (NJ) analysis respectively. GenBank accession numbers are shown in parentheses (scale bar: 0.02 nucleotide substitutions per site).

Gangjeong-Goryeong weir at Dalsung-gun, Daegu-city, Nakdong River (35°51′31.6″N 128°23′23.0″E).

Specimens: NIBRCY0000001021 (collected by E.H. Bae, no. EHB20200513-1, 35°51′31.6″N 128°23′23.0″E, 13 May 2020), NIBRCY0000001022 (collected by E.H. Bae, no. EHB20200513-2, 35°51′31.6″N 128°23′23.0″E, 13 May 2020), NIBRCY0000001023 (collected by E.H. Bae, no. EHB20200513-3, 35°51′31.6″N 128°23′23.0″E, 13 May 2020), NIBRCY0000001024 (collected by E.H. Bae, no. EHB20200616-1, 35°51′31.6″N 128°23′23.0″E, 16 June 2020) in KB.

Habit: *Microseira wollei* was identified as remarkably large-celled, greenish black filamentous, mat-forming characteristics, observed with naked eyes.

Morphology

Thallus a prostrate, greenish blue-black tough mat of densely entangled filaments (Fig. 1). Filaments straight to variously contorted, and infrequently false-branched. An individual filament consists of an uniseriate file of discoids to cylindrical cells. Cell division occurs throughout the length of the filament and apex is flat at immediately after trichome division. Sheath colorless, hyaline, when old distinctly thickened, roughened and colored, open at the apex. Trichomes isopolar, uniformly cylindrical, up to 70 μm wide; not or slightly constricted at the cross-walls. Cells discoid, coffee bean shaped in lateral view; contents finely granular, without aerotopes; apical cells rounded,

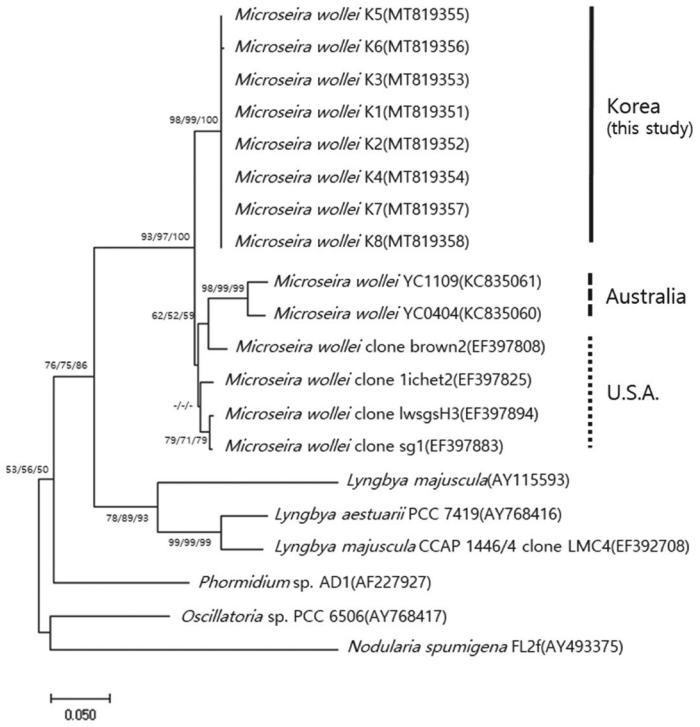

Fig. 5. Phylogenetic tree based on the *nifH* gene partial sequences aligned 318 bp showing the genetic position of *Microseira wollei* of Korea along with other geographical populations. Numbers indicate bootstrap value (>50%) from 1,000 replicates maximum parsimony (MP), maximum likelihood (ML), neighbor-joining (NJ) analysis respectively. GenBank accession numbers are shown in parentheses (scale bar: 0.05 nucleotide substitutions per site).

without a calyptra and thickened outer cell wall (Fig. 2).

Ultrastructures by transmission electron microscopy (TEM)

Filaments of *M. wollei* were cross-sectioned to observe their ultrastructural patterns of thylakoids and intracellular contents by TEM. The trichome filaments were covered by cell wall and outer thick sheath (Fig. 3A). The cytoplasm was filled with numerous thylakoids (Fig. 3B). Membranous thylakoids were extended from near cell membrane and densely stacked as bundle (Fig. 3C, D). The bundles of thylakoids were located irregularly, not parietally nor radially.

Molecular phylogeny

A total of 1,267 bp of the 16S rRNA gene and 318 bp *nifH* (nitrogenase iron protein) gene sequences of were analyzed. All sequences of eight isolates from Korea were identical based on these two genes. Among 1,267 bp the 16S rRNA gene sequences, sequences of the Korean population were different from 0.02% (2 bp) to the Japanese population (NIES-4236), 1.2−1.3% to the Australian population, and 2.5−3.7% to USA population and other geographical populations in NCBI database. While, nucleotides of *nifH* gene showed distance as 8.4−8.7% to Australian samples and 3.5−3.8% to other populations. The neighbor joining trees are presented for 16S rRNA gene (Fig. 4) and *nifH* gene (Fig. 5). All trees supported our conspecific speculation of the Korean population of *M. wollei*.

Cyanotoxin analysis by PCR of *cyr* genes: The *cyrF* gene of 447 bp and *cyrJ* gene of 626−657 bp were not positive in all Korean samples.

Discussion

Microseira wollei is known as a producer of the cyanotoxin PST and CYN. In the North America, it is commonly observed in the metaphyton of rivers and lakes. Massive benthic blooms of *M. wollei* occurred in northern Florida (Cowell and Botts, 1994), the Tennessee River (Doyle and Smart, 1998), and Lake Erie (Bridgeman and Penamon, 2009). While toxin producing cyanobacteria have been studied in the planktonic taxa such as *Microcystis*, *Dolichospermum* and *Aphanizomenon*, toxic benthic filamentous cyanobacteria are less studied in Korea (Park, 2012). In this study, we report *M. wollei*, benthic cyanobacteria, for the first time in Korea.

CYNs are excreted by benthic cyanobacterial cells more extensively and extracellular concentrations often exceed intracellular contents (Bormans *et al*., 2014). To find out if Korean samples have a CYN synthetase gene, we amplified the *cyrF* and *cyrJ* gene. The analysis was not positive in all Korean samples but additional research is needed to analyze the intracellular and extracellular compounds for toxin production.

Microseira wollei, first discovered near in Gangjeong-Goryeong weir, is characterized by long filaments of indeterminate length and very resilient mats like the specimens of the United States and Australia. Recently, in the United States, there is increasing concern about the growth of *M. wollei* affecting water quality. Moreover, since the mat of *M. wollei* has been reported to be maintained for several years, it is necessary further investigation of seasonal and regional distribution of the species is also needed in Korea.

Molecular phylogenetic analysis of *M. wollei* living in Korea revealed monophyly with the geographical populations of U.S.A., Australia, and other geographical populations. However, the intraspecific genetic variation of this cosmopolitan freshwater species is still under research.

In this study, transmission electron microscopy of *M. wollei* revealed that ultrastructural pattern of thylakoids in detail for the first time. Thylakoid arrangement of *M. wollei* was irregular, not radical nor circular parallel to its cell membrane. The pattern of thylakoid arrangement was regarded as important character to distinguish families among order Oscillatoriales (Komárek *et al*., 2014). The irregular arrangement of thylakoids of *M. wollei* was corresponded to synapomorphy of family Oscillatoriaceae.

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