Journal of Species Research 11(3):162-168, 2022

New record of the cold freshwater dinoflagellate *Palatinus apiculatus* (Dinophyceae) from the Paldang Reservoir, Korea

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Compared to marine dinoflagellates, freshwater species are rarely recorded in Korea. In the present study, we isolated a freshwater dinoflagellate, *Palatinus*, from the Paldang Reservoir, Korea, in December 2021. The overall cell shape was ovoid, and the cell size was 34.3 μm in length (25.8−39.5 μm) and 28.4 μm in width (21.5−34 μm). An eyespot was usually observed near the sulcal region. The Kofoidian plate formula of the species was determined to be 4′, 2a, 7″, 6c, 5s, 5‴, and 2⁗. Apical pore complex was not observed. However, variations in the cingular plate caused by the fusion of 3C and 4C were observed. Analyses of 28S rDNA sequences revealed that the unidentified species is 100% similar to *Palatinus apiculatus*, and clustered together in the same lineage in the phylogenetic tree (100% bootstrap value). Our findings confirmed that the isolated dinoflagellate is *Palatinus apiculatus*, which was discovered for the first time in Korean freshwaters.

Keywords: 28S rDNA, freshwater dinoflagellates, morphology, *Palatinus apiculatus*, Paldang Reservoir

DOI:10.12651/JSR.2022.11.3.162

Introduction

Dinoflagellates are one of the dominant groups of phytoplankton in aquatic environments. They are widely distributed in marine and freshwater, with different species occurrences depending on habitats (Taylor *et al*., 2008). The genus *Peridinium* Ehrenberg 1830 is the most common thecate dinoflagellate found in freshwater ecosystems like rivers and lakes. The classification of this genus has been revised several times. At first, all *Peridinium* species were thought to have similar morphological characteristics. Investigations into the apical pore complex (APC), however, led to a major revision in the genus *Peridinium* during the twentieth century. For example, Lemmermann (1910) proposed two morphological sections (*Peridinium* setc. *Cleistoperidinium* and *Proroperidinium*) in the genus *Peridinium*, depending on the presence or absence of an APC. The typical *Peridinium* members like *P. cinctum* lack an APC, and they were classified as *Cleistoperidinium* Lemmermann 1910. In contrast, other *Peridinium* species such as *P. bipes*, had a distinct APC, and they were classified as *Proroperidinium* Lemmermann 1910. Nonetheless, taxonomist Édouard Lefèvre did a detailed review of the genus *Peridinium* in which he retained the name of the genus; however, he placed the *Cleistoperidinium* and *Proroperidinium* into two subgenera within the genus (Lefèvre, 1932). In addition, Lefèvre divided these subgenera into sets of morphological "groups" having similar thecal plate arrangements. These "groups" have been used to identify peridinioid species, but they do not have any official taxonomic position (Craveiro *et al*., 2009).

Recent advances in microscopy and molecular techniques enable deep analysis of the taxonomic system of the dinoflagellate *Peridinium*, and some of them have been classified into independent genera such as *Parvodinum* and *Chimonodinium* (Carty, 2008; Craveiro *et al*., 2011). The "group palatinum" was erected to the new genus *Palatinus* because it is clearly distinguished from the typical *Peridinium* in terms of thecal plate number and ornamentations (Craveiro *et al*., 2009). In brief, the six cingular plates and smooth plate surface without ridges are notable features distinguishing *Palatinus* from typical *Peridinium* (Craveiro *et al*., 2009).

Freshwater dinoflagellates have been described from Korean waters, and most species are reported through ecological monitoring. For these reasons, the taxonomic identities and systems are not accurately verified by morphological and molecular comparisons (Kim *et al*., 2020). Only five freshwater dinoflagellates with appropriate taxonomic analyses have been reported from Korean fresh-

waters (Ki *et al*., 2005; Ki and Han, 2005; 2008; Kim *et al*., 2020; Kim and Ki, 2021). These include peridinioids species (e.g., *Apocalathium aciculiferum*, *Parvodinium umbonatum*, *Peridinium bipes* f. *occultatum*, *Unruhdinium penardii* var. *robustum*, and *U. kevei*) from Korean reservoirs and lakes, and some were described by using dinoflagellate cysts (Li *et al*., 2015; Kim and Ki, 2022).

When compared to global dinoflagellates (Guiry and Guiry, 2022), very few freshwater dinoflagellates have been reported in Korea, and a large number seem to remain unrecorded (Kim and Ki, 2021). This is supported by our recent studies conducted on the Hangang River, showing the high molecular diversity of freshwater dinoflagellates, including unidentified peridinioids (Boopathi and Ki, 2016; Muhammad *et al*., 2021). In recent studies, we identified two unrecorded species from the Paldang Reservoir, and reported their ecological features and seasonal occurrence patterns (Kim *et al*., 2020; Kim and Ki, 2021; 2022). These dinoflagellates were common in the Hangang River in the spring and autumn. However, some others appeared in the cold season and their species identities are unknown.

In the present study, we isolated a dinoflagellate *Palatinus* during the cold season from the Paldang Reservoir and succeeded in culturing the species in laboratory conditions. We examined the morphological characteristics of the isolate and analyzed the DNA sequences of 18S and 28S rDNA. With these data, we identified it to the species level as *P. apiculatus*.

Materials and Methods

Sampling and culture

Water samples were collected from the Paldang Reservoir (GPS code: 37°39′15.63″N, 127°17′15.89″E) on 12 December 2021 (Fig. 1). Additional phytoplankton cells in the surface water were collected using a 20-μm plankton net. The net samples were sieved with a 200-μm mesh to remove large-sized zooplankton. The collected samples were placed in an ice box and transported to a laboratory. For unialgal cultures, single cells were isolated by using the micropipette technique under an inverted microscope (KI-450, MDM INSTRUMENTS, Suwon, Korea). The isolated cells were washed with URO medium (Kimura and Ishida, 1985) and transferred into a 24-well plate (SPL, Pocheon, Korea). The culture cells were maintained at 16°C under a 12 : 12 h light-dark cycle with a photon flux density of approximately 65 μmol photons/m^2/s.

Morphological observations

Cell shapes of the isolated dinoflagellates were observed with a light microscope (LM; Carl Zeiss Axioskop, Oberkochen, Germany) under 400× magnification. Digital images were taken with a ProgRes® CF Scan CCD camera (Jenoptik, Jena, Germany), and analyzed with ProgRes CapturePro 2.10.0.1 software (Jenoptik, Jena, Germany). Average cell lengths and widths were calculated by measuring 30 cells.

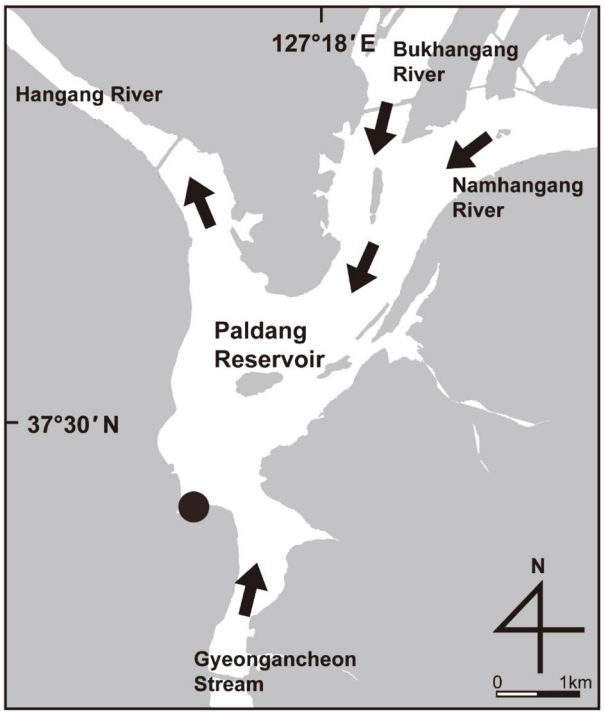

Fig. 1. A map of the Paldang Reservoir, Korea. A black circle represents the sampling site. Black arrows represent direction of water flow.

The formula and shape of the thecal plates were analyzed using a scanning electron microscope (SEM; JSM 5410, JEOL, Tokyo, Japan). The dinoflagellate species of this study was identified according to previous literature (Craveiro *et al*., 2009; Kretschmann *et al*., 2018). The taxonomic classification of freshwater dinoflagellates followed Moestrup *et al*. (2018) and Algaebase accessed on April 13, 2022 (Guiry and Guiry, 2022).

DNA extraction and PCR

Exponential phase cells were harvested via centrifugation (3,000 rpm), mixed with 0.8 mL of extraction buffer [100 mM Tris-HCl, 100 mM Na_2-EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% cetyltrimethylammonium bromide (CTAB)], and stored at −20°C until further analysis. Total genomic DNA was extracted by the modified CTAB method by Faria *et al*. (2014). Sequences of the 18S to 28S rDNA region were amplified by the long PCR with two dinoflagellate-specific primers (forward: 18F01, 5′-CACCTGGTTGATCCTGCCAGTAG-3′; reverse:

PM28-R1318, 5′-TCGGCAGGTGAGTTGTTACACAC-3′) (Ki *et al*., 2011). PCR was carried out in 20 μL reaction volumes containing 11.8 μL of sterile distilled water, 2 μL of 10 × Ex PCR buffer (TaKaRa, Shiga, Japan), 2 μL of dNTP mix (4 mM each), 1 μL of each primer (final concentrations of 500 nM), 0.2 μL Ex Taq polymerase (2.5 U), and 2 μL of template DNA. PCR cycling was performed on a thermal iCycler (Bio-Rad, Hercules, CA) via the following program: 94°C for 3 min; followed by 40 cycles of 94°C for 30 sec, 55°C for 40 sec, and 68°C for 5 min; with a final extension at 72°C for 10 min. The resulting PCR products were electrophoresed on a 1.0% agarose gel (Promega, Madison, WI), stained with Midori^{Green} (Nippon Genetics Europe, GmbH, Germany), and visualized on a transilluminator under ultraviolet light.

The PCR amplicons were then purified with the QIA quick PCR Purification Kit (Qiagen GmbH, Germany), and DNA sequencing reactions were run with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA) using the PCR primers. After that, the remaining DNA sequences were determined by primer walking. Labeled DNA fragments were analyzed on an automated DNA sequencer (Model 3700, Applied Biosystems, Foster City, CA).

Editing and contig assembly of the rRNA sequence fragments were carried out in the Sequencher 4.7 software (Gene Codes, Ann Arbor, MI). All the sequences determined here were deposited in GenBank.

Molecular identification by DNA similarity

Molecular identification of the dinoflagellates was done using 18S (1,775 bp) and 28S (1,265 bp) rDNA sequences with Basic Local Alignment Search Tool (BLAST) searches at the National Center for Biotechnology Information (NCBI). In addition, DNA similarities of the 28S rDNA between the unidentified species and other *Palatinus* species were calculated using paired nucleotide sequences (585 bp) in BioEdit (Hall, 1999).

Phylogenetic analyses

Phylogenetic analysis was carried out using dinoflagellate 28S rDNA sequences that were retrieved from the NCBI database. We constructed a data matrix of our species and other relatives, including 18 genera and 28 species. The 28S sequences were aligned in the MAFFT software (Katoh *et al*., 2019), and ambiguous regions were removed using the Gblocks server (Castresana, 2000). A maximum-likelihood (ML) tree was constructed using the aligned 28S rDNAs (459 alignment sites) with the GTR + G + I nucleotide substitution model in PhyML 3.0 (Guindon *et al*., 2010). In this analysis, a total of 5,000 replicate bootstrap analyses were run. The phylogenetic tree was visualized in MEGA X (Kumar *et al*., 2018) and re-drawn in Adobe Illustrator CC (Adobe Systems, San Jose, CA).

Results and Discussion

Based on the morphological and molecular results, we report the first record of *Palatinus apiculatus* in Korea. We provide a taxonomic description of isolated *Palatinus apiculatus*.

Class Dinophyceae F.E.Fritsch 1927
Order Peridiniales Haeckel 1894
Family Peridiniopsidaceae Gottschling *et al*. 2017
Genus *Palatinus* Craveiro *et al*. 2009

Palatinus apiculatus (Ehrenberg) Craveiro *et al*. 2009 (Figs. 2 and 3)

Reference: Craveiro, S. C., Calado, A. J., Daugbjerg, N., & Moestrup, Ø, Journal of Phycology 45: 1178, figs. 1–13. 2009.
Basionym: *Glenodinium apiculatum* Ehrenberg, Infusionsthierchen, p. 258, pl. XXII, fig. XXIV. 1838.
Homotypic synonyms: *Peridinium apiculatum* (Ehren-

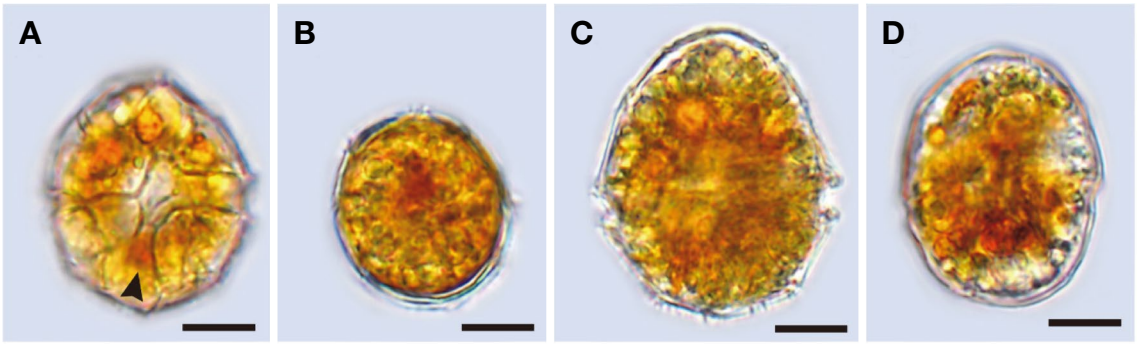

Fig. 2. Vegetative cells (A , C) and temporary cysts (B, D) of the Korean *Palatinus apiculatus* isolated from the Paldang Reservoir. An arrowhead represents an eyespot. Cell size is proportional to a given scale bar (10 μm).

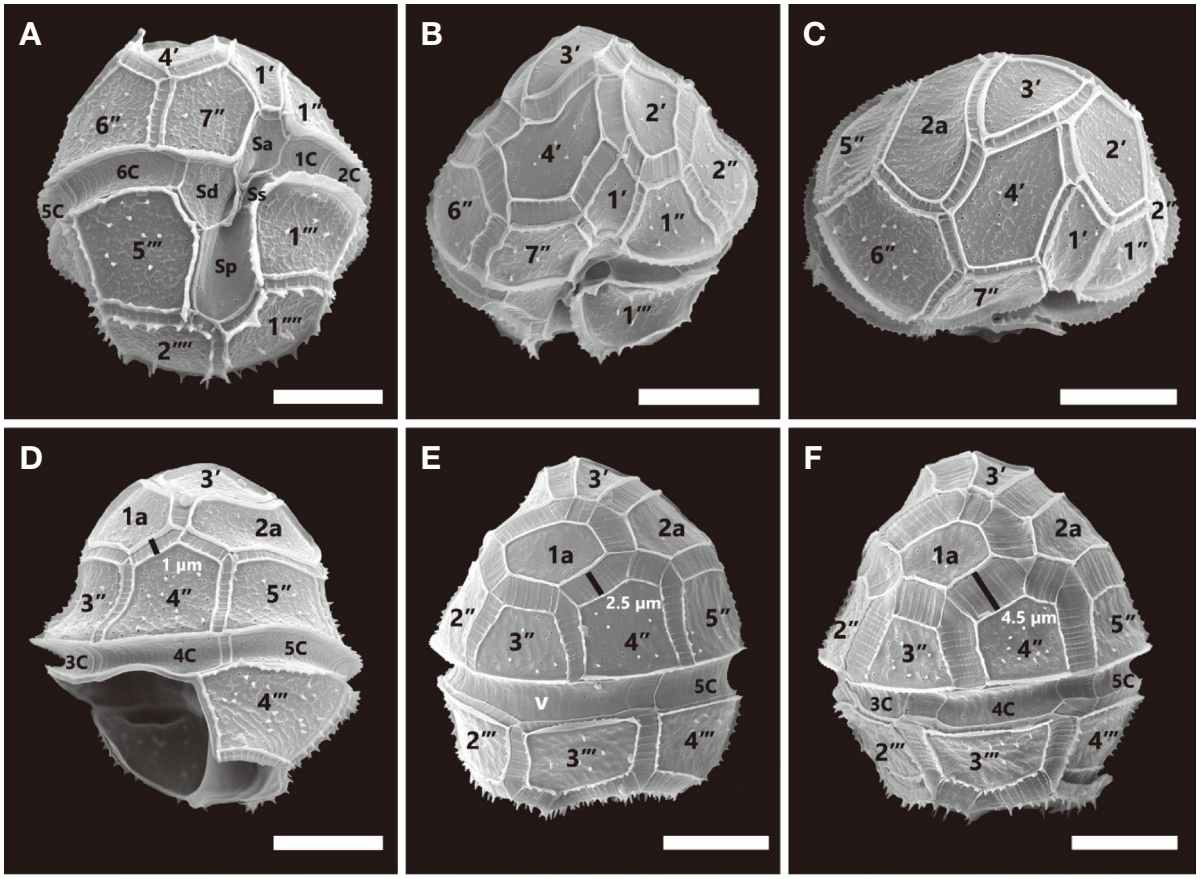

Fig. 3. Scanning electron microscrope micrographs of *Palatinus apiculatus* isolated from the Paldang Reservoir, Korea. Plates of the theca are indicated, following to Kofoidian plate formula. A: ventral view showing sulcal region; B, C: apical view from the ventral side; D–F: dorsal view showing different wide of suture and variation of the cingular plate (v). Thick black bar in D–F represents each given length. Sa: anterior sulcal plate. Sd: right sulcal plate. Sp: posterior sulcal plate. Ss: left sulcal plate. Scale bar = 10 μm.

berg) Claparède & Lachmann, Mémoires de l'Institut National Genevois 5: 404. 1859. *Properidinium apiculatum* (Ehrenberg) Meunier, Mémoires du Musée Royal d'Histoire Naturelle de Belgique 8: 60, pl. XVIII 47–52. 1919.

Description: Ovoid cell with a yellow-brownish color. 34.3 μm in length (25.8–39.5 μm) and 28.4 μm in width (21.5–34 μm). An eyespot is located near the sulcal region. The Kofoidian plates formula is 4′, 2a, 7″, 6c, 5s, 5‴, 2⁗, with no apical pore complex (APC). The epitheca twisted to the left in ventral view. The apical plate and intercalary plate are asymmetrically arranged in apical view. Plate surfaces are smooth or finely granulated, and pore is evenly placed on the surface of thecal plates. Several small spines are present on the plate surface, and they are usually clustered in the hypotheca. The suture between the thecal plates expands to 4.5 μm. A vertical pattern is present on the suture.

Taxonomic remarks: Plate variations of *Palatinus apciculatus* are mainly present in the number of intercalary and precingular plates (Craveiro *et al*., 2009; Kretschmann *et al*., 2018). In our study, new variations in cingular plates were the first to be observed. In specimens from Craveiro *et al*. (2009), plates 3C and 4C are in the down of the plates 3″ and 4″, respectively. However, present specimens showed a fusion of the 3C and 4C (Fig. 3E). The number of cingular plates is a key morphological feature that distinguishes the genus *Palatinus* and typical *Peridinium* [(e.g., *Peridinium* subg. *Cleistoperidinium* (Lemmermann) Lefèvre 1932] (Craveiro *et al*., 2009). The six cingular plates in the genus *Palatinus* are different from the five in the typical *Peridinium* group (Craveiro *et al*., 2009). Considering this, the variation of cingular plates should be carefully addressed. Therefore, detailed observation of cingular plates may be required in peridinioid species for clear classification. Compared to common variations described in the previous study (Craveiro *et al*., 2009), variations of cingular plates were rare in the present specimen. Therefore, it can be ignored for the identification of the genus *Palatinus*.

Table 1. DNA similarities of the *Palatinus* 28S rDNAs estimated among five pairs of the aligned sequence data (585 bp).

No.	Species and strain	[1]	[2]	[3]	[4]	[5]
[1]	***Palatinus apiculatus* FD-02 (ON358395)#**					
[2]	*Palatinus apiculatus* GeoM500 (KX710288)	100				
[3]	*Palatinus apiculatus* GeoB*762 (KY996787)	100	100			
[4]	*Palatinus apiculatus* AJC4cl-a (AF260394)	100	100	100		
[5]	*Palatinus pseudolaevis* AJC6-798 (AF260395)	91.4	91.4	91.4	91.4	
		DNA similarity (%)				

represents the isolate and sequence determined in the present study.

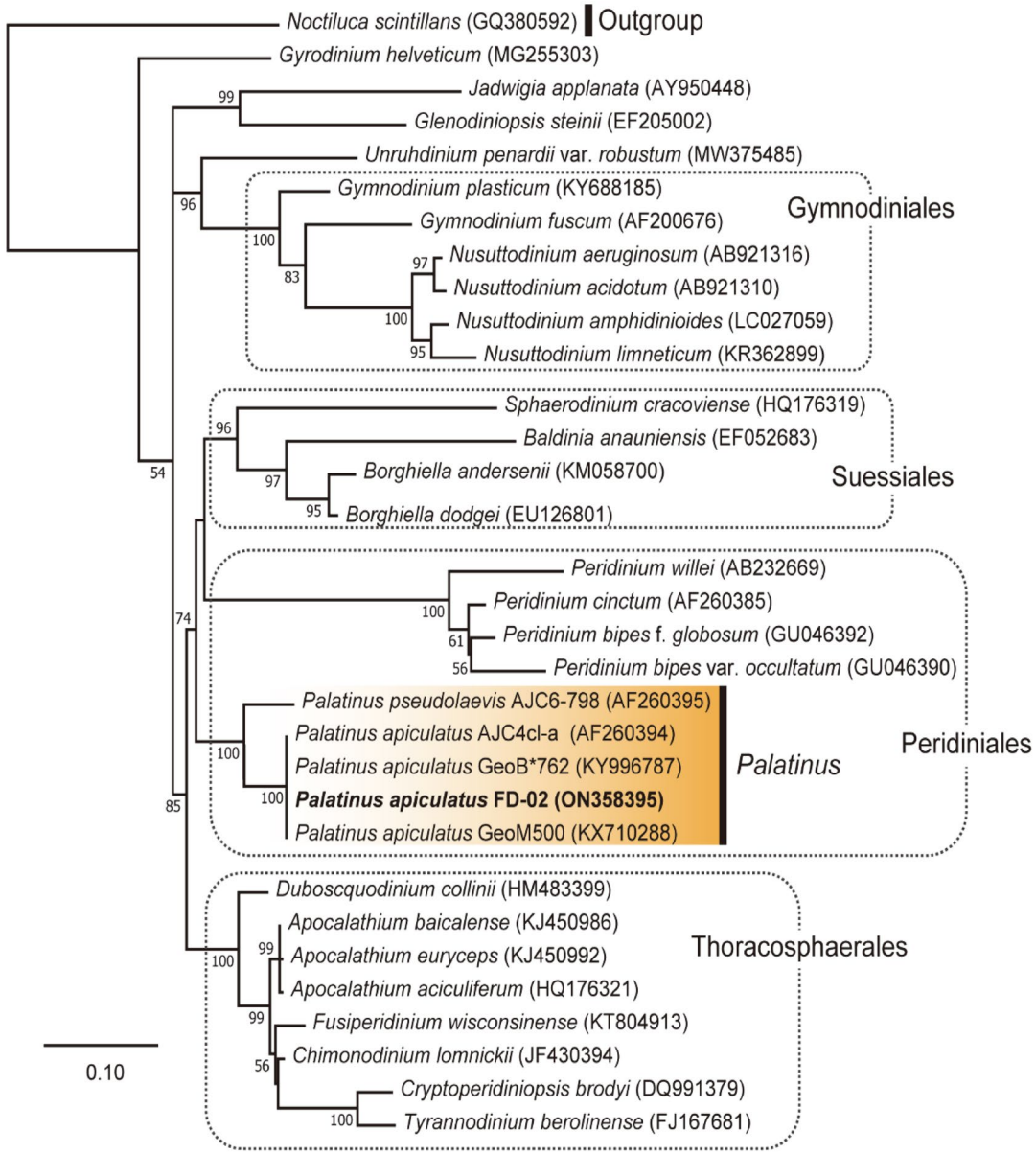

Fig. 4. A maximum likelihood tree constructed from a 28S rDNA dataset of the Korean *Palatinus apiculatus* and other freshwater dinoflagellates. A total of 5,000 replicates were run for bootstrap analyses. Members of the genus *Palatinus* are highlighted in orange. The isolate from this study and its GenBank No. are given in bold font.

Ecology: Korean *Palatinus apiculatus* was isolated from the cold water of a temperate reservoir. The water temperature of sampling site was 5.2°C. The bloom of this species was not observed.
Site of collection: Paldang Reservoir, Gyeonggi-do, Korea (GPS code: 37°39′15.63″N, 127°17′15.89″E; Fig. 1).
Date of collection: 12 December 2021.
Gene sequences: Partial 18S rRNA, whole 18S–28S ITS, and partial 28S rRNA gene sequences under GenBank Accession number ON358395.

Molecular affiliation of Korean *Palatinus apiculatus* by 18S and 28S rDNA

The region of 18S to 28S rDNA was sequenced from our *Palatinus apiculatus* isolates (GenBank Accession No. ON358395). BLAST searches showed that 18S rDNA sequence of our Korean *Palatinus apiculatus* was nearly identical (99.0% similarity) with already-known *Palatinus apiculatus* (KY996787). In addition, the sequence of 28S rDNA (585 bp) showed 100% DNA similarity with other *Palatinus apiculatus* sequences recorded in GenBank (Table 1). However, our species differed from *Palatinus pseudolaevis* with 91.4% DNA similarity in 28S rDNA. Phylogenetic relationships of *Palatinus apiculatus* and other freshwater dinoflagellates were investigated by using the 28S rDNA sequences (Fig. 4). Our 28S rDNA ML tree showed that the order Peridiniales was separated from other orders within dinoflagellates. In the Peridiniales, the genus *Palatinus* was clustered together and separated from *Peridinium* species (100% bootstrap value), indicating a difference in generic level (Craveiro *et al*., 2009; Kretschmann *et al*., 2018). Our *Palatinus* isolate clustered with other *Palatinus apiculatus* with 100% bootstrap support, forming a sister relationship with *Palatinus pseudolaevis*. These results suggest that the dinoflagellate isolated in the present study belongs to *Palatinus apiculatus* genetically.

In conclusion, we investigated the morphological and genetic traits of a cold water dinoflagellate isolated from the Paldang Reservoir. This species was identified as *Palatinus apiculatus* based on the plate formula (4′, 2a, 7″, 6c, 5s, 5‴, 2⁗), absence of APC and 28S rDNA phylogeny. This is the first record of *Palatinus apiculatus* from Korean freshwaters.

Acknowledgements

We would like to thank Dr. SD Lee for assisting in the scanning electron microscopy. This work was supported by grants from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR202212103 and NIBR 202203204).

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Submitted: May 17, 2022
Revised: July 11, 2022
Accepted: July 12, 2022