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A New Soil Ciliate, *Birojimia soyaensis* nov. spec. (Ciliophora: Urostylida) from South Korea

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Abstract. A new soil urostylid ciliate, *Birojimia soyaensis* nov. spec. was discovered from Soya Island, Incheon, South Korea. The species description is based on live and stained specimen observations, and 18S ribosomal RNA gene sequence analysis. *Birojimia soyaensis* nov. spec. is characterized by the following features: body slender, elongate, and somewhat twisted; body size *in vivo* 170–200 μm × 40–50 μm; contractile vacuole located at middle of left cell margin; cortical granules present; 37–48 adoral membranelles; 3 frontal and 2 frontoterminal cirri present; III/2 and buccal cirrus present; midventral pairs only; pretransverse ventral and transverse cirri present; 1 left and 4 right marginal rows, including 3 compound rows; 5 long dorsal kineties with 3 additional shortened kineties in anteriorly compound rows; 8–11 caudal cirri; 53–69 macronuclear nodules; and 2 or 3 micronuclei. *Birojimia soyaensis* nov. spec. is distinguished from *B. terricola* by cortical granule size (0.4–1.2 μm in diameter vs. 2–3 μm × 1–2 μm), cortical granule shape (mostly spherical vs. broadly ellipsoid to lenticular, respectively); number of caudal cirri (8–11 vs. 2–7), and number of dorsal bristle rows (8 vs. 6–7). Phylogenetic analysis suggests this new species is most closely related to the genus *Hemicycliostyla*.

Key words: *Birojimia soyaensis* nov. spec., protargol impregnation, soil urostylid, 18S rRNA gene

INTRODUCTION

The genus *Birojimia,* established by Berger and Foissner (1989), consisted of two soil ciliates, *B. terricola* Berger & Foissner, 1989 and *B. muscorum* (Kahl, 1932) Berger & Foissner, 1989. A newly recorded species, *B. litoralis* Foissner 2016, has now been added to the genus, and *B. muscorum* has been transferred to a new genus *Pseudobirojimia* Foissner, 2016. The *Birojimia* genus is characterized by the following features: continuous adoral zone of membranelles; 3 frontal cirri, buccal cirrus, frontoterminal cirri, transverse and caudal cirri present; midventral complex composed of cirral pairs only; 1–2 left and > 2 right marginal rows gradually shortened and replaced by dorsal bristles from anterior to posterior; and several dorsal kineties forming distinct suture left of body midline (Berger 2006, Foissner 2016).

Soil ciliate are widely known as an abundant group that plays an important role in nitrogen mineralization (Griffiths 1989). Their activity affects soil parameters

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such as pH, the amount of humus, and soil nutrients (Acosta-Mercado and Lynn 2004). Terrestrial and semi-terrestrial habitats are estimated to contain more than 2,000 species (Foissner *et al*. 2002, Foissner 2008). Information on soil ciliates from Asia, America, and Australia is poor compared to that for species from central Europe, Africa, and Antarctica (Foissner 1986, 1988, 1993, 1996, 1997, 2000, 2008; Blatterer and Foissner 1988; Foissner *et al*. 2002; Foissner and Xu 2007). In this study, we present a new Asian soil ciliate from Soya Island, Incheon, South Korea. We describe the new species based on its morphology and 18S rRNA gene sequences.

MATERIALS AND METHODS

Sample site and morphological identification

Specimens of the new soil ciliate were discovered on Soya Island, Incheon, South Korea (37°12'49.73" N, 126°10'33.19" E), in September 2012. Soil samples from around wetlands (freshwater, pH 6.0) were transferred to Petri dishes, mixed with clean mineral water, and allowed to stand at room temperature for a minimum of 6 h. A single raw culture of *Birojimia soyaensis* was used for this study.

Live and protargol-impregnated specimens were observed under a stereo microscope (SZH10; Olympus, Japan) and a light microscope (DM2500; Leica, Wetzlar, Germany) at magnifications ranging from 50 × to 1000 ×. Protargol impregnation was performed according to the methods of Foissner (1991). Classification and terminology followed that of Berger (2006) and Foissner (2016).

Molecular analyses

Cells were washed several times with distilled water to isolate a single cell. Genomic DNA was then extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich Co., St. Louis, MO, USA), based on the manufacturer's protocols. PCR amplification and sequencing of the 18S rRNA gene was conducted according to the methods of Jung *et al*. (2012). The 18S rRNA sequences were aligned using BioEdit (Hall 1999) and compared to sequences of closely related species retrieved from GenBank.

To determine the appropriate DNA substitution model for Maximum Likelihood (ML) and Bayesian Inference (BI) analyses, we used the jModelTest 2.1.7 (Darriba *et al*. 2012). The model selected was GTR + I (0.5570) + G (0.5310). The ML analysis was conducted using PhyML version 3.1 (Guindon *et al*. 2010) and BI assessment was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The genetic distance was calculated using MEGA 4.0 (Tamura *et al*. 2007) with the Kimura 2-parameter distance option (Kimura 1980).

RESULTS

Birojimia soyaensis nov. spec.

Diagnosis: Body size *in vivo* 170–200 μm × 40–50 μm; body slender, elongate, and somewhat twisted; cell color grayish to transparent; cortical granules present; contractile vacuole located near midbody at left margin; 53–69 macronuclear nodules; 2 or 3 micronuclei; adoral zone approximately 25–35% of body length in stained specimens; 3 frontal cirri present; III/2 and buccal cirrus present; midventral complex consists of midventral cirral pairs only; pretransverse ventral and transverse cirri present; 1 left and 4 right marginal rows, including 3 compound rows, 5 long dorsal kineties, with additional 3 shortened kineties in anteriorly compound rows; 8–11 caudal cirri.

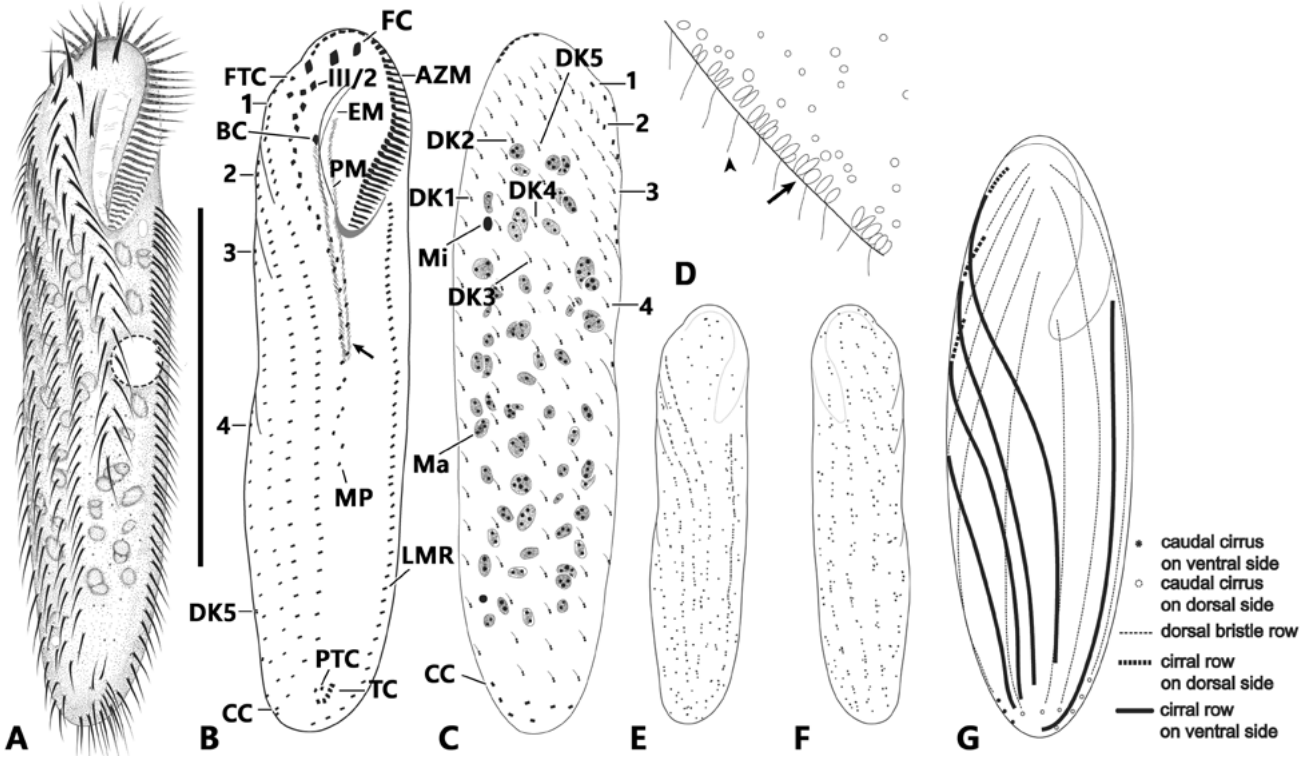
Type locality: Wetland soil on Soya Island, South Korea, 37°12' N and 126°10' E

Type specimens: The holotype slide (NIBRPR 0000107151) and single paratype slide (NIBRPR 0000107152) containing protargol-impregnated specimens, have been deposited in the National Institute of Biological Resources, South Korea.

Etymology: The name originates from the name of the island (Soya) on which the species was discovered.

Description: Body size *in vivo* 170–200 μm × 40–50 μm, with an average of 184.9 μm × 42.1 μm in stained specimens (Figs 1A, 2A, 3A). Body outline usually elongate, elliptical, and somewhat twisted at right marginal region, slightly flexible, colorless to slightly grayish at low magnification. Macronuclear nodules size 3–9 μm × 2–6 μm *in vivo* (on average 6.5 μm × 4 μm in protargol-impregnated specimens), 53–69 in number, distributed everywhere, except in the anterior and posterior portions of the cell; 2 or 3 micronuclei (on average 5 μm × 4 μm in protargol-impregnated specimens), oval to spherical shape (Figs 1C, 2I, 3A). *In vivo*, colorless cortical granules, size 0.4–1.2 μm in diameter, irregularly distributed, partially in longitudinal lines on ventral side, sparsely arranged in short longitudinal rows on dorsal side, and rod-shaped in lateral view, average size *in vivo* 3.0 μm × 1.0 μm (Figs 1D–F, 2D, F, J). Contractile vacuole in midbody near left cell margin, about 15 μm in diameter when fully extended, dividing into 2 collecting canals (Figs 2B, C).

Adoral zone composed of 37–48 membranelles, about 25–35% of body length in protargol-impregnated specimens (Table 1). Bases of largest membranelles 8-μm long, adoral zone of membranelles slightly



Figs 1A–G. Morphology of *Birojimia soyaensis* nov. spec. from live (A, D–F) and protargol-impregnated (B, C, G) specimens. **A, B, E, F** – ventral view; **C, D, E** – dorsal view; **B, C** – ventral and dorsal views of the holotype specimen: **B** – arrow indicates pharynx; **D** – extrusive cortical granules (arrow), dorsal cilia (arrowhead); **E** – arrangement of cortical granules on ventral side; **F –** arrangement of cortical granules on dorsal side; **G –** ciliary pattern of cirral rows and dorsal kineties. AZM – adoral zone of membranelles; BC – buccal cirrus; CC – caudal cirri; DK – dorsal kineties; EM – endoral membrane; FC – frontal cirri; FTC – frontoterminal cirri; LMR – left marginal cirral row; Ma – macronuclei; Mi – micronuclei; MP – midventral pairs; PM – paroral membrane; PTC – pretransverse ventral cirri; TC – transverse cirri; 1, inner right marginal cirral row; 2–4, compound rows. Scale bars: 100 µm.

curved like a "question mark" (Figs 1B, 3A, B). Paroral and endoral membranes slightly curved, crossing each other (Figs 1B, 3B). Pharynx conspicuous in stained specimens, with 2–3 µm short rods in the wall, similar to *B. terricola* and *B. litoralis* (Figs 1B, 3D) (Berger 2006; Foissner 2016).

Frontal and transverse ventral cirri about 12-µm long, and other cirri about 9–10-µm long (Figs 2A, B, D, H). Three enlarged frontal cirri, 2 frontoterminal cirri, III/2 and buccal cirrus present (Figs 1B, 3B). Midventral complex composed of 13–17 midventral cirral pairs only, beginning close to cirrus III/2 (Figs 1B, 3A, B). Marginal ciliature composed of 1 left and 4 right rows, including 3 compound ones. Three "compound" rows (with bristles anteriorly and cirri posteriorly; Foissner and Stoeck 2011) on right margin; inner right marginal cirral row and left marginal cirral rows (LMR) composed of cirri only; LMR extended to center of rear body margin as J-shape; 1 or 2 pretransverse ventral and 4 to 8 transverse cirri (Figs 1B, C, G, 3A–C, E, F).

Eight dorsal bristle rows, cilia approximately 3–4-µm long *in vivo*, all dorsal kineties associated with cirral rows or caudal cirri: 5 long dorsal kineties (DK) associated with caudal cirri at rear end of cell; 3 shortened kineties associated with marginal rows (= compound rows) (Figs 1B, C, F, 2G, 3A, C, E, F). Two bipolar kineties (DK1, DK2) and 3 shortened rows (rows 1 to 3) commencing from the anterior portion, while DK 3 to 5 and row 4 commencing near midbody, between DK 2 and row 3; 2 bipolar DK1 and 2 extending along left body margin (Figs 1C, G, 3E). First and fifth dorsal kinety associated with 3 or 4 caudal cirri, second to fourth

Table 1. Morphometric characterization of *Birojimia soyaensis* nov. spec.

Characteristic [a]	mean	M	SD	CV	Min	Max	n
Body, length	184.9	185	16.2	8.7	146.0	213.0	21
Body, width	42.1	42	7.9	18.7	33.0	65.0	21
Adoral zone, length	54.4	55	3.9	7.1	48.0	62.0	21
Macronuclear nodules, number	60.7	60	3.3	5.5	53.0	69.0	21
Macronuclear nodules, length	6.4	6	2.1	32.4	3.0	9.0	21
Macronuclear nodules, width	3.8	4	1.1	28.5	2.0	6.0	21
Adoral membranelles, number	42.1	42	2.4	5.8	37	48	21
Frontal cirri, number	3.0	3	0.0	0.0	3	3	21
Buccal cirri, number	1.0	1	0.0	0.0	1	1	21
Frontoterminal cirri, number	2.0	2	0.0	0.0	2	2	21
Midventral pairs, number	14.4	14	1.1	7.8	13	17	21
Pretransverse ventral cirri, number	1.9	2	0.3	16.6	1	2	21
Transverse cirri, number	5.5	6	1.0	18.5	4	8	21
Right marginal row 1, number of cirri	43.6	43	2.8	6.5	40	51	21
Right marginal row 2, number of cirri	34.2	34	2.9	8.4	30	41	21
Right marginal row 3, number of cirri	26.5	27	3.1	11.8	22	35	21
Right marginal row 4, number of cirri	13.1	13	3.6	27.7	8	18	21
Left marginal row, number of cirri	50.4	50	4.0	7.9	45	59	21
Dorsal kineties, number	5.0	5	0.0	0.0	5	5	21
Caudal cirri, number	10.1	10	1.1	10.9	8	11	21

[a] Data based on protargol-impregnated specimens. All measurements in μm.
CV – coefficient of variation (%); M – median; Max – maximum; Min – minimum; n – number of individuals examined; SD – standard deviation.

kineties combined 1 or 2 caudal cirri. Eight to 11 caudal cirri, conspicuous and approximately 11-μm long *in vivo* (Figs 1C, 2H, 3F).

B. soyaensis feeds on fungi, testate amoebae, soil particles, and small protozoa (Fig. 3A).

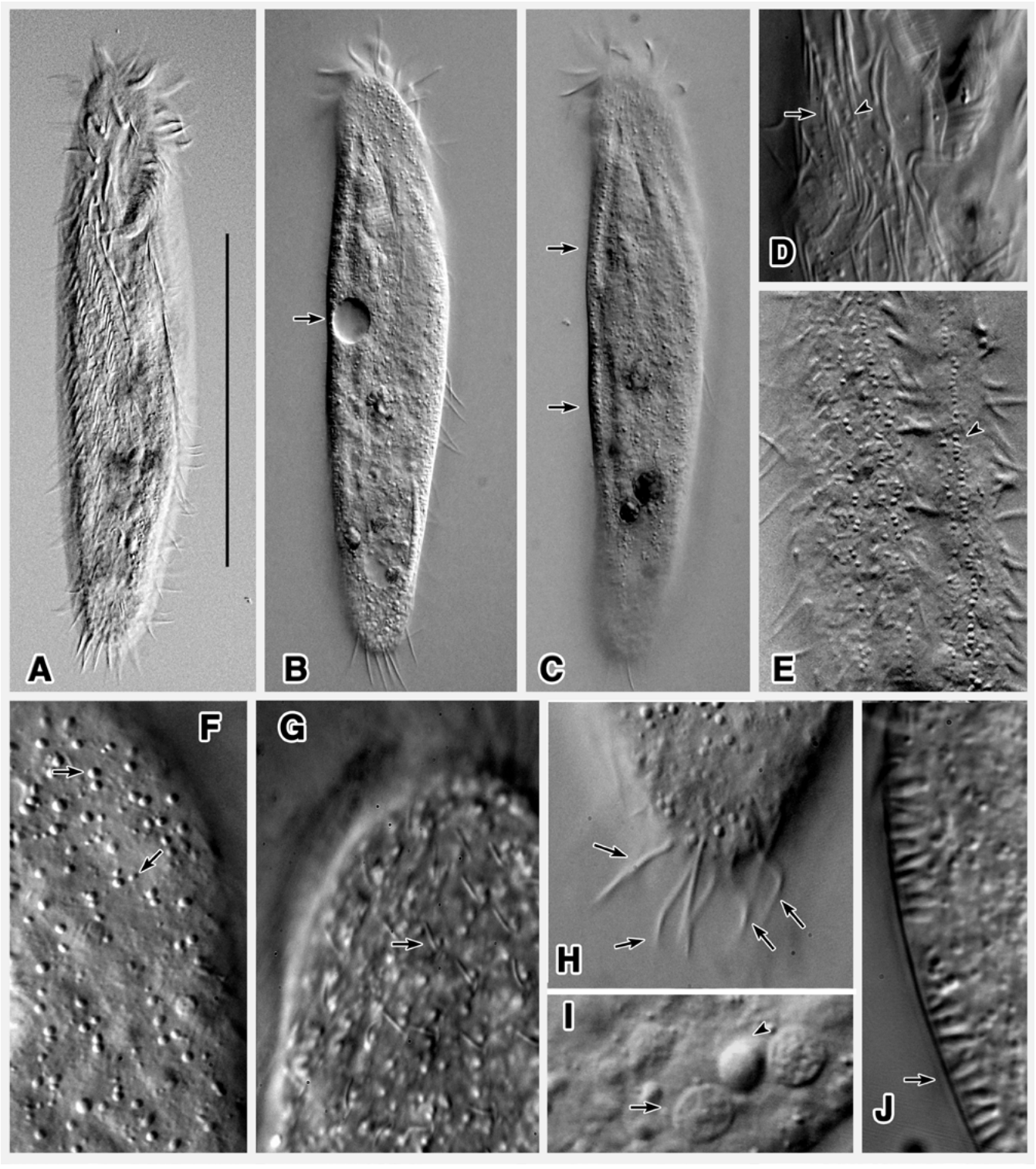
Molecular analysis: The GenBank accession number of *B. soyaensis* is KY176378 (1761bp). In the phylogenetic tree, *B. soyaensis* formed a (partial) clade with *Anteholosticha, Hemicycliostyla, Pseudokeronopsis*, *Pseudourostyla*, and *Uroleptopsis*. Of these, *B. soyaensis* showed the closest relationship with the genus *Hemicycliostyla*: *H. sphagni* and *H. franzi* (pairwise distance: *H. sphagni*, 0.17; *H. franzi*, 0.18) (Fig. 4).

DISCUSSION

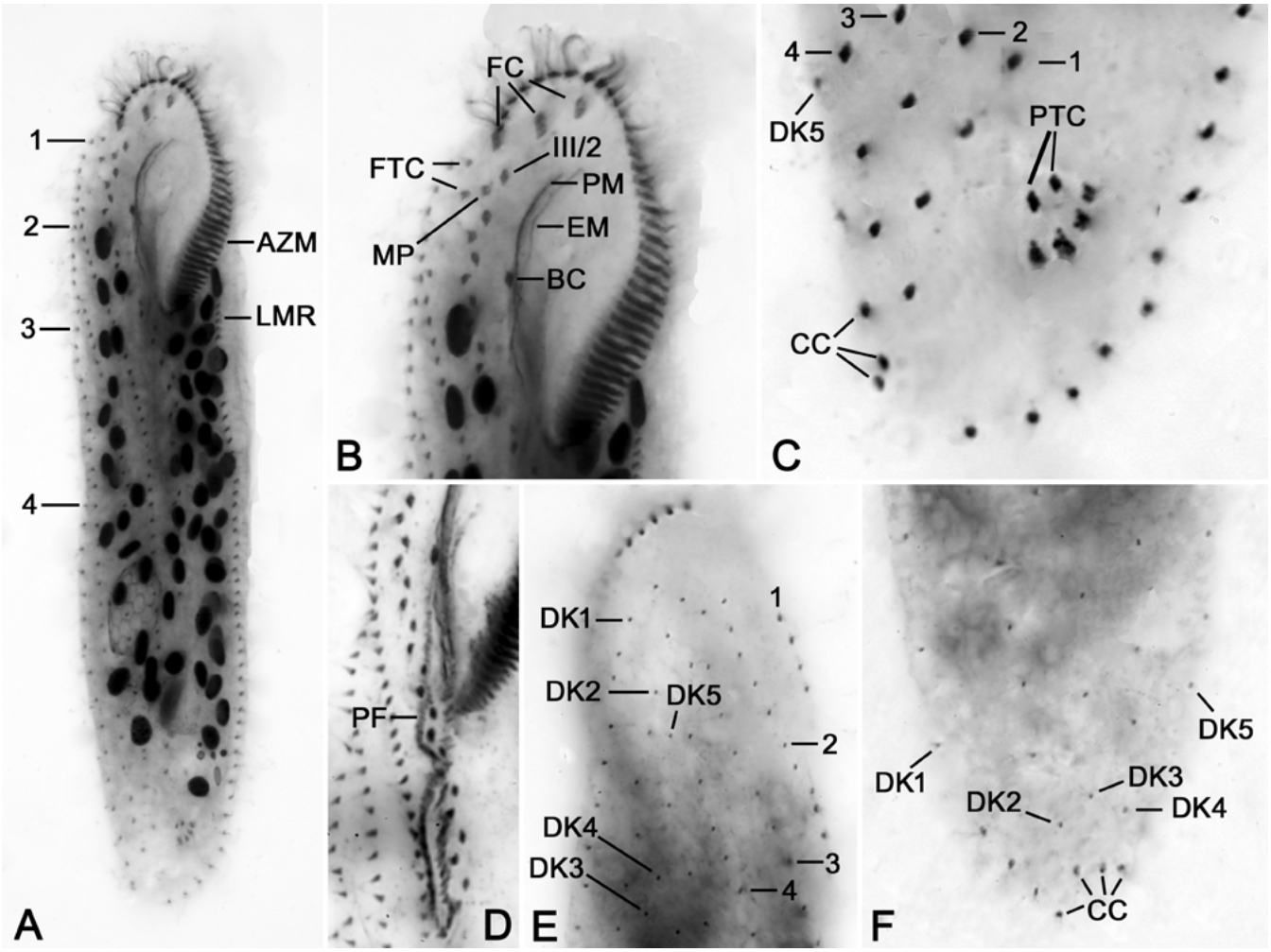
Comparison with congeners: *Birojimia* comprises two soil ciliates, *B. terricola* and *B. litoralis* (Foissner 2016). *Birojimia terricola*, the type species of the genus *Birojimia*, and *B. litoralis* have a feature typical of the Holostichidae: 3 frontal cirri, midventral pairs only. The Holostichidae applies to more than one category, a superfamily (Jankowski 1979) and a subfamily (Borror and Wicklow 1983). However, Berger (2006) used the name "Holostichidae" without any categories.

Foissner (2016) transferred *B. muscorum* to the new genus *Pseudobirojimia,* as it differed from the other species in the *Birojimia*, e.g., midventral cirral pair plus a midventral row (vs. midventral cirral pairs only), and an ordinary bipolar pattern of dorsal kineties (vs. 2 bipolar kineties) (Berger 2006, Foissner 2016). Herein, we discuss the different features of the new species against its congeners.

Pseudobirojimia muscorum differs from *B. soyaensis* as follows: cell size in protargol preparations (120–147 μm × 16–23 μm vs. 146–213 μm × 33–65 μm), number of adoral membranelles (26–32 vs. 37–48), midventral complex (midventral pairs with a midventral row vs. midventral pairs only), pretransverse ventral



Figs 2A–J. Photomicrographs of *Birojimia soyaensis* nov. spec. from live specimens. **A, D, E** – ventral view; **B, C, F, H** – dorsal view: **B, C** – arrows indicate contractile vacuole and collecting canals; **D** – distribution of cortical granules (arrowhead) and inner right cirral row (arrow); **E** – distribution of cortical granules on the ventral side, arrowhead indicates a longitudinal row of cortical granules; **F** – arrows indicate cortical granules on the dorsal side; **G** – arrow indicates dorsal cilia; **H** – arrows indicate caudal cirri; **I** – macronuclear nodules (arrow), micronuclei (arrowhead); **J** – cortical granules in lateral view (arrow). Scale bars: 100 µm.



Figs 3A–F. Photomicrographs of *Birojimia soyaensis* nov. spec. from protargol-impregnated specimens. **A, B, D** – holotype specimen. **A** – ventral view of specimen; **B** – anterior portion of ventral view; **C** – posterior portion of ventral view; **D** – pharynx; **E** – anterior portion of dorsal view; **F** – posterior portion of dorsal view. AZM – adoral zone of membranelles; BC – buccal cirrus; CC – caudal cirri; DK – dorsal kineties; EM – endoral membrane; FC – frontal cirri; FTC – frontoterminal cirri; LMR – left marginal cirral row; Ma – macronuclei; Mi – micronuclei; MP – midventral pairs; PM – paroral membrane; PTC – pretransverse ventral cirri; TC – transverse cirri; 1 – inner right marginal cirral row; 2–4 – compound rows. Scale bars: 100 μm.

cirri (absent vs. present), number of transverse cirri (2–4 vs. 4–8), and the number of right marginal cirral row (2 vs. 4) (Table 2) (Kahl 1932, Berger 2006).

Birojimia litoralis can be separated from *B. soyaensis* using body size (179–251 μm × 40–65 μm vs. 146–213 μm × 33–65 μm); lithosome in cytoplasm (present vs. absent); number of frontoterminal cirri (2–3 vs. invariable 2); number of macronuclear nodules (116–210 vs. 53–69); number of left marginal cirral rows (2 vs. 1); number of dorsal kineties (8–11, invariable 8). However, the dorsal structure is similar between the two *Birojimia* species, i.e., bipolar 1 and 2 dorsal kineties extend to the posterior end of the body, and several dorsal kineties commence near the mid-body (Foissner 2016).

Berger and Foissner (1989) missed the cortical granules of *B. terricola*, although the granules were found in deeply stained specimens (Foissner 2016). *Birojimia soyaensis* nov. spec. also has the cortical granules. The granules of *B. terricola* differ from those of *B. soyaensis* as follows: shape (broadly ellipsoid to lenticular vs. mostly spherical), size (2–3 μm × 1–2 μm vs. 0.4–1.2 μm in diameter), and pattern of distribution (within and between cirral and bristle rows vs. irregularly

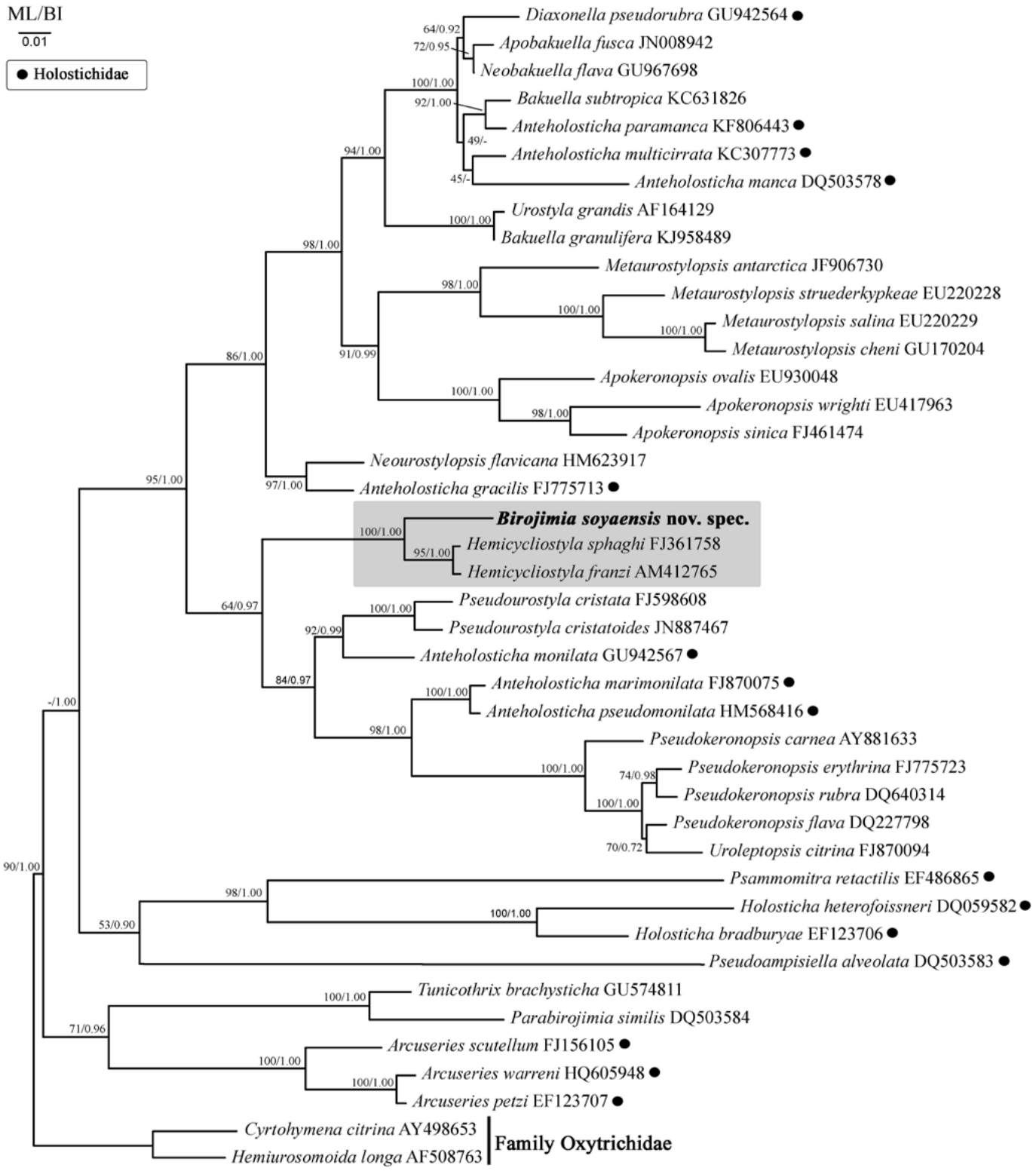


Fig. 4. Phylogenetic tree of 18S rRNA gene sequences, showing the position of *Birojimia soyaensis* nov. spec. on the basis of Maximum Likelihood (ML) and Bayesian Inference (BI). Bootstrap values for ML and posterior probability values for BI are represented on the interior branches. Black circles indicate the species of the family Holostichidae (sensu Berger 2006 system).

distributed, partially in longitudinal rows) (Foissner 2016). Cortical granules are important characteristics for classifying ciliates (Gong *et al*. 2001, Lei *et al*. 2005, Berger 2006). Furthermore, caudal cirri (CC) in *B. soyaensis* are more numerous than those in *B. terricola* (8–11 vs. 2–7 in the Japanese population) (Berger and Foissner 1989; Berger 2006; Foissner 2016) (Table 2; Figs 1C, 3E, F).

In the original description of *B. terricola*, Berger and Foissner (1989) used the terms "rows 5, 6" for the outermost right cirral rows on the ventral surface. In comparison to other compound rows, rows 5 and 6 have fewer cirri (2 or 3) and a large portion of dorsal bristles, so that Berger (2006) reported the possibility of caudal cirri. *Birojimia soyaensis* also has these ambiguous cirri, so we have used the term "caudal cirri" in this study. These were easy to find, as caudal cirri were often formed on the ventral surface, similar to row 5 of this new species (Blatterer and Foissner 1988, Foissner *et al*. 2002, Berger 2006). In *B. soyaensis*, the basis for caudal cirri is as follows: (i) formed at the rear end of the bipolar dorsal kineties; (ii) a larger proportion of bristle parts than in other compound rows; and (iii) occupying the rear end of the cell margin.

Molecular analysis: Phylogenetic analysis did not support the morphological analysis, as we could not find a relationship between the Holostichidae species in the phylogenetic tree (Fig. 4). Molecular data did not reflect the feature of the Holostichidae – 3 frontal cirri and midventral pairs only. Interestingly, our phylogenetic tree showed that *B. soyaensis* nov. spec. formed a clade with two species in the genus *Hemicycliostyla*: *H. sphagni* and *H. franzi* (Stokes 1886, Berger 2006, Paiva *et al*. 2012). *Pseudourostyla franzi* was transferred to the genus *Hemicycliostyla* on the basis of terrestrial habit, marginal rows occupying a large part of the dorsal side, via individual within-row primordia, and molecular analysis (Kumar *et al*. 2010, Paiva *et al*. 2012). According to Berger (2006), absence of transverse cirri is one of the important features used to define the genus *Hemicycliostyla*. Presence or absence of transverse cirri, however, was found to vary within populations in the Brazilian populations, and molecular results strongly supported the two species *H. sphagni* and *H. franzi* forming a clade that was separate from the *Pseudourostyla* group (Fig. 4) (Paiva *et al*. 2012). Furthermore, information of cirral patterns in *Hemicycliostyla* species is insufficient at present (Berger 2006). Thus, it should follow Paiva *et al.* (2012) until the appropriate information for *Hemicycliostyla* species is available.

Birojimia soyaensis has a different frontal ciliature (3 frontal cirri in *B. soyaensis* vs. bicoronal in *Hemicycliostyla*) and left marginal cirral row (single row in *B. soyaensis* vs. multiple rows in *Hemicycliostyla*) from the other *Hemicycliostyla* species, whereas the clade is supported by several morphological characteristics, such as presence of rod-shaped granules, a short midventral complex, and combined ciliature (= compound row in this study) (Stokes 1886, Berger 2006, Paiva *et al*. 2012) (Figs 1B, C, 3A, C, E, F). We focused on the combined ciliature, which is related to the

Table 2. Comparison of morphological features between *Birojimia litoralis, B. terricola* (Japanese population), *and B. soyaensis* nov. spec.

Characteristic [a]	*B. litoralis*	*B. terricola*	*B. soyaensis* nov. spec.
Body size	205 × 50	186 × 36.5	187 × 41
Cortical granules	present	present	present
Adoral membranelles, number	40–55	35–43	37–48
Midventral complex	midventral pairs only	midventral pairs only	midventral pairs only
Left cirral (marginal) rows, number	2	1	1
Dorsal kineties, number [b]	8–11	7–8	8
Caudal cirri, number	3	2–7	8–11
Data source	Foissner (2015)	Foissner (2015)	This study

[a] Data based on protargol-impregnated specimens. Measurements in μm.
[b] Average, includes shortened kineties in anteriorly compound rows.

morphogenetic process. In the genus *Hemicycliostyla*, marginal rows and dorsal kineties are all formed from individual within-row primordia (Paiva *et al*. 2012). Although ontogenetic data was not available, combined ciliature (compound rows) of *B. soyaensis* very likely have this feature, which formed from individual within-row primordia during morphogenesis, similar to the genus *Hemicycliostyla*, as the genera *Birojimia* and *Hemicycliostyla* showed a close relationship in the phylogenetic tree and shared the feature of combined ciliature. *Birojimia terricola* and *B. litoralis* also have compound rows, although these are not found in *P. muscorum* (Berger 2006). Furthermore, *P. muscorum* is distinguished from the other two *Birojimia* species by having a different midventral structure, which is midventral pairs with a midventral cirral row. The midventral complex is an important feature for classification of urostylids. In a previous study, *Apourostylopsis sinica*, *Neourostylopsis songi*, and *N. flavicana* were separated from their "stem genus" *Metaurostylopsis* using the following characteristics: only 2 frontoterminal cirri; midventral row absent; midventral complex extending over the buccal vertex (*Apourostylopsis* extends to the posterior end, *Neourostylopsis* extends to the mid-body level) (Berger 2006, Song *et al*. 2011, Chen *et al*. 2013). Furthermore, phylogenetic data strongly supported the results. Although molecular data from *P. muscorum* was not available, we expect that *P. muscorum* is also distinctly separate from *B. soyaensis*, *B. terricola*, and *B. litoralis* in the phylogenetic and morphological analyses, thus, we concur with Berger (2006) and Foissner (2016). Conversely, we expect that the genus *Birojimia* consists of a monophyletic group in the phylogenetic tree, which *B. terricola*, *B. litoralis*, and *B. soyaensis* strongly support by sharing morphological features, e.g., midventral cirral pairs only, dorsal bristles gradually replaced by marginal cirral row, caudal cirri present, 2 dorsal kineties (DK1, DK 2) bipolar along the left body margin, several dorsal kineties forming near the midbody (Foissner 2016). Additional molecular and morphogenetic data of *Birojimia* and *Hemicycliostyla* species are needed for more thorough analysis.

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