

ORIGINAL ARTICLE

Morphology and Molecular Phylogeny of Two New Brackish Water Ciliates of *Bakuella* (Ciliophora: Urostylida: Bakuellidae) from South Korea

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Keywords

Bakuella incheonensis; *Bakuella litoralis*; ciliature; new species; SSU rRNA gene.

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ABSTRACT

Two new species of *Bakuella*, *B. (B.) incheonensis* n. sp. and *B. (Pseudobakuella) litoralis* n. sp., were discovered in brackish water near Aamdo Shore Park, Incheon, South Korea. We conducted a morphological study based on live observations and protargol-impregnated specimens, and a molecular analysis using nuclear SSU rRNA gene sequences. *Bakuella (B.) incheonensis* is diagnosed by: body size 70–105 × 20–40 μm in vivo, 21–25 adoral membranelles, three or four frontoterminal cirri, midventral complex composed of 7–10 midventral pairs with one or two rows and terminating at about 62% of body length, 20–28 left and 25–32 right marginal cirri, 58–87 macronuclear nodules, and yellowish cortical granules. *Bakuella (Pseudobakuella) litoralis* is diagnosed by: body size 90–125 × 30–40 μm in vivo, 25–33 adoral membranelles, 3–5 buccal cirri, midventral complex composed 10–15 midventral pairs with one or two rows and terminated at 70% of body length, one or two pretransverse cirri, 3–6 transverse cirri, 26–39 left and 29–47 right marginal cirri, 49–84 macronuclear nodules, and two types of cortical granules. Molecular phylogeny using SSU rRNA gene sequences shows a nonmonophyletic relationship among *Bakuella* species and emphasizes the need for further morphogenetic studies of this genus and other related hypotrichs.

THE genus *Bakuella* Agamaliyev and Alekperov 1976 is characterized by continuous adoral zone of membranelles, three enlarged frontal cirri, one or more buccal cirri, two or more frontoterminal cirri, midventral complex composed of obliquely arranged midventral pairs and midventral rows, transverse cirri, one left and one right marginal cirral row, and an absence of caudal cirri (Agamaliyev and Alekperov 1976; Berger 2006; Song et al. 1992). To date, 16 species/subspecies have been originally assigned to *Bakuella* (Berger 2001; Chen et al. 2013; Foissner 2004; Foissner et al. 2002; Kumar et al. 2010). Song et al. (1992) synonymized *B. imbricata* with *B. marina* and *B. polycirrata* with *B. crenata*, and suggested *B. variabilis* needs a genus of its own. Berger (2006) considered *Bakuella kreukampii* and *Bakuella muensterlandii* were junior synonyms of *B. agamaliyevi*. Consequently, there are 11 species/subspecies that belong to this genus at present. Berger (2006) divided the genus *Bakuella* into two subgenera; *Bakuella (Bakuella)* and *B. (Pseudobakuella)* based on the

number of frontoterminal cirri. The former has more than two frontoterminal cirri, while the latter has two. The nuclear small subunit ribosomal RNA (SSU rRNA) gene sequences of two *Bakuella* species, *B. granulifera* and *B. subtropica*, are available from GenBank; however, these do not indicate a sister relationship between the two species (Lv et al. 2015). Unfortunately, the sequence of type species for *Bakuella*, *B. marina*, is unavailable to resolve this problematic situation.

Bakuella species are known to inhabit marine, brackish, limnetic, and terrestrial environments. *Bakuella marina* is found in extreme environments (e.g. mineral and hot springs), and can endure even 370 practical salinity units (psu) (Berger 2006; Chaouite et al. 1990; Chen et al. 2013; Hammer 1986; Kumar et al. 2010). *Bakuella* species feed on bacteria, protists, and small eukaryotes (Chen et al. 2013; Eigner and Foissner 1992; Foissner 1998, 2004; Foissner et al. 2002; Mihailowitsch 1989; Mihailowitsch and Wilbert 1990; Song et al. 2002).

In this study, we describe two new brackish water ciliate species, *Bakuella (Bakuella) incheonensis* n. sp. and *Bakuella (Pseudobakuella) litoralis* n. sp. from South Korea, based on morphological studies and molecular phylogeny using the SSU rRNA gene.

MATERIALS AND METHODS

Sample collection and identification

Bakuella (Bakuella) incheonensis n. sp. and *B. (Pseudobakuella) litoralis* n. sp. were discovered on different dates and at different sites in brackish water near Aamdo Shore Park, Incheon, South Korea. *Bakuella (B.) incheonensis* was collected on 23 June 2014 at 37°24'30"N, 126°38'43" E (salinity, 4.1 psu; temperature, 26.9 °C) and *B. (P.) litoralis* was collected on 16 December 2012 at 37°24'48"N, 126°38'26"E (salinity, 15.0 psu; temperature unavailable). Samples of each were cultured in Petri dishes with few rice grains enriching bacterial growth. A single clonal culture of *B. (B.) incheonensis* and raw culture of *B. (P.) litoralis* were used for following studies. Protargol impregnation was performed to reveal the ciliature and nuclear apparatus (Foissner 1991). Protargol-impregnated and living specimens were observed under a light microscope (Leica DM2500, Wetzlar, Germany) at magnifications of 50X to 1,000X. Terminology and classification follows Berger (2006). Here, we considered parabuccal cirri as the cirri positioned between the rightmost frontal cirrus and the first midventral cirral pair.

DNA extraction, PCR amplification, and sequencing

After several washes of each living individual with sterilized saline water, genomic DNA was extracted using a RED-Extract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO) according to the manufacturer's protocol. Modified new EukA (5'-CTG GTT GAT YCT GCC AGT-3') (Medlin et al. 1988) and LSU rev4 (Sonnenberg et al. 2007) primers were used to amplify the SSU rRNA gene and ITS1-5.8S-ITS2-partial LSU region. PCR amplification and sequencing was performed according to Jung et al. (2011), and SSU rRNA gene was used for phylogenetic analysis.

Phylogenetic analysis

For phylogenetic analyses, 53 SSU rRNA gene sequences were downloaded from the GenBank database. *Strombidium purpureum* and *Tintinnidium mucicola* were used as outgroups. These 53 sequences were aligned with those of the two new species by using Clustal Omega (Sievers et al. 2011) and manually trimmed both ends in BioEdit 7.1.11 (Hall 1999). Pairwise distances were calculated using MEGA 5.2.2 (Tamura et al. 2011). The program jModelTest 2.1.1 (Darriba et al. 2012) was used to choose the best model, the TIM3 + I (0.4750) +G (0.5030) model, using the Akaike information criterion. A maximum likelihood (ML) tree was inferred by PhyML 3.1

(Guindon et al. 2010) with 1,000 bootstrap replicates. A Bayesian inference (BI) tree was obtained using MrBayes 3.2.2 (Ronquist et al. 2012) with 1,000,000 Markov chain Monte Carlo iterations, of which the first 30% were burn-in. Convergence was assessed by MrBayes' the average standard deviation of split frequencies dropping below 0.01, and the potential scale reduction factor approaching 1.0.

Tree topology tests

We performed the approximately unbiased, weighted Shimodaira–Hasegawa, and weighted Kishino–Hasegawa tests to assess statistical significance of topological constraints by using CONSEL ver. 0.20 (Shimodaira and Hasegawa 2001). The best (i.e. unconstrained) tree and the topologically constrained trees were inferred in PAUP* v4.0b10 (Swofford 2003), using the ML criterion and a heuristic search with TBR branch swapping and 10 random sequence addition replicates. The topologies of the best tree found here was identical to that inferred previously using PhyML (see above). PAUP* was used to calculate per-site log likelihoods of the best and constrained ML trees under the best-fit model. The following constraints were statistically tested: (1) monophyly of the family Bakuellidae (*Apobakuella fusca*, *Bakuella incheonensis*, *Bakuella granulifera*, *Bakuella litoralis*, *Bakuella subtropica*, *Metaurostyloopsis antarctica*, *Metaurostyloopsis cheni*, *Metaurostyloopsis salina*, and *Metaurostyloopsis struederkypkeae*); (2) monophyly of the genus *Bakuella* (*B. incheonensis*, *B. granulifera*, *B. litoralis*, and *B. subtropica*); and (3) monophyly of the subgenus *Bakuella* (*B. incheonensis*, *B. granulifera*, and *B. subtropica*).

RESULTS

Bakuella (Bakuella) incheonensis n. sp.

Morphological description

Size 70–105 × 20–40 μm in vivo (Fig. 1A, 2A–C), on average 82 × 37 μm in protargol preparations (Fig. 1D, E, 3A, B). Body flexible and slightly contractile; dorsoventrally flattened, slender to elliptical shape; cell yellowish to colorless. Cortical granules mainly distributed along cirral rows and dorsal kineties; yellowish, spherical to slightly ellipsoidal, about 0.7 × 0.5 μm in dorsal view, while ellipsoidal and about 1.0 × 0.7 μm in lateral view (Fig. 1B, C, 2D, F–H). On average 69 macronuclear nodules, measuring 2.3–7.0 × 1.3–2.0 μm in stained specimens, scattered throughout the whole body; irregularly ellipsoidal in shape (Fig. 1E, 3D, arrow). About five oval micronuclei measuring 2.3–3.0 × 1.7–2.0 μm in stained specimens (Fig. 1E, 3D, arrowhead). Contractile vacuole located on left side at about 40% of body length, 10–12 μm in diam. during diastole (Fig. 1A, 2C, arrows). Crawling on the bottom of Petri dish at moderately fast speed.

Adoral zone about 34% (29–45%) of body length in stained specimens, with 21–25 membranelles (Table 1).

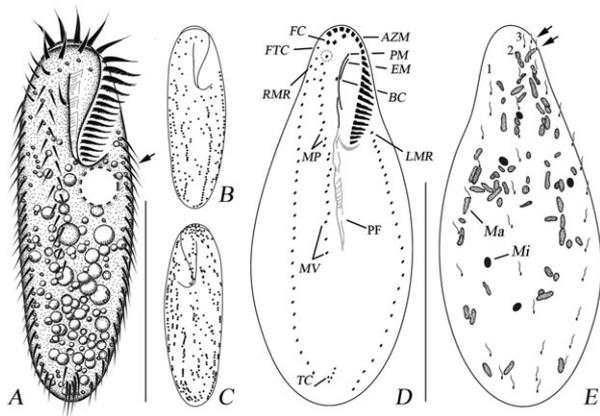


Figure 1 Morphology of *Bakuella (Bakuella) incheonensis* n. sp. in vivo (A–C) and after protargol impregnation (D, E). **A**. Ventral view of a representative individual, arrow indicates the contractile vacuole. **B, C**. Ventral (B) and dorsal (C) views showing cortical granulation. **D, E**. Ventral (D) and dorsal (E) views of the holotype specimen, dotted circle marks the parabuccal cirrus, arrows indicate two dikinetics anterior to the right marginal row. AZM = adoral zone of membranelles; BC = buccal cirrus; EM = endoral membrane; FC = frontal cirri; FTC = frontoterminal cirri; LMR = left marginal row; Ma = macronuclear nodules; Mi = micronuclei; MP = midventral pairs; MV = midventral row; PF = pharyngeal fibers; PM = paroral membrane; RMR = right marginal row; TC = transverse cirri; 1–3 = dorsal kineties. Scale bars: 50 μ m.

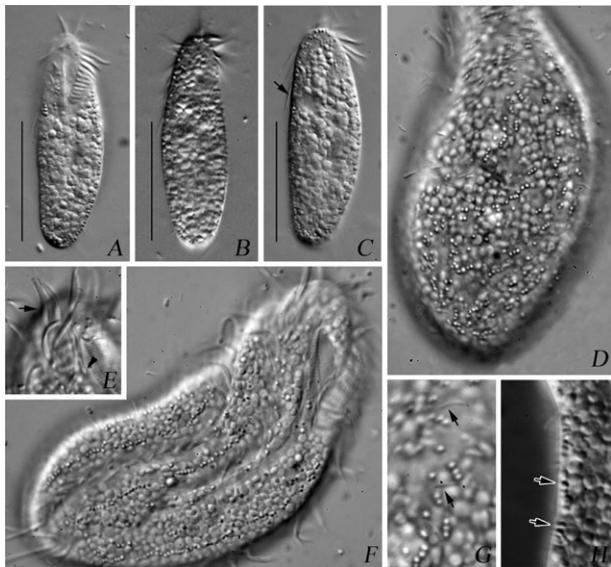


Figure 2 Photomicrographs of *Bakuella (Bakuella) incheonensis* n. sp. in vivo. **A–C**. Ventral (A) and dorsal (B, C) views, showing elliptical body shape, arrow in (C) indicates the contractile vacuole. **D**. Distribution of cortical granules on dorsal side. **E**. Ventral view of the anterior body portion, right frontal cirrus is marked by arrow and the single buccal cirrus by arrowhead. **F**. Arrangement of cortical granules on the ventral side. **G**. Dorsal bristles (arrows) and cortical granules. **H**. Lateral view of cortical granules (arrows). Scale bars: 50 μ m.

Table 1. Morphometric data for *Bakuella (B.) incheonensis* n. sp. (first line of each characteristic) and *B. (Pseudobakuella) litoralis* n. sp. (second line of each characteristic)

Characteristics	Mean	M	SD	SE	CV	Min	Max	n
Body, length (μ m)	81.8	81	9.6	2.2	11.8	60	100	19
	88.5	90	9.8	2.0	11.1	70	106	25
Body, width (μ m)	37.4	37	5.9	1.4	15.8	29	50	19
	36.0	36	6.9	1.4	19.1	22	49	25
Adoral zone, length (μ m)	27.7	27	2.1	0.5	7.7	25	31	19
	36.3	36	5.8	1.2	16.0	25	47	25
Adoral membranelles, no.	22.4	23	1.3	0.3	5.8	21	25	19
Frontal cirri, no.	3.0	3	0.0	0.0	0.0	3	3	19
	3.0	3	0.0	0.0	0.0	3	3	25
Buccal cirri, no.	1.0	1	0.0	0.0	0.0	1	1	19
	3.6	4	0.6	0.1	16.4	3	5	25
Parabuccal cirri, no.	1.0	1	0.0	0.0	0.0	1	1	19
	3.0	3	0.9	0.2	30.4	2	4	25
Frontoterminal cirri, no.	3.6	4	0.5	0.1	14.2	3	4	19
	2.0	2	0.0	0.0	0.0	2	2	25
Midventral pairs, no.	8.2	8	1.0	0.2	11.7	7	10	19
	12.6	13	1.4	0.3	11.2	10	15	25
Midventral rows, no.	1.9	2	0.2	0.1	11.8	1	2	19
	1.1	1	0.3	0.1	29.6	1	2	25
Cirri in midventral row 1, no.	3.0	3	0.0	0.0	0.0	3	3	19
	3.4	3	0.6	0.1	19.0	3	5	25
Cirri in midventral row 2, no.	3.3	3	0.5	0.1	14.1	3	4	18
	3.7	4	0.6	0.3	15.7	3	4	3
Pretransverse cirri, no.	0.5	1	0.5	0.1	97.5	0	1	19
	1.1	1	0.3	0.1	25.6	1	2	25
Transverse cirri, no.	4.7	5	0.5	0.1	10.2	4	5	19
	4.6	5	0.7	0.1	15.6	3	6	25
Left marginal cirri, no.	24.1	24	2.3	0.5	9.4	20	28	19
	30.4	29	3.6	0.7	11.8	26	39	25
Right marginal cirri, no.	28.2	28	2.4	0.6	8.6	25	32	19
	33.8	33	4.1	0.8	12.0	29	47	25
Macronuclear nodules, no.	69.3	66	11.2	4.2	16.2	58	87	7
	67.0	66	11.3	3.4	16.8	49	84	11
Micronuclei, no.	4.9	5	0.9	0.3	19.0	3	6	9
	4.7	4	1.3	0.4	28.5	3	8	11
Dorsal kineties, no.	3.0	3	0.0	0.0	0.0	3	3	19
	3.0	3	0.0	0.0	0.0	3	3	25
Dorsal bristles on anterior part of right marginal row, no.	1.9	2	0.2	0.1	12.1	1	2	18
	2.0	2	0.4	0.1	17.2	1	3	25

All data are from protargol-impregnated specimens. CV = coefficient of variation (%); M = median; Max = maximum; mean = arithmetic mean; Min = minimum; n = number of individuals examined; SD = standard deviation; SE = standard error of arithmetic mean.

Distal end of adoral zone extended only slightly on right body margin forming a question mark as in other urostyids. Buccal cavity deep and moderately wide. Undulating membranes long, slightly curved; optically intersecting at about mid-portion of paroral; paroral approximately three-quarters of endoral membrane length (Fig. 1D).

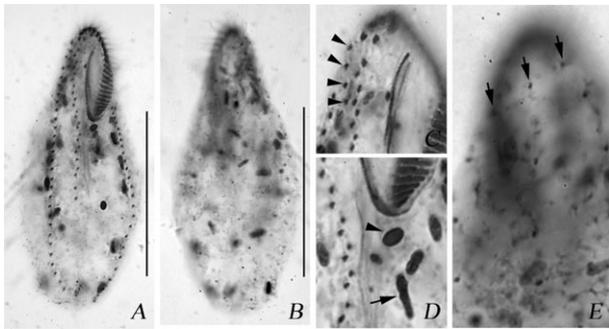


Figure 3 Photomicrographs of *Bakuella (Bakuella) incheonensis* n. sp. after protargol impregnation. **A, B.** Ventral (A) and dorsal (B) views of the holotype specimen, showing the entire cirral pattern, oral apparatus, and dorsal kineties. **C.** Ventral view of ciliature of the anterior body portion, arrowheads indicate frontoterminal cirri. **D.** Macronuclear nodule (arrow) and micronucleus (arrowhead). **E.** Close up of the anterior part of three dorsal kineties (arrows). Scale bars: 50 μ m.

All cirri relatively fine, mostly 10- to 13- μ m long. Three enlarged frontal cirri (Fig. 1D, 2E, arrow), invariably one buccal cirrus on right side of paroral membrane (Fig. 1D, 2E, arrowhead) and one parabuccal cirrus (Fig. 1D, dotted circle). Three or four frontoterminal cirri near distal end of adoral zone (Fig. 1D, 3C, arrowheads). Midventral complex composed of 7–10 inconspicuously zigzagging midventral pairs with one or two midventral rows each comprising three or four cirri and terminating at about 62% of body length (Fig. 1D). Of the 19 examined specimens, 10 had one pretransverse cirrus, while the remainder lacked the cirrus (Fig. 1D, no pretransverse cirrus). Four or five transverse cirri (Fig. 1D), protruding beyond rear end of body. One left and one right marginal row, consisting of 20–28 and 25–32 cirri, respectively (Fig. 1D). Marginal rows terminated at the level of transverse cirri and not overlapped posteriorly.

Three complete dorsal kineties (Fig. 1E, 3E, arrows); usually two dikinetids at anterior part of right marginal cirral row (Fig. 1E, arrows). Dorsal cilia 3- to 4- μ m long in vivo. Caudal cirri lacking.

Bakuella (Pseudobakuella) litoralis n. sp.

Morphological description

Size 90–125 \times 30–40 μ m in vivo (Fig. 4A, 5A–D), on average 89 \times 36 μ m in protargol preparations (Fig. 4E, F, 5J, K). Body flexible and slightly contractile; dorsoventrally flattened, slender to elliptical shape; cell slightly yellowish at low magnification due to cortical granules. Two types of cortical granules: type I granules yellowish, larger and about 0.6 \times 0.4 μ m in size, oval in dorsal and lateral views (Fig. 5G–I, arrows) and type II granules yellowish to colorless, smaller and about 0.2 μ m in diam., subglobular in dorsal and lateral views (Fig. 5G–I, arrowhead); both types distributed along cirral rows and dorsal kineties as well as between kineties as longitudinal rows (Fig. 4B–D, 5G–I).

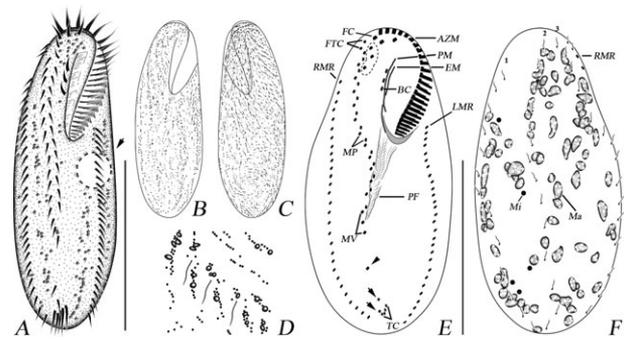


Figure 4 Morphology of *Bakuella (Pseudobakuella) litoralis* n. sp. in vivo (A–D) and after protargol impregnation (E, F). **A.** Ventral view of a representative individual, arrow indicates the contractile vacuole. **B, C.** Ventral (B) and dorsal (C) views of cortical granulation. **D.** Detail of cortical granulation on dorsal body side. **E, F.** Ventral (E) and dorsal (F) views of the holotype specimen, arrows indicate pretransverse cirri, arrowhead shows cirrus between the midventral row and pretransverse cirri, the dotted circle marks parabuccal cirri. Nuclear apparatus in (F) was illustrated from another typical specimen because of faint impregnation of those of the holotype. AZM = adoral zone of membranelles; BC = buccal cirri; EM = endoral membrane; FC = frontal cirri; FTC = frontoterminal cirri; LMR = left marginal row; Ma = macronuclear nodules; Mi = micronuclei; MP = midventral pairs; MV = midventral row; PF = pharyngeal fibers; PM = paroral membrane; RMR = right marginal row; TC = transverse cirri; 1–3 = dorsal kineties. Scale bars: 50 μ m.

On average 67 macronuclear nodules, measuring 2.8–8.0 \times 1.9–2.3 μ m in stained specimens, scattered throughout the whole body; irregularly ellipsoidal in shape (Fig. 4F, 5M, arrow). Three to eight spherical or oval micronuclei, measuring 1.7–2.0 \times 1.3–2.0 μ m in stained specimens, distributed throughout the body (Fig. 4F, 5M, arrowhead). Contractile vacuole located on left side of mid-body, about 12.5 μ m in diam. (Fig. 4A, 5C, D, arrows). Crawling on the bottom of Petri dish at moderately fast speed.

Adoral zone about 41% (27–49%) of body length in stained specimens, with 25–33 membranelles (Table 1). Distal end of adoral zone extended only slightly on right body margin forming a question mark as in other urostyids. Buccal cavity deep and moderately wide. Undulating membranes long, slightly curved; optically intersecting in mid-portion of paroral; paroral and endoral membranes almost equal in length (Fig. 4E).

All cirri relatively fine, mostly 10- to 15- μ m long in vivo. Three enlarged frontal cirri (Fig. 4E, 5L, arrows), 3–5 buccal cirri arrayed in a longitudinal row right of the paroral membrane (Fig. 1E). Parabuccal cirri, numbering 2–4, arranged with two or three cirri below rightmost frontal cirrus and no or one cirrus below first midventral cirral pair (Fig. 4E, dotted circle). Invariably two frontoterminal cirri near the distal end of the adoral zone (Fig. 4E, 5L, arrowheads). Midventral complex composed of 10–15 zigzagging midventral pairs with one or two midventral rows each comprising 3–5 cirri and terminating at about 70%

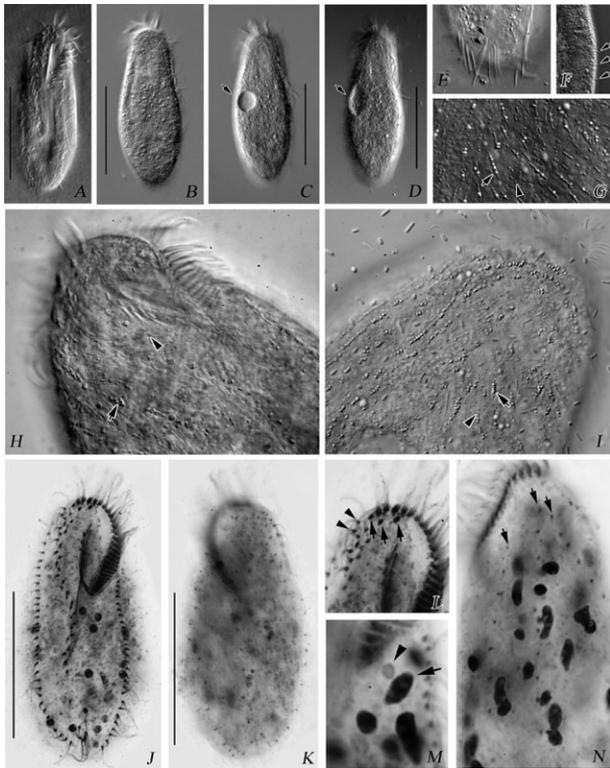


Figure 5 Photomicrographs of *Bakuella (Pseudobakuella) litoralis* n. sp. in vivo (A–I) and after protargol impregnation (J–N). **A–D.** Ventral (A) and dorsal (B–D) views, showing elliptical body shape, arrows in (C, D) indicate the contractile vacuole. **E.** Ventral view of the posterior body portion, arrows mark the two pretransverse cirri. **F.** Dorsal bristles (arrows). **G–I.** Ventral (H) and dorsal (G, I) patterns of cortical granulation, arrows mark the larger granules and arrowheads mark the smaller granules. **J, K.** Ventral (J) and dorsal (K) views of the holotype specimen, showing the entire cirral pattern, oral apparatus, and dorsal kineties. **L.** Ventral view of the ciliature of the anterior body portion, arrows indicate three frontal cirri and arrowheads indicate two frontoterminal cirri. **M.** Macronuclear nodule (arrow) and micronucleus (arrowhead). **N.** Close up of the anterior part of three dorsal kineties (arrows). Scale bars: 50 μ m.

of the body length (Fig. 4E). Peculiarly, no midventral row observed in few specimens (2 of 25), but no other differences from typical specimens. One or two pretransverse cirri (Fig. 4E, arrow, 5E, arrows) ahead of 3–6 transverse cirri (Fig. 4E). All transverse cirri protruding beyond rear end of body. One or two cirri situated between midventral complex and pretransverse/transverse cirri in most specimens (23 of 25 specimens) (Fig. 4E, arrowhead). One left and one right marginal row, consisting of 26–39 and 29–47 cirri, respectively (Fig. 4E). Anterior part of right marginal cirral row located on dorsal side (Fig. 4F). Marginal rows not connected and overlapped posteriorly.

Three complete dorsal kineties (Fig. 4F, 5N, arrows); usually two dikinetids ahead of anterior part of right marginal row that extends onto dorsal side. Dorsal cilia 3- to 4- μ m long in vivo. Caudal cirri lacking.

Molecular analysis of *Bakuella (Bakuella) incheonensis* n. sp. and *Bakuella (Pseudobakuella) litoralis* n. sp.

The length of SSU rRNA gene sequences is 1,746 bp in *B. (B.) incheonensis* (KR024011) and *B. (P.) litoralis* (KR024010). The congeners of *Bakuella* showed pairwise similarities of 96.8–99.9%. In the phylogenetic tree (Fig. 6), the four congeners were not contained in a single cluster. There was strong support (BI = 1.00, ML = 85) for *Bakuella (B.) incheonensis* clustering with *B. (B.) subtropica*, while *B. (B.) granulifera* did not cluster with the other members of *Bakuella (Bakuella)*. The sole member of the subgenus *Pseudobakuella*, *B. litoralis*, clustered with *Neobakuella flava* (pairwise similarity of 99.8%) and *A. fusca* (pairwise similarity of 99.7%). The clade was moderately well supported by the phylogenetic analysis (BI posterior probability 1.00; ML bootstrap support 78%). In addition, the tree topology test rejected the following hypotheses: (1) monophyly of the family Bakuellidae, (2) monophyly of the *Bakuella*, and (3) monophyly of the subgenus *Bakuella* (Table 4).

DISCUSSION

According to Berger (2006) and recent studies (Chen et al. 2013; Kumar et al. 2010), there are 10 species included in the genus *Bakuella*: *B. agamalievi* Borror and Wicklow, 1983; *B. crenata* Agamaliev and Alekperov 1976; *B. edaphoni* Song et al. 1992; *B. granulifera* Foissner et al., 2002; *B. marina* Agamaliev and Alekperov 1976; type species; *B. nilgiri* Kumar et al. 2010; *B. pampinaria* Eigner and Foissner 1992; *B. salinarum* Mihailowitsch and Wilbert 1990; *B. subtropica* Chen et al. 2013; and *B. walibonensis* Song et al. 1992;. The genus *Bakuella* consists of two subgenera discriminated by the number of frontoterminal cirri (more than two vs. exactly two). The key diagnostic characters for distinguishing *Bakuella* species are body size, cirral patterns, nuclear apparatus, cortical granules, and habitat (Berger 2006; Song et al. 1992).

Comparison of *Bakuella (Bakuella) incheonensis* n. sp. with congeners

Two of the seven previously described species in the subgenus *Bakuella* have one buccal cirrus in the same manner as *B. (B.) incheonensis* n. sp.: *B. (B.) agamalievi* Borror & Wicklow, 1983 and *B. (B.) subtropica* Chen et al. 2013 (Table 2).

Here, we compare the new species, *B. (B.) incheonensis* n. sp., to the previously described populations of *B. agamalievi* Borror and Wicklow, 1983 (Agamaliev 1972, 1974; Berger 2006; Mihailowitsch and Wilbert 1990; Song et al. 2002). According to the combined data from two populations well described by Mihailowitsch and Wilbert (1990) and Song et al. (2002; neotype population), *B. (B.) agamalievi* can be distinguished from *B. (B.) incheonensis* by body size (97–176 \times 40–70 μ m vs. 60–100 \times 29–50 μ m in protargol-impregnated specimens), number of adoral membranelles (26–37 vs. 21–25), frontoterminal cirri

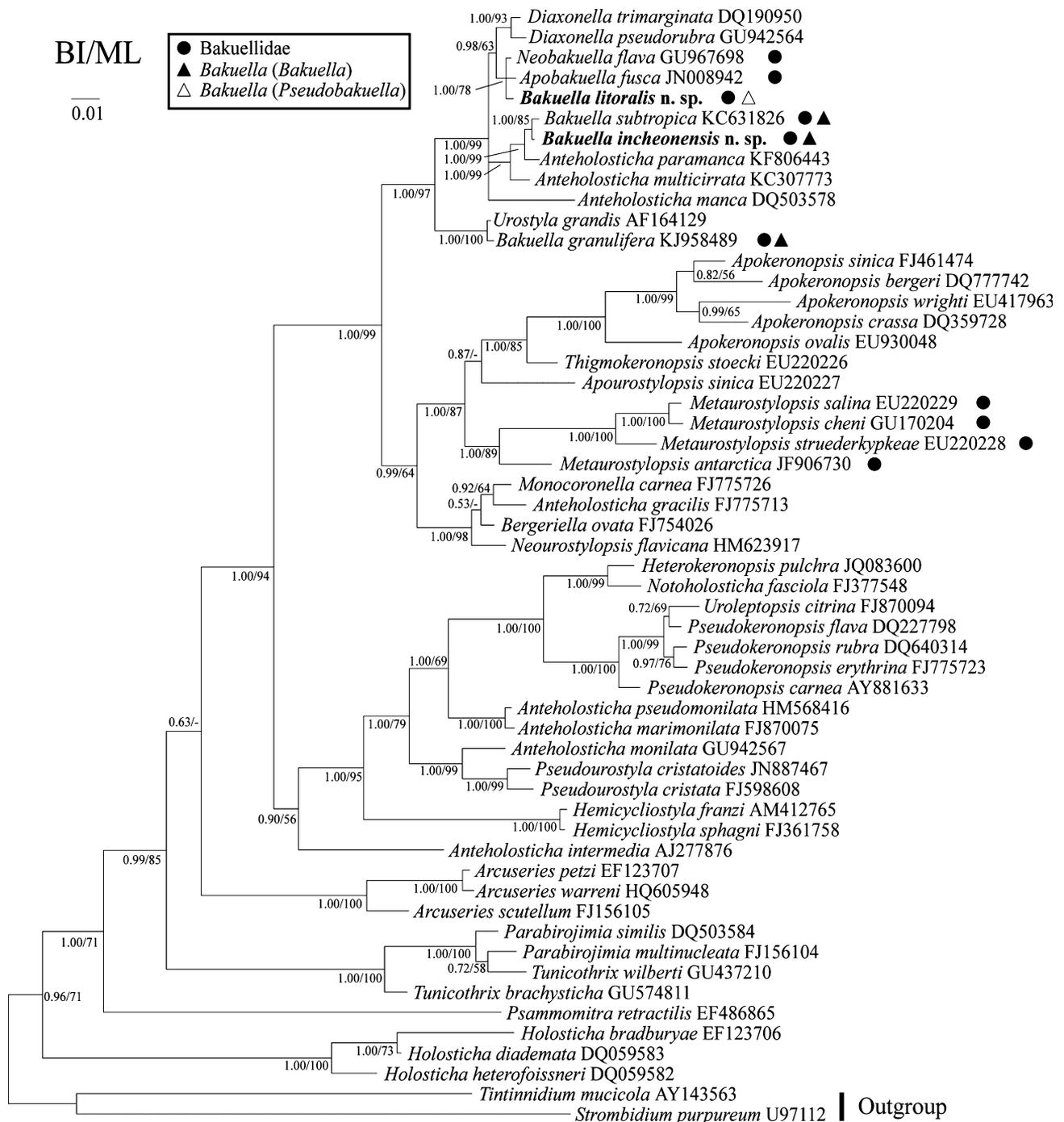


Figure 6 Majority consensus tree of the Bayesian inference (BI) constructed from SSU rRNA gene sequences, showing the position of *Bakuella* (*Bakuella*) *incheonensis* n. sp. and *Bakuella* (*Pseudobakuella*) *litoralis* n. sp. The names of the species investigated are indicated by bold type. The first values on nodes are posterior probabilities for BI tree, and the latter are bootstrap values for ML trees. A dash indicates a value less than 0.50 (BI) or 50% (ML).

(4–7 vs. 3 or 4), midventral pairs (9–18 vs. 7–10), left and right marginal cirri (30–40 vs. 20–28, 34–47 vs. 25–32, respectively), and cortical granules (colorless or slightly greenish vs. yellowish) (Mihailowitsch and Wilbert 1990; Song et al. 2002). This excludes the type population (Agamaliev 1972) because an inappropriate impregnation

method was used, causing misinterpretation of its morphology (Berger 2006).

Bakuella (*B.*) *subtropica* Chen et al. 2013 is distinguished from *B.* (*B.*) *incheonensis* by body size (116–208 × 43–125 μm vs. 60–100 × 29–50 μm in protargol-impregnated specimens), number of adoral

membranelles (25–44 vs. 21–25), frontoterminal cirri (4–12 vs. 3 or 4), midventral pairs (9–23 vs. 7–10), length of midventral complex (80% vs. 62% of body length), left and right marginal cirri (30–54 vs. 20–28, 28–64 vs. 25–32, respectively), macronuclear nodules (68–144 vs. 58–87), and cortical granules (1–2 μm , yellow-brownish to yellow-greenish vs. 0.7 μm , yellowish) (Chen et al. 2013). In addition, there are two nucleotide substitutions (K2P distance of 0.12%) in the SSU rRNA gene sequences. While each of the congeners has distinct morphology, there is relatively low genetic variation among the congeners and even among genera: *B. litoralis* vs. *A. fusca* (0.31%) and *B. litoralis* vs. *N. flava* (0.19%). This low interspecific variation, calculated using the SSU rRNA gene, is similar to that observed in other hypotrichs (e.g. *Pseudokeronopsis*) and emphasizes the need to identify more variable gene regions.

Comparison of *Bakuella* (*Pseudobakuella*) *litoralis* n. sp. with congeners

Among the 10 species in *Bakuella*, two species have been designated in the subgenus *Pseudobakuella* by Berger (2006): *B. (P.) salinarum* Mihailowitsch and Wilbert 1990 and *B. (P.) walibonensis* Song et al. 1992; *Bakuella nilgiri* Kumar et al. 2010 was not designated in any subgenera in the original description, but it usually has two frontoterminal cirri (ranging 2–4) (Kumar et al. 2010). This suggests the species could be assigned to the subgenus *Pseudobakuella*; therefore, here we compare it to *B. (P.) litoralis* (Table 3).

Bakuella nilgiri Kumar et al. 2010 is differentiated from *B. (P.) litoralis* by habitat (soil vs. brackish water), body size (124–158 \times 44–77 μm vs. 70–106 \times 22–49 μm in protargol-impregnated specimens), number of adoral membranelles (42–54 vs. 25–33), transverse cirri (6–11 vs. 3–6), midventral pairs (18–23 vs. 10–15), length of midventral complex (extended to near transverse cirri vs. to 70% of body length), left and right marginal cirri (38–56 vs. 26–

39, 49–65 vs. 29–47, respectively), and cortical granules (one type, colorless vs. two types, yellowish or yellowish to colorless) (Kumar et al. 2010).

Bakuella (P.) salinarum Mihailowitsch and Wilbert 1990 is clearly distinguished from *B. (P.) litoralis* by body size (272–348 \times 87–145 μm vs. 70–106 \times 22–49 μm in protargol-impregnated specimens), number of adoral membranelles (47–63 vs. 25–33), buccal cirri (6–8 vs. 3–5), transverse cirri (7–12 vs. 3–6), midventral pairs (22–38 vs. 10–15), midventral rows (13–21 vs. 1 or 2), length of midventral complex (terminated close to transverse cirri vs. at 70% of body length), left and right marginal cirri (45–60 vs. 26–39, 55–66 vs. 29–47, respectively), and macronuclear nodules (more than 100 vs. 49–84) (Mihailowitsch and Wilbert 1990).

Bakuella (P.) walibonensis Song et al. 1992 can be discriminated from *B. (P.) litoralis* by body size (180–229 \times 62–83 μm vs. 70–106 \times 22–49 μm in protargol-impregnated specimens), buccal cirri (5 or 6 vs. 3–5), length of midventral complex (54% vs. 70% of body length), pretransverse cirri (absent vs. present), left and right marginal cirri (42–50 vs. 26–39, 51–66 vs. 29–47, respectively), posterior part of marginal rows (slightly overlapped vs. clearly separated), and macronuclear nodules (more than 100 vs. 49–84) (Mihailowitsch and Wilbert 1990; Song et al. 1992).

Phylogeny

Morphologically, Bakuellidae is characterized as Urostyloidea with three frontal cirri and midventral complex composed of cirral pairs and row(s) (Berger 2006). Seven genera have been assigned to the family Bakuellidae (*Australothrix*, *Bakuella*, *Birojimia*, *Holostichides*, *Metaurostypopsis*, *Parabirojimia*, and *Paragastrostyla*; Berger 2006). Recently, *Parabirojimia* was transferred to Parabirojimidae as type genus (Dai and Xu 2011) and two new genera,

Table 2. Morphological comparison of *Bakuella* (*Bakuella*) *incheonensis* n. sp. with two closely related species

Characters	<i>B. incheonensis</i> n. sp.	<i>B. agamalievi</i> (neotype)	<i>B. agamalievi</i> (Germany)	<i>B. subtropica</i>
Body size (μm)	60–100 \times 29–50	97–131 \times 48–70	136–176 \times 40–51	116–208 \times 43–125
Adoral membranelles, no.	21–25	26–37	30–37	25–44
Frontoterminal cirri, no.	3–4	4–7	6 (in illustration)	4–12
Midventral pairs, no.	7–10	9–13	10–18	9–23
Midventral rows, no.	1–2	3–6	3–5	1–2
Midventral complex, % of body length	62	67	61 ^a	80
Cirri in LMR, no.	20–28	30–38	30–40	30–54
Cirri in RMR, no.	25–32	40–47	34–46	28–64
Macronuclear nodules, no.	58–87	47–60	> 100	68–144
Cortical granules	0.7 μm , yellowish	0.8 μm , colorless or slightly greenish	No data	1–2 μm , yellow–brownish to yellow–greenish
Data source	This study	Song et al. (2002)	Mihailowitsch and Wilbert (1990)	Chen et al. (2013)

Morphometric data are based on protargol-impregnated specimens. Data on cortical granules are from in vivo observations.

LMR = left marginal row; RMR = right marginal row.

^aThis value was measured from illustration provided by Mihailowitsch and Wilbert (1990).

Table 3. Morphological comparison of four species in *Bakuella* (*Pseudobakuella*)

Characters	<i>B. litoralis</i> n. sp.	<i>B. nilgiri</i>	<i>B. salinarum</i>	<i>B. walibonensis</i>
Body size (µm)	70–106 × 22–49	124.1–157.7 × 44.1–77.0	272–348 × 87–145	180–229 × 62–83
Adoral membranelles, no.	25–33	42–54	47–63	33–47
Buccal cirri, no.	3–5	4–8	6–8	5–6
Midventral pairs, no.	10–15	18–23	22–38	13–14
Midventral rows, no.	1–2	2–3	13–21	2–5
Midventral complex, length	70% of body length	Extended to near transverse cirri	Extended to near transverse cirri	54% of body length (in illustration)
Pretransverse cirri, no.	1–2	2, rarely 3	Absent	Absent
Transverse cirri, no.	3–6	6–11	7–12	4–6
Cirri in LMR, no.	26–39	38–56	45–60	42–50
Cirri in RMR, no.	29–47	49–65	55–66	51–66
Macronuclear nodules, no.	49–84	Around 100	> 100	> 100
Cortical granules	Type I: larger, yellowish; Type II: smaller, yellowish to colorless	Colorless	No data	No data
Habitat	Brackish water	Soil	Salt water	Salt water
Data source	This study	Kumar et al. (2010)	Mihailowitsch and Wilbert (1990)	Mihailowitsch and Wilbert (1990)

Morphometric data are from protargol-impregnated specimens. Data on cortical granules are from in vivo observations.

LMR = left marginal row; RMR = right marginal row.

Apobakuella and *Neobakuella*, were established in Bakuellidae (Jiang et al. 2013; Li et al. 2011). The main diagnostic morphological combination distinguishing *Bakuella* from the seven other genera assigned to Bakuellidae is two marginal rows and absence of caudal cirri.

Of the eight genera in Bakuellidae, only the following four have been subjected to analyses of their phylogenetic relationships using SSU rRNA gene sequences: *Apobakuella*, *Bakuella*, *Metaurostylopsis*, and *Neobakuella* (Chen et al. 2013; Huang et al. 2014; Lv et al. 2015). Of the four genera, *Metaurostylopsis* clustered separately from the clades *A. fusca*–*B. litoralis*–*N. flava* and *B. granulifera*, and showed a sister relationship with *Apokeronopsis*–*Apourostylopsis*–*Thigmokeronopsis*. In common with previous reports, our phylogenetic tree does not support monophyly of Bakuellidae (Table 4). The sister clade of *Metaurostylopsis*, *Apokeronopsis*–*Thigmokeronopsis*–*Apourostylopsis* belonging to Urostylidae, lacks the three frontal cirri and midventral complex composed of cirral pairs and row(s) characteristic of bakuellids (Fig. 6). Eight percent (2 of 25 cells) of the examined population of *B. (P.) litoralis* lacked the cirral row,

suggesting we need to investigate this character state more precisely using phylogeny based on multiple genes and morphogenesis to discriminate Bakuellidae from Urostylidae.

The clade *A. fusca*–*B. litoralis*–*N. flava* (Fig. 6) shows commonalities in morphological characters such as cortical granules, midventral complex composed of cirral pairs and rows, and parabuccal cirri. However, they can be distinguished from *Bakuella*, at the genus level, by the presence of two left marginal rows in *Neobakuella* (vs. one left marginal row in *Bakuella*), and two right marginal rows and no frontoterminal cirri in *Apobakuella* (vs. one right marginal row, two or more frontoterminal cirri in *Bakuella*) (Jiang et al. 2013; Li et al. 2011).

Interestingly, the subgenus *Bakuella* was inferred to be nonmonophyletic relationship, and one of the three consubgenera clustered with a species of Urostylidae, i.e. in the clade *B. granulifera*–*Urostyla grandis*. *Bakuella granulifera* has a conspicuously longer midventral cirral row than the other two consubgenera (number of cirri in the rightmost midventral row, 9–14 vs. 3 or 4 in *B. incheonensis*, 3–5 in *B.*

Table 4. Log likelihoods and associated *p*-values from approximately unbiased (AU), weighted Shimodaira–Hasegawa (WSH), and weighted Kishino–Hasegawa (WKH) tests of different topological scenarios on phylogenetic trees

Topology	Log likelihood (–ln L)	Difference to best tree (–ln L)	AU (<i>p</i>)	WSH (<i>p</i>)	WKH (<i>p</i>)	Conclusion
Best maximum likelihood tree (unconstrained)	11,979.378	–	1.000	1.000	1.000	–
Monophyly of the family Bakuellidae	12,301.915	322.537	2e-45	0.000	0.000	Rejected
Monophyly of the genus <i>Bakuella</i>	12,209.150	229.772	2e-54	0.000	0.000	Rejected
Monophyly of the subgenus <i>Bakuella</i>	12,162.539	183.161	2e-59	0.000	0.000	Rejected

Significant differences (*p* < 0.05) between the best unconstrained and constrained topologies are in bold.

subtropica). *Bakuella granulifera* and *Urostyla grandis* can be discriminated from each other by the number of marginal rows (two vs. more than two), and frontoterminal cirri (present vs. absent).

SSU rRNA gene sequences of two *Bakuella* species are available from GenBank (*B. granulifera*, KJ958489; *B. subtropica*, KC631826). These two species, along with the two new species described here are congeners, but the four showed a nonmonophyletic relationship (Fig. 6; Table 4). In addition, the subgenus *Bakuella* composed of *B. granulifera*, *B. incheonensis*, and *B. subtropica* were also nonmonophyletic. The clade *B. (B.) subtropica*–*B. (B.) incheonensis* showed a sister relationship with *Anteholosticha paramanca*, which belongs to Holostichidae. The genus *Anteholosticha* is well known for polyphyly (Berger 2003, 2006; Park et al. 2013; Shao et al. 2011; Yi and Song 2011). Park et al. (2013) suggested nine groups within *Anteholosticha*, using a combination of molecular and morphogenetic data to resolve the polyphyly. In our phylogenetic tree, *A. paramanca* and *B. (B.) subtropica* appeared to have a close relationship with *Anteholosticha* group I (*A. manca* and *A. multicirrata*; Park et al. 2013). These four species have similar morphogenetic processes, for instance: (1) in the proter, parental adoral membranelles are completely replaced by oral primordium; (2) in the opisthe, oral primordium commences near the left midventral pairs; (3) new marginal rows and dorsal kineties are formed within the parental one; and (4) the macronuclear nodules fuse into a single mass (Chen et al. 2013; Fan et al. 2014; Li et al. 2008; Park et al. 2013). The main diagnostic character for discriminating the two genera is the midventral cirral row (absent in *Anteholosticha* vs. present in *Bakuella*).

To understand the phylogeny of the genus *Bakuella*, it is necessary to utilize both morphogenetic studies and multiple gene-based approaches. In addition, other *Bakuella* species have to be analyzed based on gene sequences including *B. marina*, type species of the genus *Bakuella*.

TAXONOMIC SUMMARY

Phylum Ciliophora Doflein, 1901
 Class Spirotrichea Bütschli, 1889
 Order Urostylida Jankowski, 1979
 Family Bakuellidae Jankowski, 1979
 Genus *Bakuella* Agamaliyev and Alekperov 1976
 Subgenus *Bakuella (Bakuella)* Agamaliyev and Alekperov 1976

Bakuella (Bakuella) incheonensis n. sp

Diagnosis. Size about 70–105 × 20–40 μm in vivo; body flexible and slightly contractile with slender to elliptical shape. 58–87 macronuclear nodules with 3–6 micronuclei. Yellowish cortical granules distributed beside cirral rows and along dorsal bristles as loose longitudinal rows, but some granules scattered irregularly between rows; spherical to slightly ellipsoidal and measuring 0.7 × 0.5 μm in dorsal view, ellipsoidal and measuring 1.0 × 0.7 μm in lateral view. Exactly, 21–25 adoral membranelles, three enlarged frontal cirri, one buccal cirrus, one parabuccal cir-

rus, three or four frontoterminal cirri, usually one pretransverse cirrus, and four or five transverse cirri. Midventral complex composed of 7–10 midventral cirral pairs with one or two midventral cirral rows, each row composed of three or four cirri. Exactly, 20–28 left and 25–32 right marginal cirri. Three dorsal kineties.

Type locality. Brackish water near Aamdo Shore Park, Incheon, South Korea (37°24'30"N, 126°38'43"E).

Type slides. One slide containing the holotype specimen (NIBRPR0000105679) and one paratype slide (NIBRPR0000105680) with protargol-impregnated specimens have been deposited in the National Institute of Biological Resources (NIBR), South Korea. The holotype and other relevant specimens have been marked with circles on the bottom of the slides.

Etymology. The name is derived from the city (Incheon) where the species was discovered.

Gene sequence. SSU rRNA gene sequence deposited in GenBank under the accession number KR024011.

Zoobank registration number. urn:lsid:zoobank.org:act:DC7F3192-CCA3-492A-9259-2D12503ABCFD.

Subgenus *Pseudobakuella* Alekperov, 1992

Bakuella (Pseudobakuella) litoralis n. sp

Diagnosis. Size about 90–125 × 30–40 μm in vivo; body flexible and slightly contractile with elliptical shape. Exactly, 49–84 macronuclear nodules with 3–8 micronuclei. Two types of cortical granules widely distributed on body surface; type I, larger, yellowish and oval; type II, smaller, yellowish to colorless and subglobular. Exactly, 25–33 adoral membranelles, three enlarged frontal cirri, 3–5 buccal cirri, 2–4 parabuccal cirri, invariably two frontoterminal cirri, one or two pretransverse cirri, and 3–6 transverse cirri. Midventral complex composed of 9–15 midventral cirral pairs with one or two midventral cirral rows, each row composed of 3–5 cirri. Exactly, 26–39 left and 29–47 right marginal cirri. Three dorsal kineties.

Type locality. Brackish littoral zone of Aamdo Shore Park, Incheon, South Korea (37°24'48"N, 126°38'26"E).

Type slides. One slide containing the holotype specimen (NIBRPR0000105675) and three paratype slides (NIBRPR0000105676–NIBRPR0000105678) of protargol-impregnated specimens have been deposited in the National Institute of Biological Resources (NIBR), South Korea. The holotype and other relevant specimens have been marked with circles on the bottom of the slides.

Etymology. The name is derived from the location (littoral zone) where the species was discovered.

Gene sequence. SSU rRNA gene sequence deposited in GenBank under the accession number KR024010.

Zoobank registration number. urn:lsid:zoobank.org:act:C49B3732-06D2-4BC0-B67D-B49468686CA5.

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