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New record of four spirotrichs (Protozoa, Ciliophora) from South Korea

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During a field survey of Korean ciliates, we collected four newly recorded species from different habitats, namely, *Apokeronopsis sinica* Liu *et al*., 2009; *Diophrys japonica* Hu, 2008; *Neokeronopsis asiatica* Foissner *et al*., 2010; and *Pseudoamphisiella lacazei* (Maupas, 1883) Song, 1996. The morphology of these species was investigated based on the observation of living cells, protargol-impregnated specimens and in scanning electron microscope. In this study, we provide a brief diagnosis, remarks, and photomicrographs for each species. Furthermore, we studied the phylogenetic position of *P. lacazei* because the Korean population has pretransverse cirri (vs. lacking in the authoritative Chinese population).

Keywords: freshwater, marine, protargol impregnation, taxonomy

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Introduction

Ciliates are one of the most abundant protist groups in all habitats including freshwater, marine, and terrestrial environments (Jung *et al*., 2017). According to National Institute of Biological Resources (2023), 718 ciliate species have been recorded in Korea. Despite this substantial number of recorded species, the ciliate diversity in South Korea remains relatively underexplored. To address this gap, we conducted research from 2022 to 2024 and collected water samples containing four ciliates (belonging to the genera *Apokeronopsis*, *Diophrys*, *Neokeronopsis*, and *Pseudoamphisiella*), previously unrecorded in Korea. Among these, three *Apokeronopsis* species (*A. bergeri* Li *et al*., 2008; *A. ovalis* (Kahl, 1932) Shao *et al*., 2009; and *A. wrighti* Long *et al*., 2008), five *Diophrys* species (*D. appendiculata* (Ehrenberg, 1838) Schewiakoff, 1893; *D. oligothrix* Borror, 1965; *D. pauciciliata* Choi *et al*., 2022; *D. quadrinucleata* Zhang *et al*., 2020; and *D. scutum* (Dujardin, 1841) Kahl, 1932), and one *Pseudoamphisiella* species (*P. alveolata* (Kahl, 1932) Song and Warren, 2000) have been recorded in Korea (Kwon and Shin, 2006; Kwon *et al*., 2008; Jung and Min, 2009; Jung *et al*., 2011; Kim *et al*., 2020; Chae and Min, 2022; Choi *et al*., 2022). The discovery of ciliate species in South Korea not only enhances our understanding of ciliate diversity, but also emphasizes the ecological roles they may play in their respective environments (Rosario *et al*., 2011; Kim *et al*., 2021; Liu *et al*., 2022). Future studies should focus on the distribution patterns of these species to provide deeper insights into the biodiversity of protists in South Korea.

In this study, we provide brief description including micrographs of living cells and protargol impregnated specimens and comparison of the closest relatives of these newly recorded species. These species are as follows: 1) *Apokeronopsis sinica* Liu *et al*., 2009; 2) *Diophrys japonica* Hu, 2008; 3) *Neokeronopsis asiatica* Foissner *et al*., 2010; and 4) *Pseudoamphisiella lacazei* (Maupas, 1883) Song, 1996.

Materials and Methods

Sample collection and identification

The four species were collected from various habitats from South Korea. The detailed information about sample locality is described in the section 'Material examined' for each species. Water samples were collected and transferred to the laboratory within a few days. All water samples were kept in Plant culture dishes at room temperature (ca. 20°C) for few months. Also, sterilized 1−2 rice grains were supplied for each culture to increase the number of bacteria as food resource. The morphology

of each species was studied using a stereomicroscope (SZ11; Olympus, Tokyo, Japan) and a light microscope (BX53; Olympus) with differential interference contrast at magnifications of 40−1000×. Micrographs were captured using a digital camera (DP74; Olympus). The protargol impregnation followed the 'procedure A' method of Foissner (2014) and the powder was synthesized using the method of Pan *et al*. (2013) and Kim and Jung (2017). To prepare scanning electron microscopy specimens, we followed the protocol of Foissner (2014). Image stacking of the protargol-impregnated specimens was conducted using the software of Helicon Focus 8.1.0 (HeliconSoft Ltd, Kharkiv, Ukraine). The basic terminology and taxonomic classification mainly followed Lynn (2008).

DNA extraction, PCR amplification, and sequencing

Five cells of *Pseudoamphisiella lacazei* were isolated from the raw culture using microcapillary under the stereomicroscope. Cells were washed using culture water filtered by a 0.2 μm syringe filter at least three times to remove other eukaryotes and each cell was transferred to a 1.5 mL centrifuge tube with a minimum volume of water. REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, Mo, USA) was used to extract genomic DNA. Slightly modified primers, namely New Euk A (5′-CTG GTT GAT YCT GCC AGT-3′) (Jung and Min, 2009) and LSU rev 4 (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) (Sonnenberg, 2007), were used to amplify 18S rDNA sequence. The PCR conditions were as follows: initial denaturation at 94°C for 1 min 30 s, followed by 40 cycles of denaturation at 98°C for 10 s, annealing at 58.5°C for 30 s, and extension at 72°C for 3 min, and a final extension step at 72°C for 7 min. After the amplification, the PCR products were purified using MG PCR Purification Kit (MGmed, Korea). Three internal primers were used for DNA sequencing: 18SF790v2, 18SF1470, 18SR300 (Jung *et al*., 2018). DNA sequencing was performed using an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA). Sequence fragments were assembled using Geneious 9.1.8 (Kearse *et al*., 2012).

Phylogenetic analyses

To determine the phylogenetic position of *Pseudoamphisiella lacazei* (PQ436461), 18S rDNA sequences of 40 ciliate species were obtained from the GenBank database, including *Blepharisma americanum* (M97909) as outgroup taxon. The sequences were aligned using Clustal W (Tompson *et al*., 1994) and both ends were trimmed in BioEdit 7.2.5.0 (Hall, 1999). jModeltest 2.1.7 (Darriba *et al*., 2012) was used to select best-fit substitution model (GTR+I+G) of alignment based on the Akaike information criterion (AIC). The maximum likelihood (ML) tree was constructed using IQ-Tree 1.6.12 with 100,000 bootstrap replicates. Bayesian inference (BI) analyses was constructed using MrBayes 3.1.7 (Ronquist *et al*., 2012) with Markov chain Monte Carlo (MCMC) for 1,000,000 generations with a sampling frequency of every 100 generations and the first 3,000 trees were discarded as burn-in. Geneious 2024.0 was used for calculating the similarity between the Korean and Chinese population of *P. lacazei*. FigTree 1.4.4 (Rambaut, 2012) was used to visualize the phylogenetic trees.

Results and Discussion

Phylum Ciliophora Doflein, 1901
Class Spirotrichea Bütschli, 1889
Order Urostylida Jankowski, 1979
Family Pseudokeronopsidae Borror and Wicklow, 1983
Genus *Apokeronopsis* Shao *et al*., 2007

1. *Apokeronopsis sinica* Liu *et al*., 2009 (Fig. 1)

Material examined. Marine water (salinity 32.9‰, temperature 20.1°C) collected from Boritgol-gil, Gampo-eup, Gyeongju-si, Gyeongsangbuk-do, South Korea (35°49′35″N, 129°30′37″E) on October 22, 2023.

Diagnosis. Size 152−192×68−77 μm in vivo and 81−129×36−54 μm after protargol impregnation (n=11); body elliptical with narrow posterior end; cytoplasm colorless; body flexible and non-contractile (Fig. 1A−C); contractile vacuole at 40% of body length (Fig. 1C); two types of cortical granules: smaller one colorless, about 0.2 μm in diameter, arranged in longitudinal rows, and larger one slightly yellowish, about 2×1 μm in size, scattered in whole body (Fig. 1D, E); adoral zone, composed of 45−61 membranelles, occupies about 50% of body length in staining specimens; frontal cirri (bicorona) consist of 17−26 cirri total, 8−12 in anterior corona, and 9−14 in posterior corona; two frontoterminal, 2−5 buccal, and 9−11 transverse cirri; midventral complex composed of 20−31 left and 22−33 right midventral cirri; one left (27−41 cirri) and one right (33−46) marginal cirral rows (Fig. 1F); three dorsal kineties (Fig. 1G); and about 100 macronucleus nodules, 4−7 micronuclei, distributed in whole body (Fig. 1F, G).

Distribution. Hong Kong (type locality, Liu *et al*., 2009), Korea (in this study).

Remarks. The Korean population of *Apokeronopsis sinica* is similar to the type population described by Liu *et al*. (2009) in most features except for body width (36−54 μm vs. 72−105 μm after protargol impregnation; very likely resulted from different impregnation methods) (Liu *et al*., 2009). Among *Apokeronopsis* species, *A. antarc-*

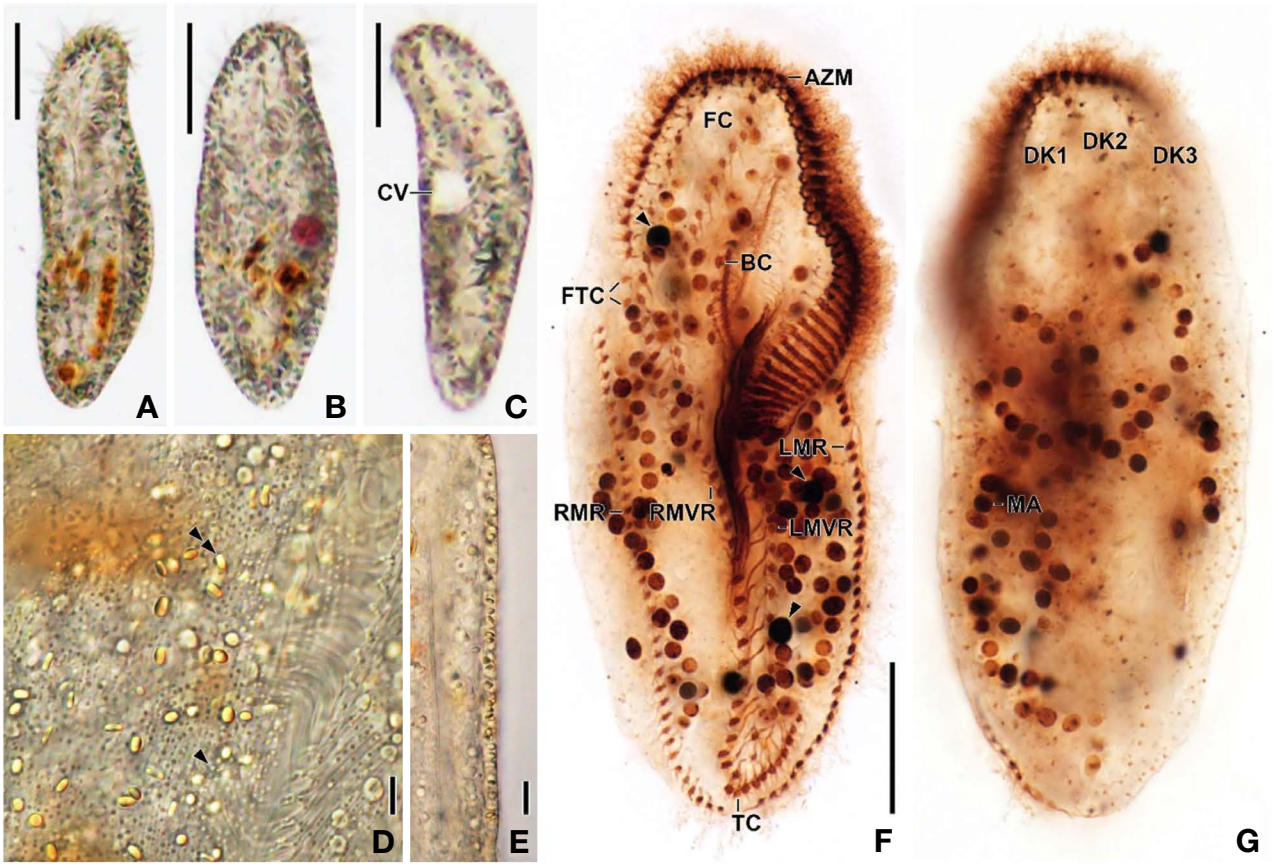


Fig. 1. *Apokeronopsis sinica* in vivo (A–E) and after protargol impregnation (F, G). A, B. Ventral view showing the body shape. C. Dorsal view showing a contractile vacuole. D, E. Two types of cortical granules: smaller one (arrowhead) arranged in the longitudinal rows, and larger one (double-arrowhead) scattered in the whole body. F, G. Ventral (F) and dorsal (G) view showing the adoral zone of membranelles, midventral complex, the nuclear pattern, and three dorsal kineties. Arrowheads denote micronuclei. AZM, adoral zone of membranelles; BC, buccal cirri; CV, contractile vacuole; DK, dorsal kineties; FC, frontal cirri; FTC, frontoterminal cirri; LMR, left marginal row; LMVR, left midventral row; MA; macronuclear nodules; RMR, right marginal row; RMVR, right midventral row; TC, transverse cirri. Scale bars: 50 μm (A, B); 40 μm (C); 20 μm (F); 10 μm (E); 5 μm (D).

tica (Petz, 1995) Shao *et al*., 2007, *A. crassa* (Claparède and Lachmann, 1858) Shao *et al*., 2007, and *A. wrighti* can be easily distinguished from *A. sinica* by possessing more than 20 transverse cirri (vs. less than 20) (Shao *et al*., 2007; Long *et al*., 2008). Considering the number of transverse cirri, *A. sinica* is similar to *A. bergeri* and *A. ovalis*, which were already recorded in Korea by Jung *et al*. (2011). However, the Korean population of *A. sinica* differs from *A. bergeri* by the number of adoral membranelles (45–61 vs. 72–106), buccal cirri (2–5 vs. 9–15), transverse cirri (9–11 vs. 11–22), and left and right marginal cirri (27–41 vs. 44–70; 33–46 vs. 54–88) (Li *et al*., 2008; Jung *et al*., 2011). Also, the body shape of *A. bergeri* differs from that of *A. sinica* in having an elongate to fusiform body with both ends broadly rounded (vs. elliptical body with posterior end narrowed) (Li *et al*., 2008). On the other hand, *A. sinica* can be distinguished from *A. ovalis* by the body size after protargol impregnation (81–129 × 36–54 μm vs. 120–175 × 55–90 μm) (Shao *et al*., 2008, population I; Jung *et al*., 2011), the body shape (elliptical body with posterior end narrowed vs. oval to elliptical), and the color of the large cortical granules (slightly yellowish vs. green to yellow-greenish) (Shao *et al*., 2008).

Voucher slides. Three slides with protargol-impregnated specimens were deposited at the National Institute of Biological Resources (NIBRPR0000111269, NIBRPR0000111270, NIBRPR0000111271).

Family Urostylidae Bütschli, 1889
Genus *Pseudoamphisiella* Song, 1996

2. *Pseudoamphisiella lacazei* (Maupas, 1883) Song, 1996 (Fig. 2, 3)

Material examined. Marine water (salinity 33.9‰, tem-

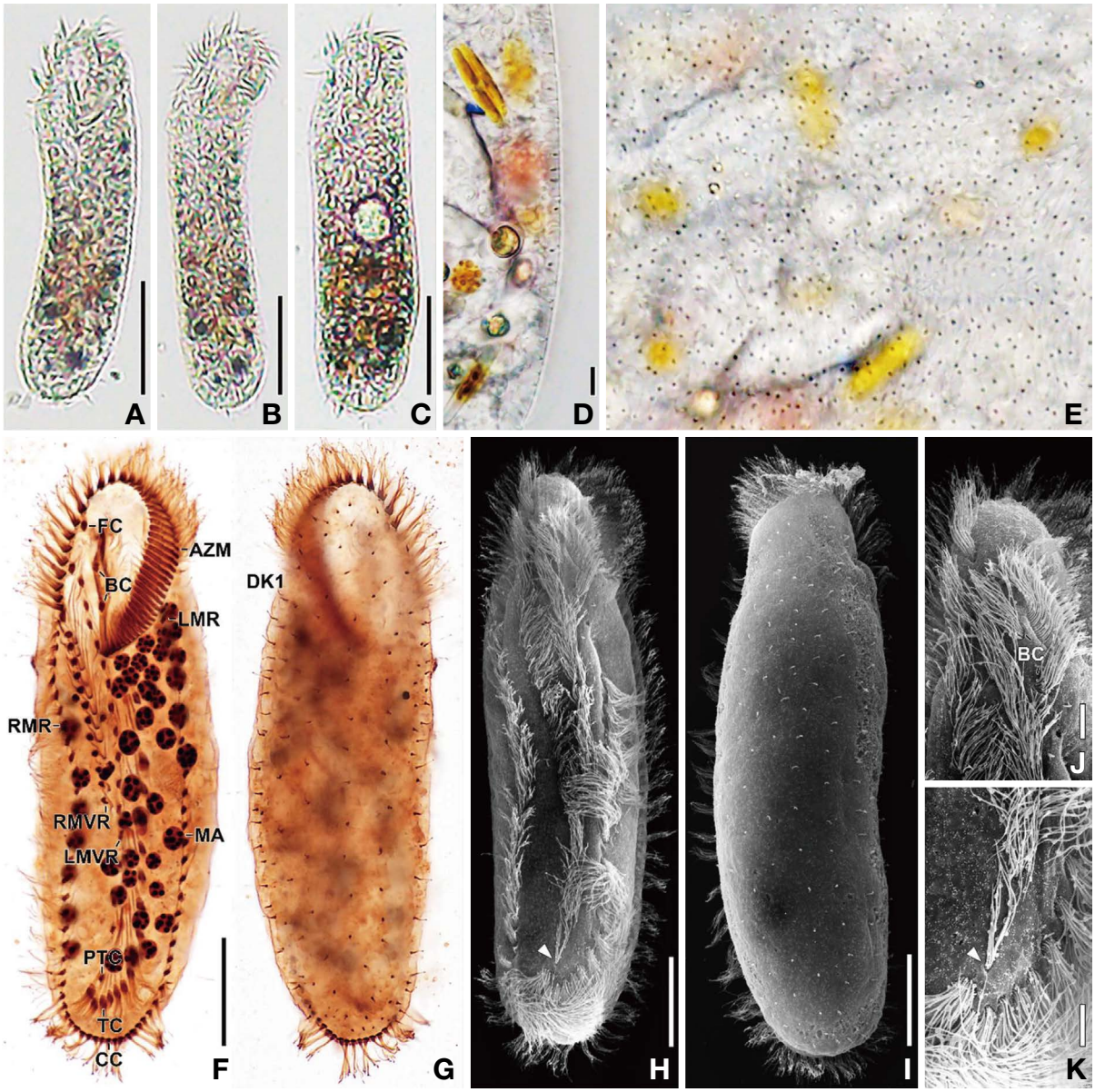


Fig. 2. *Pseudoamphisiella lacazei* in vivo (A–E), after protargol impregnation (F, G), and in the scanning electron microscope (H–K). A–C. Ventral views showing various body shape. D, E. Bar shaped extrusomes, loosely distributed throughout the cortex. F, G. Ventral (F) and dorsal (G) view showing the adoral zone of membranelles, macronucleus nodules, pretransverse cirri and dorsal kineties. H, I. Ventral (H) and dorsal (I) view showing typical morphology and ciliature. Arrowhead in H denotes pretransverse cirrus. J, K. Ventral views showing the buccal cirri and pretransverse cirrus (arrowhead). AZM, adoral zone of membranelles; BC, buccal cirri; CC, caudal cirri; DK, dorsal kineties; FC, frontal cirri; LMR, left marginal row; LMVR, left midventral row; MA, macronuclear nodules; PTC, pretransverse cirri; RMR, right marginal row; RMVR, right midventral row; TC, transverse cirri. Scale bars: 50 μm (A–C); 20 μm (F, H, I); 10 μm (D); 5 μm (J, K).

perature 10.5°C) collected from Sodol beach, Jumun-ri, Junumjin-eup, Gangnueng-si, Gangwon-do, South Korea (37°54′24″N, 128°49′40″E) on January 31, 2023.

Diagnosis. Size 165–202 × 37–53 μm in vivo and 93–155 × 34–45 μm after protargol impregnation (n = 11); elliptical body with both ends broadly rounded; cytoplasm colorless; body flexible and slightly contractile (Fig. 2A–C); extrusomes bar shaped, about 2 μm long, loosely distributed throughout the cortex (Fig. 2D, E); adoral zone, composed of 40–52 membranelles, occupies about 30% of body length in staining specimens; two buccal, three frontal, and 1–2 pretransverse cirri, and 13–20 transverse cirri arranged in very long J-shape (Fig. 2F, H, J, K); two midventral rows: right row relatively short, composed of 9–15 cirri; and left row relatively long, composed of 14–21 cirri; one left (23–32

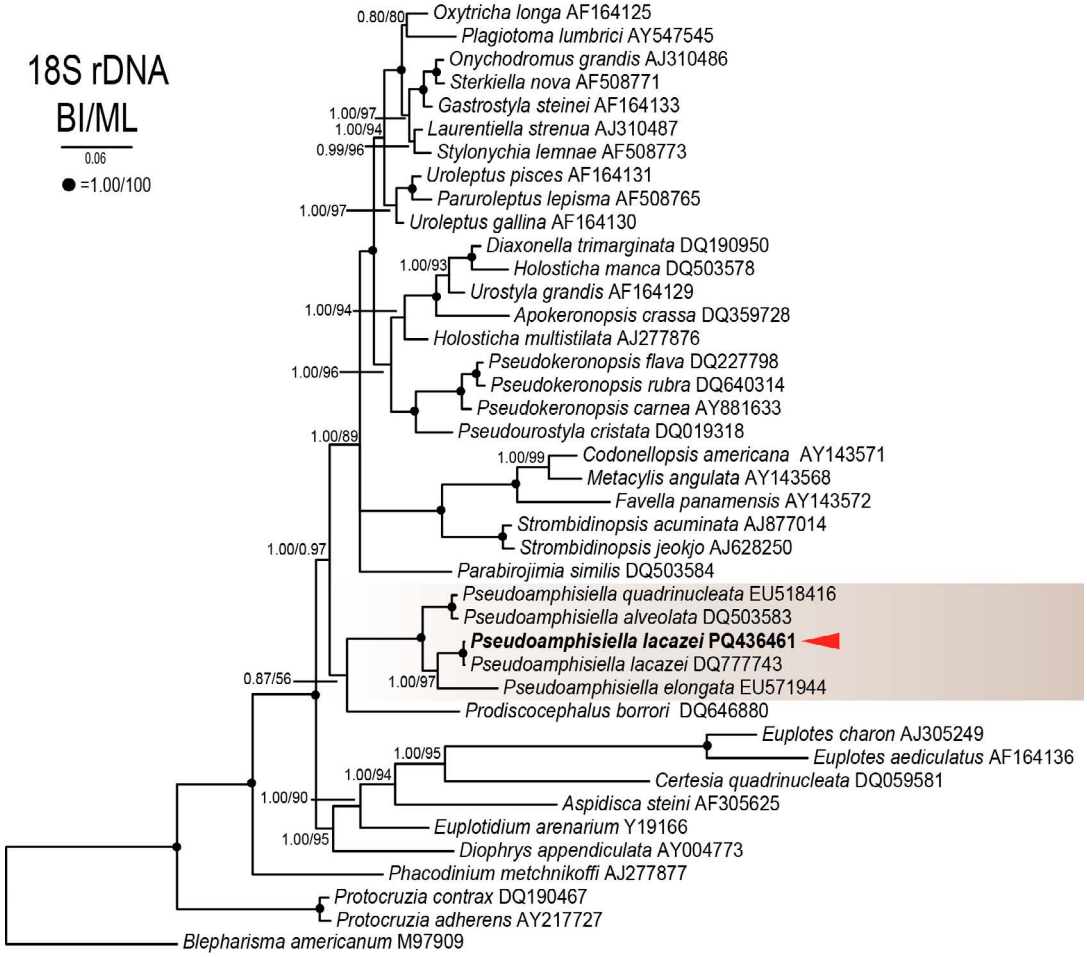


Fig. 3. Bayesian inference (BI) tree based on 18S rDNA sequences, showing the phylogenetic position of the Korean population of *Pseudoamphisiella lacazei* (arrowhead). GenBank accession numbers follows species names. Numbers at nodes represent the Bayesian inference (BI) posterior probabilities and the maximum likelihood (ML) bootstrap values. The scale bar represents six nucleotide substitutions per 100 nucleotides.

cirri) and one right (23–30) marginal cirral rows (Fig. 2F, H); 11–20 caudal cirri, between posterior ends of marginal rows (Fig. 2F); 9–11 dorsal kineties (Fig. 2G, I); about 50 macronuclear nodules, each 4–12 × 4–5 μm after protargol impregnation; micronucleus not observed (Fig. 2F).

Distribution. Algeria (type locality, Maupas, 1883), Baltic Sea (Telesh *et al*., 2009), Brazil (Kattar, 1970), China (Song *et al*., 1996), Germany (Kahl, 1932), USA (Torch, 1960), Korea (in this study).

Remarks. Up to date, four species are assigned to the genus *Pseudoamphisiella*, namely, *P. alveolata*; *P. elongata* Li *et al*., 2010; *P. lacazei*; and *P. quadrinucleata* Shen *et al*., 2008 (Song, 1996; Song and Warren, 2000; Berger, 2006; Shen *et al*., 2008; Li *et al*., 2010). The Korean population of *P. lacazei* is similar to the Chinese population described by Song (1996) in all aspects except for the presence of pretransverse cirri. Song (1996) reported that the Chinese population of *P. lacazei* lacks pretransverse cirri, however, the Korean population has usually one and occasionally two pretransverse cirri (Fig. 2F, H, K) (Song, 1996). Despite this morphological difference, the two populations show a similarity of 99.94% in the 18S rDNA sequence and cluster together with full supporting values (100% ML and 1.00 BI) in the phylogenetic trees (Fig. 3). However, since the difference between the Chinese and the Korean population is only one nucleotide, the establishment of a new species or subspecies based on the presence (vs. absence) of pretransverse cirri seems not justified at the present state of knowledge. *Pseudoamphisiella elongata* is most similar to *P. lacazei*, except for the number of right midventral cirri (15–21 vs. 9–15), caudal cirri (6–7 vs. 11–20), and dorsal kineties (6–7 vs. 9–11) (Li *et al*., 2010). Additionally, *P. alveolata* differs from *P. lacazei* by the number of left and right marginal cirri (14–20 vs. 23–32 and 12–14 vs. 23–30), and macro-

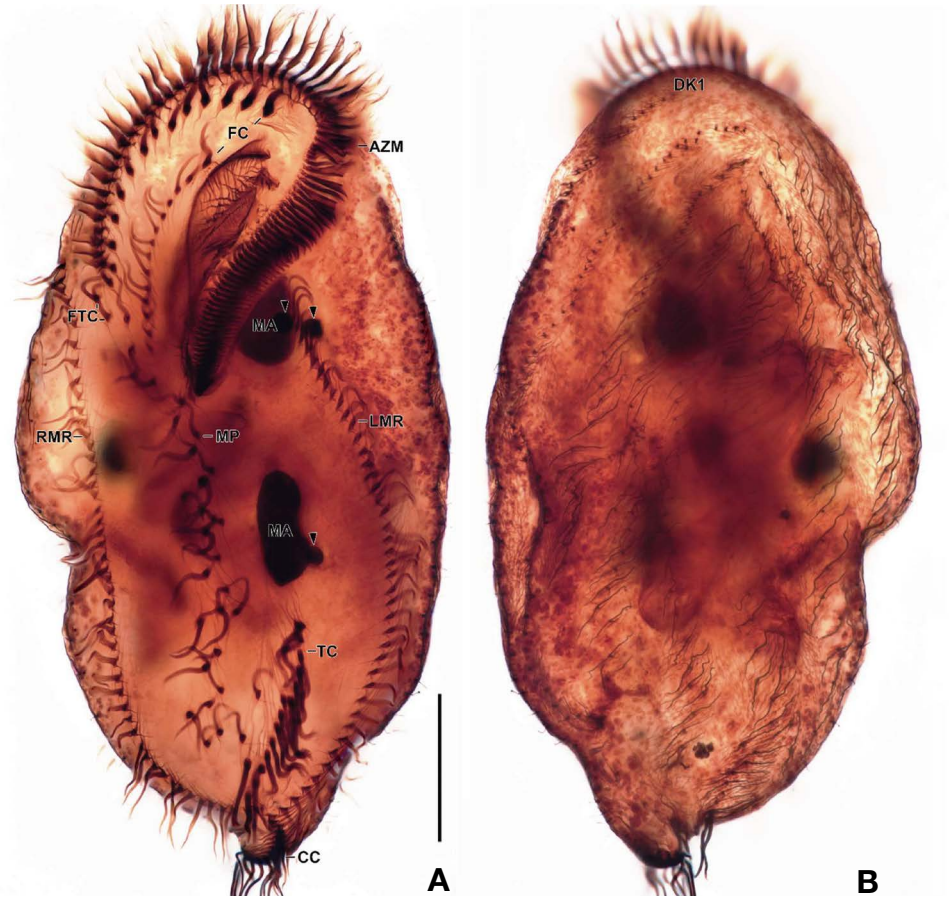


Fig. 4. *Neokeronopsis asiatica* after protargol impregnation (A, B). A. Ventral view showing the adoral zone of membranelles, frontal, frontoterminal, caudal, and transverse cirri, and left and right marginal rows. Arrowheads denote micronuclei. B. Dorsal view showing dorsal kineties. AZM, adoral zone of membranelles; CC, caudal cirri; DK, dorsal kineties; FC, frontal cirri; FTC, frontoterminal cirri; LMR, left marginal row; MA, macronuclear nodules; MP, midventral pairs; RMR, right marginal row; TC, transverse cirri. Scale bar: 50 μm.

nuclear nodules (2 vs. about 50) (Song and Warren, 2000). Also, *P. quadrinucleata* differs from *P. lacazei* by the number of adoral membranelles (52−69 vs. 40−52) and macronuclear nodules (4 vs. about 50) (Shen *et al*., 2008).
Voucher slides. Three slides with protargol-impregnated specimens were deposited at the National Institute of Biological Resources (NIBRPR0000111272, NIBRPR 0000111273, NIBRPR0000111274).

Order Sporadotrichida Fauré-Fremiet, 1961
Family Oxytrichidae Ehrenberg, 1830
Genus *Neokeronopsis* Warren *et al*., 2002

3. *Neokeronopsis asiatica* Foissner *et al*., 2010 (Fig. 4)

Material examined. Freshwater (salinity 0.1‰, temperature 6.1°C) collected from Namdaechoen, Seongnea-dong, Gangnueng-si, Gangwon-do, South Korea (37°44′46″N, 128°52′52″E) on March 11, 2024.
Diagnosis. Size 222−267 × 110−144 μm after protargol impregnation (n = 3); body elliptical with indentation at site of caudal cirri and slightly flexible; yellowish under low magnification; adoral zone occupies about 40% of body length in staining specimens and composed of 81−86 membranelles (Fig. 4A), bases of longest membranelles about 16−17 μm long; bicorona consists of 18−28 frontal cirri, 10−14 cirri in anterior corona and 8−14 cirri in posterior corona (Fig. 4A); buccal cirrus lacking; 2−3 frontoterminal, and 15−17 transverse cirri; 12−15 midventral pairs; one left (43−48 cirri) and one right (38−49) marginal cirral rows (Fig. 4A); three caudal cirral rows consist of 9−14 cirri; 11−12 dorsal kineties (Fig. 4B); two circular to ellipsoidal macronuclear nodules, each 29−39 × 17−21 μm after protargol impregnation; 1−3 globular micronuclei, about 7 μm in diameter after protargol impregnation (Fig. 4A).
Distribution. Antarctica (Jung *et al*., 2015), China (type locality, Foissner *et al*., 2010), Korea (in this study).
Remarks. In the genus *Neokeronopsis*, two species have been recorded, *N. asiatica* and *N. spectabilis* (Kahl, 1932)

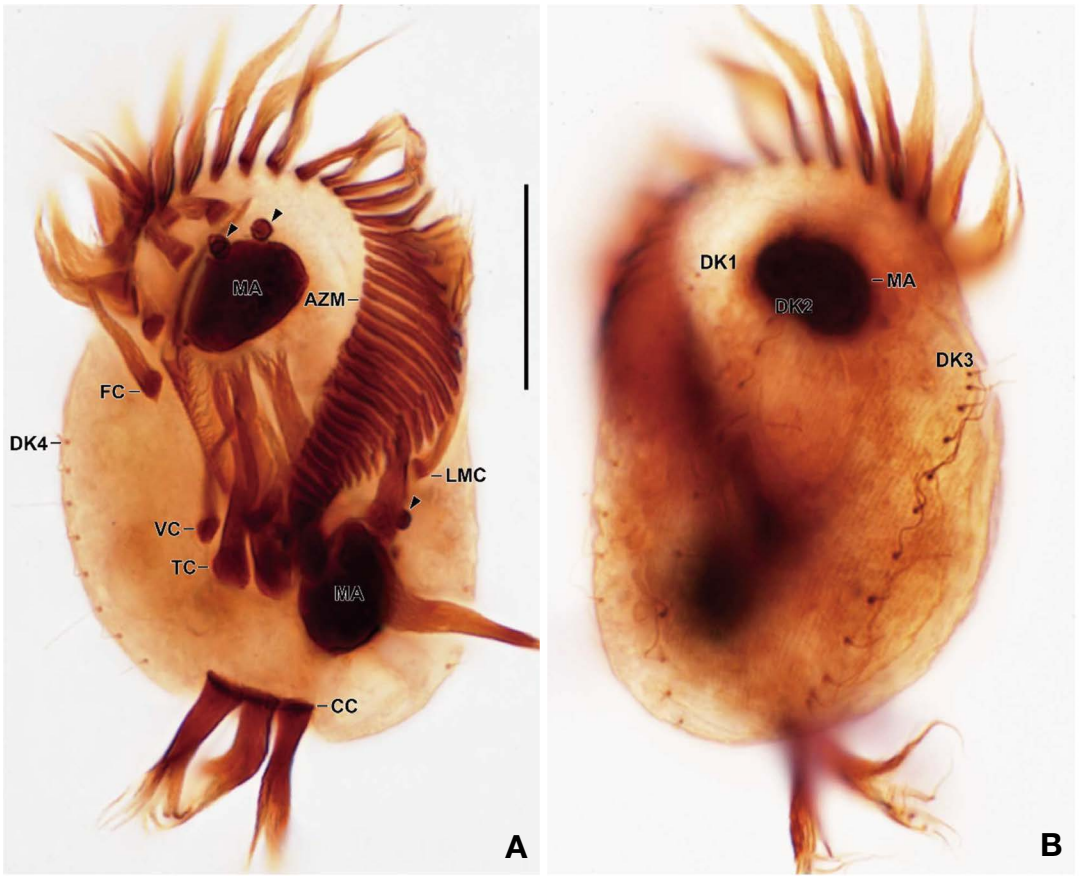


Fig. 5. *Diophrys japonica* after protargol impregnation (A, B). A. Ventral view showing the body shape, adoral zone of membranelles, frontal, ventral, transverse, caudal and leftmarginal cirri, and nuclear apparatus. Arrowheads denote micronuclei. B. Dorsal view showing dorsal kineties. AZM, adoral zone of membranelles; CC, caudal cirri; DK, dorsal kineties; FC, frontal cirri; LMC, left marginal cirrus; MA, macronuclear nodules; TC, transverse cirri; VC, ventral cirri. Scale bar: 20 μm.

Warren *et al*., 2002 (Warren *et al*., 2002; Foissner *et al*., 2010). The Korean population of *Neokeronopsis asiatica* corresponds to the type population described by Foissner *et al*. (2010) and the Antarctica population described by Jung *et al*. (2015) in all available aspects. However, unfortunately, since we were unable to observe enough materials in vivo for the Korean population, some features cannot be compared with those of the Antarctic population described by Jung *et al*. (2015). *Neokeronopsis asiatica* differs from *N. spectabilis* by the body size (222–267 × 110–144 μm vs. 360–500 × 150–200 μm after protargol impregnation), the shape of posterior body end (indentation at the site of caudal cirri vs. broadly rounded without indentation), the bases of the longest adoral membranelles (16–17 μm vs. 22–35 μm), and the number of caudal cirri (9–14 vs. 6–9) (Warren *et al*., 2002; Berger, 2006; Foissner, 2010).

Voucher slides. Two slides with protargol-impregnated specimens were deposited at the National Institute of Biological Resources (NIBRPR0000111275, NIBRPR 0000111276).

Order Euplotida Small and Lynn, 1985
Family Uronychiidae Jankowski, 1975
Genus *Diophrys* Dujardin, 1841

4. *Diophrys japonica* Hu, 2008 (Fig. 5)

Material examined. Marine water (salinity unknown, temperature 17.0°C) collected from Gangdong-myeon, Gangneung-si, Gangwon-do, South Korea (37°45′48″N, 128°57′53″E) on August 24, 2022.

Diagnosis. Size 47–76 × 35–53 μm after protargol impregnation (n = 11); body broadly elliptical with distinct concavity at right posterior portion (Fig. 5A); adoral zone, composed of 36–43 membranelles, occupies about 70% of body length in staining specimens (Fig. 5A); five frontal, two ventral, and five transverse cirri; always one left marginal and three caudal cirri (Fig. 5A); four continuous dorsal kineties (Fig. 5B); two ovoidal to ellipsoidal macronuclear nodules, 11–15 × 8–11 μm each after protargol impregnation (Fig. 5A, B); 2–5 globular micronuclei, 1.3–2.2 μm in diameter, attached to the macronuclear nodules

(Fig. 5A).

Distribution. Japan (type locality, Hu, 2008), Korea (in this study).

Remarks. The Korean population of *Diophrys japonica* corresponds to the Japanese population in all aspects except for the body length (47−76 μm vs. 91−142 μm after protargol impregnation) (Hu, 2008, population I). Hu (2008) provided the morphometric data based on three populations (i.e., populations I, II, IV) and the populations II and IV shows similar body length to the Korean one (60−82 μm, 58−112 μm). Among the twelve species belonging to the genus *Diophrys*, excluding the poorly-known species *D. salina* Ruinen, 1938 and *D. peloetes* Borror, 1965, five species have sausage-shaped macronucleus nodules (*D. apoligothrix* Song *et al*., 2009; *D. appendiculata*; *D. parappendiculata* Shen *et al*., 2011; *D. oligothrix*; and *D. scutum*), while five species have ovoidal to ellipsoidal macronucleus nodules (*D. blakeneyensis* Hu *et al*., 2012; *D. japonica*; *D. pauciciliata*; *D. peculiaris* Luo *et al*., 2014; and *D. quadrinucleata*) (Zhang *et al*., 2020; Choi *et al*., 2022). Considering the shape of the macronucleus nodules, *D. japonica* should be compared with the latter five species. *Diophrys japonica* can be easily distinguished from *D. blakeneyensis* and *D. quadrinucleata* by the number of macronucleus nodules (2 vs. 7−23 in *D. blakeneyensis* and 4 in *D. quadrinucleata*) (Hu *et al*., 2012; Zhang *et al*., 2020). Additionally, *D. blakeneyensis* differs from *D. japonica* by the number of frontal cirri (7 vs. 5) and dorsal kineties (5 vs. 4) (Hu *et al*., 2012). *Diophrys pauciciliata* and *D. peculiaris* are also similar to *D. japonica*. However, *D. pauciciliata* differs from *D. japonica* by having a bipartite adoral zone of membranelles (vs. continuous) and five fragmented dorsal kineties (vs. four continuous dorsal kineties) (Choi *et al*., 2022). Also, *D. peculiaris* differs from *D. japonica* by the number of left marginal cirri (2 vs. 1) (Luo *et al*., 2014).

Voucher slides. Two slides with protargol-impregnated specimens were deposited at the National Institute of Biological Resources (NIBRPR111183, NIBRPR111184).

Conflicts of Interest

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

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