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# Taxonomic Revision of *Notohymena gangwonensis* (Protozoa: Ciliophora), with Notes on Its Cortical Granules and Scanning Electron Micrographs

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## ABSTRACT

From a moss sample, we isolated and identified *Notohymena gangwonensis* Kim et al., 2019 based on morphological and molecular data. The moss and type population has completely identical 18S rRNA (nuclear small subunit ribosomal RNA) gene sequences and both are highly similar in morphological and morphometric attributes, except for the diameter and arrangement of the cortical granules. Thus, we reexamined the type materials (i.e., micrographs and gDNA) and resulted in finding mistakes made by the authors of the species. Based on these data and supporting materials newly obtained (i.e., internal transcribed spacer [ITS] 1, ITS2, 5.8S, and partial 28S rDNA sequences, and scanning electron micrographs), we provide improved diagnosis of the species to clarify its identity. In addition, a key for *Notohymena* species is provided.

**Keywords:** cortical granulation, hypotrich, Korea, terrestrial ciliate, type material

## INTRODUCTION

The species belonging to the genus *Notohymena* Blattterer and Foissner, 1988 mainly occur in terrestrial habitats and up to date, eight species have been assigned into this genus, including *N. gangwonensis* Kim et al., 2019 (see Berger, 1999; Küppers et al., 2007; Kamra and Kumar, 2010; Foissner, 2016; Naqvi et al., 2016; Kim et al., 2019). They are characterized by a distinct paroral membrane among typical 18-cirri oxytrichid ciliates (Berger, 1999). According to Kumar and Foissner (2015) who defined seven patterns of paroral membrane in oxytrichids, the paroral membrane of *Notohymena* curves upward at distal end that is easily recognizable after protargol impregnation.

Cortical granulation is an important diagnostic key to identify soft oxytrichids at a species-level and can be classified based on a combination of the following features: (1) presence/absence, (2) size, (3) shape, (4) arrangement, and (5) color (Berger, 1999). Thus, insufficient/incorrect description of the cortical granules might cause a misidentification. Recently, based on a further study on the cortical granules, Foissner (2016) synonymized *N. pampasica* Küppers et al., 2007 as a junior synonym of *N. antarctica* Foissner, 1996. Of the congeners (even among oxytrichids), *N. gangwonensis* peculiarly has the largest cortical granules, i.e., about 2.5 μm across (Kim et al., 2019), that is unusual among oxytrichids having a mucocyst-type (Berger, 1999).

By studying Korean ciliate diversity, we found *N. gangwonensis*-like population that has similar morphological features and morphometrics, and completely identical 18S rDNA sequence with the type population, except for the size and arrangement of the cortical granules. To clarify this discrepancy between these molecular and morphological data, we reexamined type materials (i.e., gDNA and micrographs) of *N. gangwonensis* and analyzed the data newly obtained from this study, that is four ribosomal genes (internal transcribed spacer [ITS] 1, ITS2, 5.8S, and partial 28S), protargol preparations, and scanning electron micrographs.



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## MATERIALS AND METHODS

### Sample collection and identification

*Notohymena gangwonensis* was collected and isolated from a terrestrial moss from Mt. Oedaesan, South Korea (Table 1). The moss was air-dried for two months and rewetted with mineral water to induce excystment of the ciliate using the non-flooded Petri dish method (Foissner et al., 2002). This raw culture was maintained at room temperature for morphometry and DNA analyses.

From the raw culture, cells were examined *in vivo* under a stereomicroscope (SZ11; Olympus, Japan) and light microscope (BX53; Olympus), using both bright field and differential interference contrast at magnifications of 50–1,000×. Protargol staining was conducted using acetone developer and synthesized protargol (Foissner, 2014; Kim and Jung, 2017). According to the method provided by Foissner (2014), cells were observed using scanning electron microscope. The fixed cells were attached on a coverslip using poly-L-lysine rather than using the Foissner (2014)'s brass chamber. The general terminology/classification followed Lynn (2008) and the specific terms of oxytrichids followed Berger (1999). For the type of buccal lips, we followed Foissner and Al-Rasheid (2006).

### DNA extraction, PCR amplification, and sequencing

Four cells of *Notohymena gangwonensis* were isolated from the raw culture and transferred to distilled water more than five times to remove other eukaryotes from ambient water. Each cell was then transferred to a 1.5 mL tube. Genomic DNA was extracted using a RED-Extract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO, USA) using 1/10 volume of the instruction. The conditions for PCR were as follows: 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. The PCR product that nearly covered the entire 18S rRNA gene to patial 28S rRNA gene was amplified using slightly modified versions of two primers (New Euk A and LSU rev3/4) that were described by Sonnenberg et al. (2007). After the amplification, the PCR products were purified using a MEGAquickspin Total Fragment DNA Purification Kit (iNtRON, Korea), and DNA sequencing was performed using internal primers (18SF790v2: 5′-AAA TTA KAG TGT TYM ARG CAG-3′, 18SF810: 5′-GCC GGA ATA CAT TAG CAT GG-3′, 18SR300: 5′-CAT GGT AGT CCA ATA CAC TAC-3′, and 18SF1470: 5′-TCT GTG ATG CCC TTA GAT GTC-3′) and an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA). The anterior 18S rRNA gene sequences (about 800 bp) of the four cells were completely identical; thus, one of them was completely sequenced using the above internal primers. For type population of *N. gangwonensis*, the same genetic materials studied by Kim et al. (2019) was amplified to get the concatenated genes ITS1-5.8S-ITS2-partial 28S (Table 1). Considering a mitochondrial gene marker, we failed to amplify a cytochrome *c* oxidase subunit 1 (CO1) gene using primers developed by Park et al. (2019). Sequence fragments were assembled using Geneious 9.1.8 (Kearse et al. 2012).

## SYSTEMATIC ACCOUNTS

Phylum Ciliophora Doflein, 1901
Class Spirotrichea Bütschli, 1889
Order Sporadotrichida Fauré-Fremiet, 1961
Family Oxytrichidae Ehrenberg, 1830
Genus *Notohymena* Blatterer and Foissner, 1988

***Notohymena gangwonensis* Kim, Jung and Min, 2019**
*Notohymena gangwonensis* Kim et al., 2019: 740.

**Improved diagnosis.** Size 70–105×20–35 μm *in vivo*; body outline elongated elliptical; two macronuclear nodules and 1–4 micronuclei; contractile vacuole left and slightly anterior to mid-body; two types of globular colorless cortical granules, large one, measuring about 1.5 μm, along dor-

**Table 1.** List of *Notohymena gangwonensis* populations analysed in this study

| Population | Date | Latitude/longitude | GenBank accession No. | Reference |
|---|---|---|---|---|
| Type population isolated from dried soil with twigs | 8 Nov 2014 | 38°20′11″N/128°31′01″E | MK482065[a], **MN977116**[b] | Kim et al. (2019), this study |
| Moss population | 15 Apr 2017 | 37°48′21″N/128°41′32″E | **MN977117**[c] | This study |

A sequence newly obtained is in bold.
ITS, internal transcribed spacer.
[a]18S, 1,749 bp.
[b]Partial 18S–ITS1–5,8S–ITS2–partial 28S, 1,120 bp.
[c]18S–ITS1–5,8S–ITS2–partial 28S, 2,869 bp.

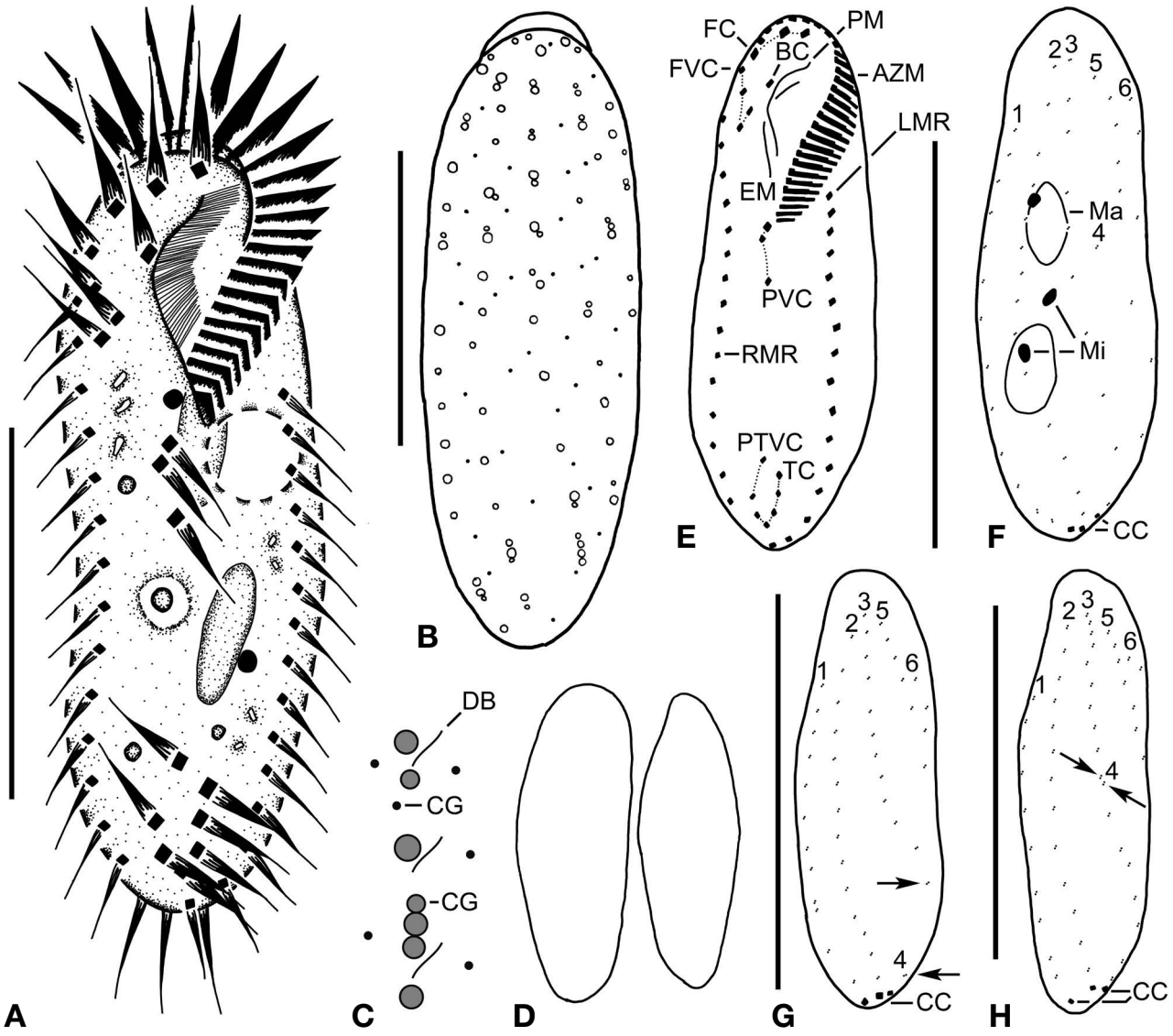


**Fig. 1.** *Notohymena gangwonensis*, moss population from life (A–D) and after protargol impregnation (E–H). A, Ventral view of a representative specimen; B, C, Cortical granulation on dorsal sides; D, Variability of body shape; E, Dorsal views showing variable body shape; E, F, Ventral and dorsal view of a typical specimen; G, H, Dorsal views showing abnormal fragmentation of dorsal kinety 3 (arrows). AZM, adoral zone of membranelles; BC, buccal cirrus; CC, caudal cirri; CG, cortical granules; DB, dorsal bristles; EM, endoral membrane; FC, frontal cirri; FVC, frontoventral cirri; LMR, left marginal row; Ma, macronuclear nodules; Mi, micronuclei; PM, paroral membrane; PTVC, pretransverse ventral cirri; PVC, postoral ventral cirri; RMR, right marginal row; TC, transverse cirri; 1–6, dorsal kinety 1–6. Scale bars: A, B, E–H, 50 μm.

sal kineties, small one, measuring about 0.4–0.5 μm, sparely distributed; 22–30 adoral membranelles; three frontal cirri, one buccal cirrus, four frontoventral cirri, three postoral ventral cirri, two pretransverse ventral cirri, and 3–5 transverse cirri; 13–19 left and 12–18 right marginal cirri; six dorsal kineties including two dorsomarginal kineties; and three caudal cirri.

**Description.** Kim et al. (2019), as an original description, provided morphological and morphometric data of *N. gangwonensis* in detail with its molecular phylogeny. However, cortical granulation, a key diagnosis in ciliates, was relatively poorly described in the type population. In this section, we focus on new observations of cortical granulation of the moss population. Additionally, we observed cell surface structure by scanning electron microscope.

Two types of colorless globular cortical granules can be easily distinguished by size and arrangement (Figs. 1B, C, 2F, G). Larger granules, with a diameter of 1.0–1.7 μm (usually

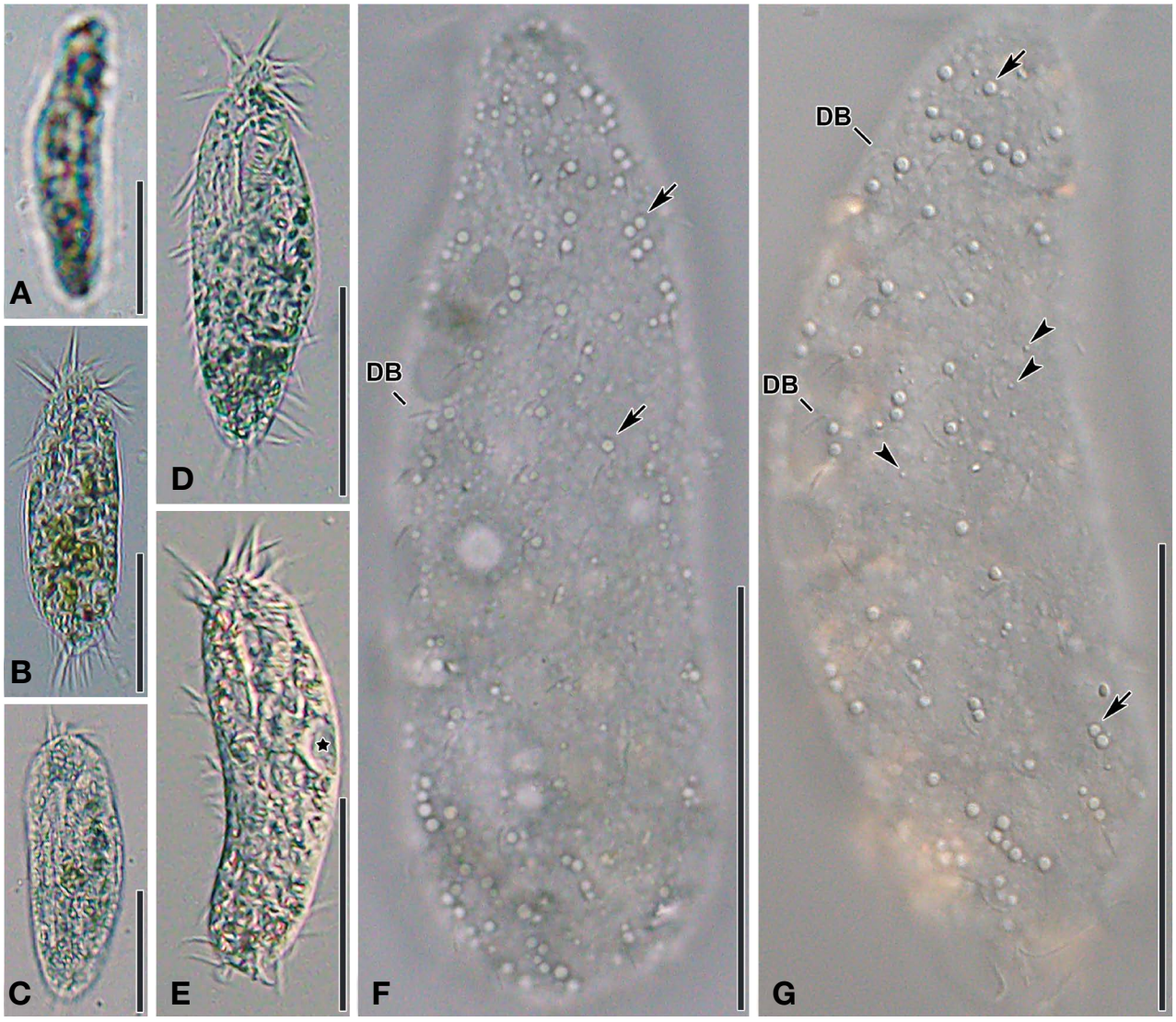


**Fig. 2.** *Notohymena gangwonensis*, moss population from life. A, Lateral view showing body outline flattened dorsoventrally; B–E, Ventral views with variable body outline. Asterisk denotes a contractile vacuole; F, G, Cortical granulation on ventral sides. Arrows and arrowheads denote larger and smaller cortical granules, respectively. DB, dorsal bristles. Scale bars: A–G = 50 μm.

1.5 μm), are arranged in groups between dorsal bristles along each kinety. Each group consists of one to several granules. Smaller granules, with a diameter of 0.3–0.5 μm (usually 0.4 μm), are less than the number of the larger granules and irregularly scattered on the cortex. Usually they do not form a group. These two types of granules are rare on ventral side.

Dorsal kineties usually consist of six longitudinal rows. Dorsal kineties 1, 4, and 6, are shortened anteriorly (slightly for kineties 1 and 6, distinctly for kinety 4) while kineties 3, 5, and 6 are shortened posteriorly (slightly for kinety 3, distinctly for kineties 5 and 6) (see Figs. 1F, 3B). Of the protargol-impregnated specimens (n = 21), we found two specimens with abnormal fragmentation of the dorsal kinety 3 (Figs. 1G, H, 3C, D). One has only one dikinetid in the kinety 4 (Figs. 1G, 3C). The other has incomplete fragmentation. Thus, the kineties 3 and 4 show a combined longitudinal row (Figs. 1H, 3D). Dorsal kineties 3 and 4 consist of 8–13 and 1–9 bristles, respectively (Table 2).

Based on the scanning electron microscopy of nine specimens, here we provide morphological data not shown in Table 2 (Fig. 4). Cells have a body size of 56–79 × 21–33 μm with an elliptical to slightly elongated elliptical body outline (Fig. 4A, B). Adoral zone occupies about 38.7% of body length on average. The cilia of frontal membranelles are up to 16 μm long. The length of the paroral cilia increases gradually from < 1 μm at proximal end to 7 μm at distal end

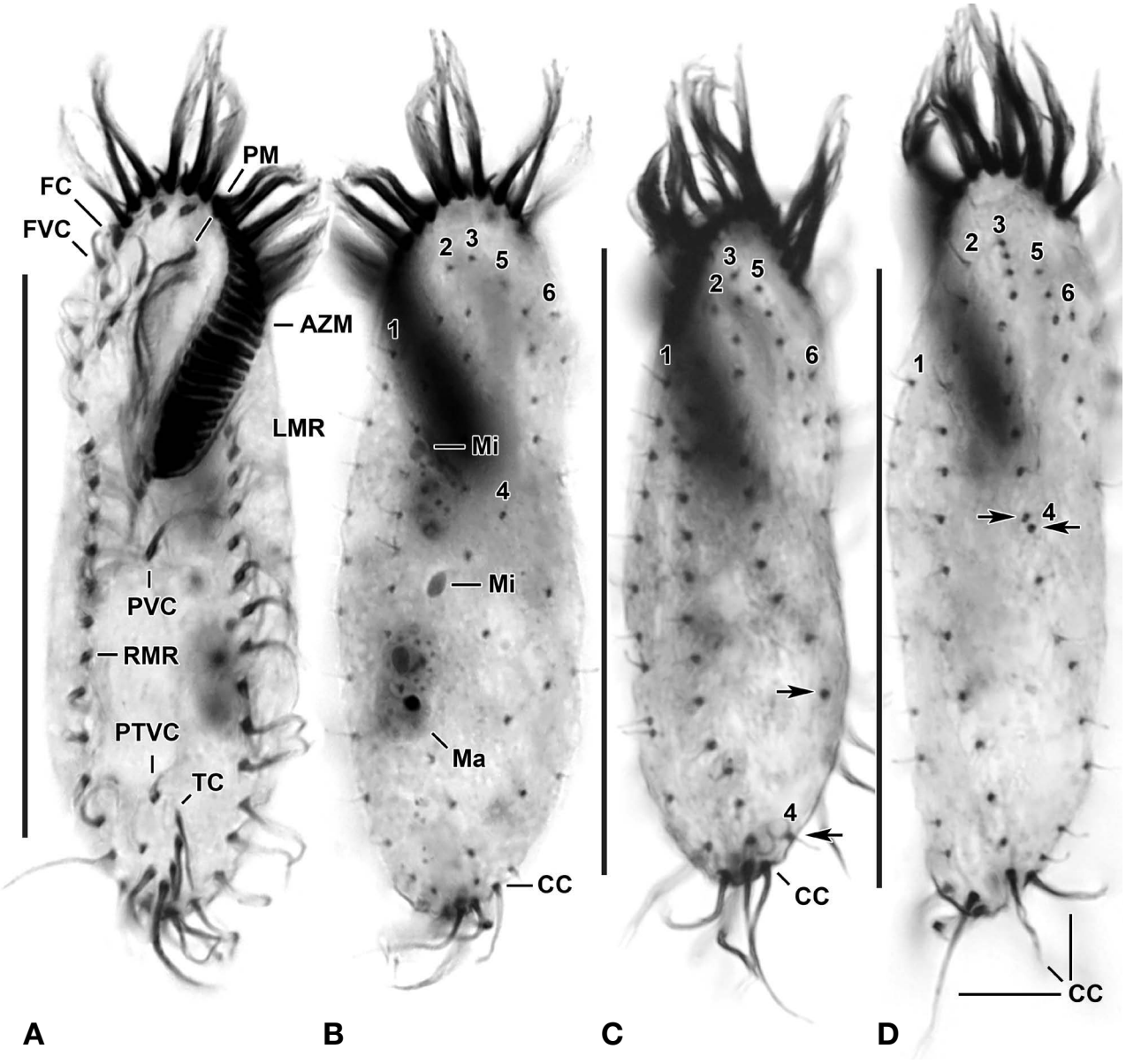


**Fig. 3.** *Notohymena gangwonensis*, moss population after protargol impregnation. A, B, Ventral and dorsal view of a representative specimen; C, D, Dorsal views showing abnormal fragmentation of dorsal kinety 3 (arrows). AZM, adoral zone of membranelles; CC, caudal cirri; FC, frontal cirri; FVC, frontoventral cirri; LMR, left marginal row; Ma, macronuclear nodules; Mi, micronuclei; PM, paroral membrane; PTVC, pretransverse ventral cirri; PVC, postoral ventral cirri; RMR, right marginal row; TC, transverse cirri; 1–6, dorsal kinety 1–6. Scale bars: A–D = 50 μm.

(Fig. 4A, C, D). The buccal vertex and lip show angular pattern; that is, they show angular section and optically crossing over each other. The cilia of the frontal cirri (about 10 μm) are slightly longer than those of the buccal cirrus (about 7 μm), frontoventral cilia (about 9 μm), postoral ventral cilia (7–10 μm), and marginal cilia (7–9 μm). The pretransverse ventral and transverse cilia are 9–12 μm and 10–16 μm long, respectively. The anteriormost pretransverse ventral cirrus locates at 16.5–21.9% (19.7% on average) of body length from posterior end. The dorsal bristles and caudal cilia are about 2.5 μm and 11–15 μm in length, respectively.

**DNA sequences.** Five regions of unclear DNA including ribosomal RNA genes (i.e., 18S, ITS1, 5.8S, ITS2, partial 28S; 2,869 bp) were analyzed and the two *Notohymena gangwonensis* populations have completely identical sequences (Table 1).

**Distribution.** The type population of *Notohymena gang-*

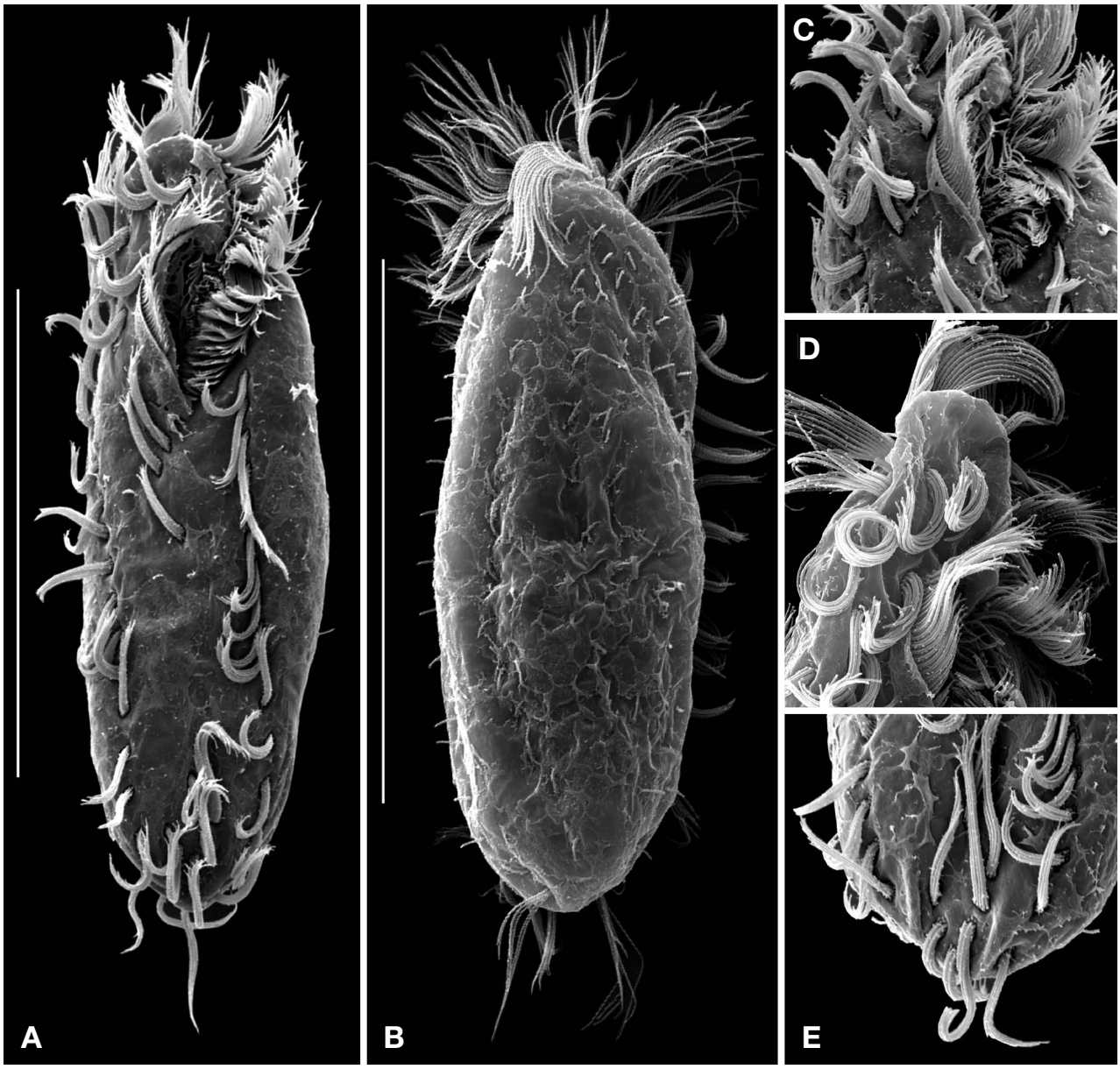


**Fig. 4.** *Notohymena gangwonensis*, moss population in the scanning electron microscope. A, Ventral view of a representative specimen; B, Dorsal view showing dorsal bristles and caudal cirri; C, D, Ventral views showing oral apparatus; E, Ventral view showing pretransverse ventral and transverse cirri. Scale bars: A, B = 50 μm.

*wonensis* is discovered from dried soil near Songjiho Lagoon in Gangwon-do, South Korea (Kim et al., 2019). In this study, the moss population was collected from Mt. Oedaesan in Gangwon-do.

**Remarks.** Considering our new observations of the cortical granulation, we should carefully compare them with those of the type population because there are some differences. According to Kim et al. (2019), *Notohymena gangwonensis* has two types of colorless globular granules with irregular distribution on the cortex. However, the diameter and arrangement differ from this study. In terms of the larger granules, the moss population differs from the type by the diameter (1.0−1.7 μm vs. about 2.5 μm) and arrangement (along dorsal kineties vs. irregularly distributed). The tiny granules of the moss population are smaller in size than those of the type population (0.3−0.5 μm vs. about 1.0 μm) (Kim et al., 2019). Our morphometric data and the concatenated rDNA sequences show that they are conspecific except for the

**Table 2.** Morphometric data on *Notohymena gangwonensis* specimens obtained from moss (upper line; this study) and type population (lower line; from Kim et al., 2019)

| Characteristic[a] | Mean | Med | SD | SE | CV | Min | Max | n |
|---|---|---|---|---|---|---|---|---|
| Body, length (μm) | 64.3 | 64 | 7.5 | 1.6 | 11.6 | 52 | 78 | 21 |
| | 74.3 | 70 | 6.7 | 1.5 | 9.0 | 68 | 84 | 21 |
| Body, width (μm) | 20.0 | 20 | 1.9 | 0.4 | 9.4 | 17 | 24 | 21 |
| | 25.3 | 25 | 2.6 | 0.6 | 10.2 | 22 | 30 | 21 |
| Adoral zone, length (μm) | 25.4 | 25 | 2.2 | 0.5 | 8.6 | 21 | 29 | 21 |
| | 25.9 | 25 | 2.6 | 0.6 | 10.1 | 23 | 30 | 21 |
| Adoral membranelles, number | 26.3 | 26 | 1.9 | 0.4 | 7.2 | 23 | 30 | 21 |
| | 25.1 | 26 | 1.5 | 0.3 | 6.0 | 22 | 26 | 21 |
| Largest adoral membranelles, width (μm) | 5.2 | 5 | 0.6 | 0.1 | 11.4 | 4 | 6 | 20 |
| | | | | NA | | | | |
| Frontal cirri, number | 3.0 | 3 | 0.0 | 0.0 | 0.0 | 3 | 3 | 21 |
| | 3.0 | 3 | 0.0 | 0.0 | 0.0 | 3 | 3 | 21 |
| Buccal cirrus, number | 1.0 | 1 | 0.0 | 0.0 | 0.0 | 1 | 1 | 21 |
| | 1.0 | 1 | 0.0 | 0.0 | 0.0 | 1 | 1 | 21 |
| Frontoventral cirri, number | 4.0 | 4 | 0.0 | 0.0 | 0.0 | 4 | 4 | 21 |
| | 4.0 | 4 | 0.0 | 0.0 | 0.0 | 4 | 4 | 21 |
| Postoral ventral cirri, number | 2.9 | 3 | 0.5 | 0.1 | 16.7 | 1 | 3 | 21 |
| | 3.0 | 3 | 0.0 | 0.0 | 0.0 | 3 | 3 | 21 |
| Pretransverse ventral cirri, number | 2.0 | 2 | 0.0 | 0.0 | 0.0 | 2 | 2 | 21 |
| | 2.0 | 2 | 0.0 | 0.0 | 0.0 | 2 | 2 | 21 |
| Posteriormost postoral ventral cirrus to anteriormost pretransverse ventral cirrus, distance (μm) | 19.7 | 19.7 | 4.9 | 1.1 | 24.8 | 12 | 31 | 21 |
| | 19.9 | 20.5 | 3.2 | 1.0 | 16.3 | 14 | 23 | 10 |
| Posterior body end to anteriormost pretransverse ventral cirrus, distance (μm) | 11.3 | 11 | 2.2 | 0.5 | 19.2 | 8 | 16 | 21 |
| | 10.4 | 10 | 1.3 | 0.4 | 13.0 | 9 | 13 | 10 |
| Transverse cirri, number | 4.5 | 5 | 0.6 | 0.1 | 13.4 | 3 | 5 | 21 |
| | 4.2 | 4 | 0.4 | 0.1 | 8.8 | 4 | 5 | 21 |
| Left marginal cirri, number | 15.1 | 15 | 1.4 | 0.3 | 9.3 | 13 | 19 | 21 |
| | 14.8 | 15 | 1.2 | 0.3 | 7.8 | 13 | 16 | 21 |
| Right marginal cirri, number | 14.7 | 14 | 1.7 | 0.4 | 11.5 | 12 | 18 | 21 |
| | 14.8 | 15 | 1.9 | 0.4 | 13.0 | 12 | 17 | 21 |
| Dorsal kineties, number[b] | 6.0 | 6 | 0.0 | 0.0 | 0.0 | 6 | 6 | 21 |
| | 6.0 | 6 | 0.0 | 0.0 | 0.0 | 6 | 6 | 21 |
| Dorsal bristles in dorsal kinety 1, number | 11.9 | 12 | 1.5 | 0.3 | 12.6 | 9 | 15 | 21 |
| | 13.6 | 14 | 1.1 | 0.2 | 7.7 | 12 | 15 | 21 |
| Dorsal bristles in dorsal kinety 2, number | 13.4 | 14 | 2.8 | 0.6 | 20.9 | 5 | 17 | 21 |
| | | | | NA | | | | |

Mean, arithmetic mean; Med, median; SD, standard deviation; SE, standard error of the arithmetic mean; CV, coefficient of variation (%); Min, minimum; Max, maximum; n, number of specimens examined; NA, not available.

[a]Data based on protargol-impregnated, randomly selected specimens from raw culture.

[b]Two of the specimens examined have abnormal kinety fragmentation. So, we considered them having six dorsal kineties.

**Table 2.** Continued

| Characteristic[a] | Mean | Med | SD | SE | CV | Min | Max | n |
|---|---|---|---|---|---|---|---|---|
| Dorsal bristles in dorsal kinety 3, number[b] | 10.8 | 11 | 1.6 | 0.4 | 15.0 | 8 | 13 | 21 |
| | | | | NA | | | | |
| Dorsal bristles in dorsal kinety 4, number[b] | 6.7 | 7 | 1.7 | 0.4 | 25.4 | 1 | 9 | 21 |
| | | | | NA | | | | |
| Dorsal bristles in dorsal kinety 5, number | 6.9 | 7 | 1.0 | 0.2 | 15.1 | 5 | 9 | 21 |
| | | | | NA | | | | |
| Dorsal bristles in dorsal kinety 6, number | 2.6 | 2 | 0.8 | 0.2 | 31.5 | 1 | 4 | 21 |
| | | | | NA | | | | |
| Caudal cirri, number | 3.0 | 3 | 0.2 | 0.0 | 7.2 | 3 | 4 | 21 |
| | 3.0 | 3 | 0.0 | 0.0 | 0.0 | 3 | 3 | 21 |
| Macronuclear nodules, number | 2.0 | 2 | 0.0 | 0.0 | 0.0 | 2 | 2 | 21 |
| | | | | NA | | | | |
| Anterior macronuclear nodule, length (μm) | 10.5 | 10 | 1.7 | 0.4 | 15.8 | 8 | 14 | 21 |
| | 10.8 | 10 | 1.4 | 0.3 | 13.5 | 9 | 13 | 21 |
| Anterior macronuclear nodule, width (μm) | 5.5 | 5 | 0.7 | 0.1 | 12.4 | 4 | 7 | 21 |
| | 5.7 | 6 | 0.5 | 0.1 | 8.7 | 5 | 6 | 21 |
| Micronuclei, number | 2.4 | 2 | 0.9 | 0.2 | 35.8 | 1 | 4 | 21 |
| | 2.2 | 2 | 0.7 | 0.2 | 31.2 | 1 | 3 | 21 |
| Micronuclei, length (μm) | 2.2 | 2 | 0.3 | 0.1 | 13.5 | 2 | 3 | 21 |
| | | | | NA | | | | |
| Micronuclei, width (μm) | 1.7 | 1.5 | 0.6 | 0.1 | 36.4 | 1 | 4 | 21 |
| | | | | NA | | | | |

granulation. Thus, we reexamined micrographs of the type population and additional micrographs overlooked by Kim et al. (2019), and we found that the authors mismeasured the diameter of the cortical granules by using an incorrect scale bar. According to the reexamination, the larger granules of the type population are 0.7−1.8 μm in diameter and longitudinally arranged along dorsal kineties. The smaller granules are irregularly distributed and measuring about 0.5 μm across (Fig. 5). That is, these granules correspond to those of the moss population. To clarify the identity of *N. gangwonensis*, we provide an improved diagnosis based on the data obtained from the type and moss population (see 'Improved diagnosis').

Unfortunately, the mitochondrial *CO1* gene was not amplified, another genetic marker for discriminating closely related species (Barth et al., 2006). We tried to amplify all genomic DNA materials extracted from the two populations of *N. gangwonensis* using primer pairs designed by Park et al. (2019) twice but failed. It is very likely resulted from the primer misannealing.

**Key to seven *Notohymena* species based on new data of *Notohymena gangwonensis***

1. Length in life usually more than 130 μm ····· 2
− Length in life usually less than 130 μm ····· 3
2. Four transverse cirri ····· *N. selvatica*
− Five transverse cirri ····· *N. saprai*
3. Four macronuclear nodules ····· 4
− Two macronuclear nodules ····· 5
4. Cortical granules colorless ····· *N. limus*
− Cortical granules yellowish to citrine ··· *N. quadrinucleata*
5. Cortical granules ruby-colored ····· *N. rubescens*
− Cortical granules not ruby-colored ····· 6
6. Cortical granules colorless, between dorsal bristles; number of dorsal bristles in kinety 1 ≤ 15 ····· *N. gangwonensis*
− Cortical granules colorless, yellow or orange or yellow-green, around dorsal bristles; number of dorsal bristles in kinety 1 > 15 ····· *N. antarctica*

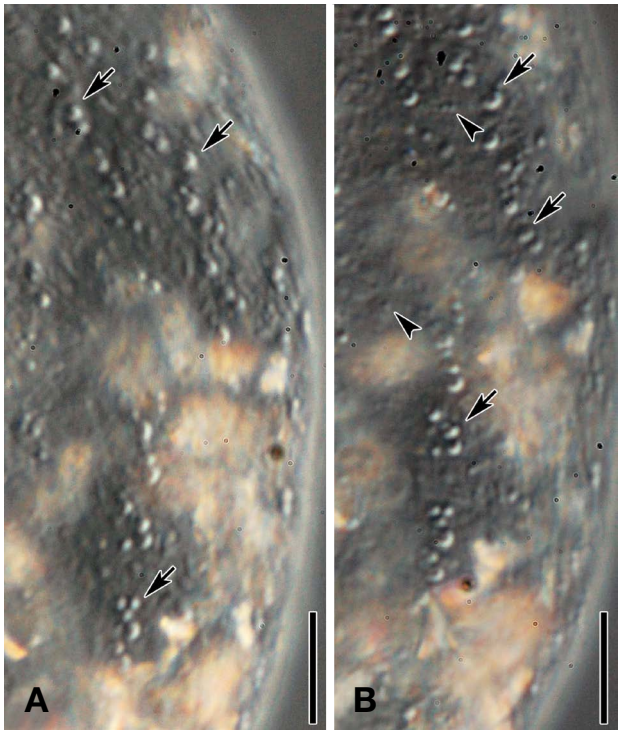


**Fig. 5.** *Notohymena gangwonensis* sensu Kim et al. (2019), type population from life. A, B, Dorsal views of the same cell showing cortical granules (arrows, larger granules; arrowheads, smaller granules). Scale bars: A, B = 10 μm.

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## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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