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Morphology and SSU rRNA gene sequence of the new brackish water ciliate, *Anteholosticha pseudomonilata* n. sp. (Ciliophora, Hypotrichida, Holostichidae) from Korea

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Abstract

The morphology and infraciliature of a hypotrichous ciliate collected from brackish-water biotope (salinity 5 ‰) in South Korea were investigated which indicate this organism should be a new form within the genus *Anteholosticha* Berger, 2003. Careful morphological comparison and SSU rRNA gene sequence alignment with similar species are performed to support the validity of *Anteholosticha pseudomonilata* **n. sp.** The main diagnostic and distinguishing characteristics of the new species include: 1) 8–11 transverse cirri; 2) midventral complex composed of 10–16 pairs of zigzagging midventral cirri, extending posteriorly to slightly ahead of pretransverse cirri; 3) cortical granules colourless and pigment-like, 0.5 μm across, longitudinally arranged in more or less short rows on whole cortex except along dorsal kineties and cirral rows; and 4) 8–12 macronuclear nodules located left of midline.

Key words: *Anteholosticha*, infraciliature, morphology, new species, SSU rRNA gene sequence

Introduction

Morphological identification is always a great challenge for the highly divergent groups of ciliates, especially for the complicated hypotrichs (Berger 2006; Borror 1972; Hemberger 1985; Kahl 1932). This situation has been remarkably improved in virtue of the staining techniques which can reveal the precise infraciliature and argyrome. However, taxonomic difficulties still exist due to the insufficient descriptions and misinterpretations in some of the previous reports. In addition, the subjective weight of characteristics used for determining differentiation of species or variation between populations was largely depended on the experience and authority of the taxonomists (Borror & Hill 1995; Song & Packroff 1997; Song *et al.* 1998; Wilbert & Song 2008). In the recent decades, small subunit rRNA gene sequence has been widely used for the phylogenetic study of ciliates and gradually becomes a conservative diagnostic feature for species definition and circumscription (Chen *et al.* 2010; Elwood *et al.* 1985; Gong *et al.* 2007; Jiang *et al.* 2010; Li *et al.* 2009; Liu *et al.* 2010; Lynn & Small 2002). Nevertheless, many forms in the molecular data were not appropriately identified or lacked corresponding morphological data which in turn may cause further confusion to the morphological and phylogenetic studies.

Anteholosticha was erected by Berger (2003) to include those species previously classified in the rather vaguely defined genus *Holosticha* Wrześniowski, 1877 which lack its apomorphies (i. e. anterior end of left marginal row curved rightwards and proximal-most membranelles widened) and caudal cirri. During the survey of the ciliate fauna inhabiting brackish biotopes in South Korea, one of our isolates was demonstrated to be an unknown form belonging to this genus. In the present work, its diagnosis, morphological description, illustrations and morphometric data are given. In addition, phylogenetic analysis based on SSU rRNA gene sequences alignments was carried out to verify the validity of the new form.

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Material and methods

Sampling. *Anteholosticha pseudomoniliata* **n. sp.** was collected from the Taehwagang River, Usan (35°32′56"N 129°20′11"E) in South Korea on 23 November 2009, with salinity 5‰, pH 6.5–7.0, and water temperature of 11.5 °C. The surface sediments (~10 cm) were collected and transferred to Petri dishes with original water, then maintained in the laboratory for several days at room temperature (about 24 °C) as a raw culture for further studies. Meanwhile, a few wheat grains were added to enrich the bacteria (Li *et al.* 2010).

Morphological investigations. The locomotion of the organisms was studied in Petri dishes under a dissecting microscope. Living cells were isolated and observed using bright-field and differential interference-contrast microscopy. The infraciliature and silverline system were revealed with the protargol impregnation method according to Wilbert (1975). Specimens were examined at 100× to 1,000× magnification and measurements were carried out with an ocular micrometer. Drawings of stained specimens were performed under oil immersion and according to photographs as a template.

Terminology. Terminology is mainly according to Berger (2006).

DNA extraction and sequencing. DNA was extracted using RED Extract-N-AmpTM PCR kit (Sigma-Aldrich Co., Brooklyn, NY) according to the manufacturer's instruction. The reaction volume was reduced to one tenth of the originally prescribed volume for each tube according to Gong *et al*. (2007). PCR amplification was performed according to Yi *et al*. (2010) and the PCR products of SSU rRNA gene were sequenced using an ABI 3700 sequencer (Applied Biosystems, Foster City, USA).

Sequence availability and alignment. The following nucleotide sequences of five *Anteholosticha* species were obtained from the NCBI/GenBank database for comparison with *A. pseudomonilata* **n. sp.**: *A. manca* (GenBank accession no. DQ503578), *A. multistilata* (AJ277876), *A. monilata* (GU942567), *A. parawarreni* (EF123707), and *A. scutellum* (FJ156105). These sequences were aligned using Clustal W, ver.1.80 (Thompson *et al*. 1994).

Results

Order Urostylida Jankowski, 1979

Family Holostichidae Fauré-Fremiet, 1961

Genus *Anteholosticha* Berger, 2003

Anteholosticha pseudomonilata n. sp.

(Figs 1–3; Tables 1–3)

Diagnosis. Grey coloured *Anteholosticha*, 110–190 µm × 40–80 µm *in vivo*; contractile vacuole mid-body positioned; 29–41 adoral membranelles; 1 buccal, 3 frontal, 2 frontoterminal, 2 pretransverse and 8–11 transverse cirri; midventral complex composed of 10–16 pairs of zigzagging midventral cirri, extending posteriorly to slightly ahead of pretransverse cirri; left and right marginal row with about 28 and 29 cirri, respectively; 4 entirely long dorsal kineties; cortical granules colourless and pigment-like, 0.5 µm across, longitudinally arranged in more or less short rows on whole cortex except along dorsal kineties and cirral rows; 8–12 macronuclear nodules located left of midline.

Type locality. Brackish water from the Taehwagang River (35°32′56"N; 129°20′11"E) near Ulsan Bay which flows into the East Sea, at Ulsan, South Korea.

Type slides. Two permanent slides of protargol-impregnated specimens are deposited as a holotype and a paratype in the collection of National Institute of Biological Resources (NIBR), Incheon, South Korea and Department of Biology, University of Ulsan, Ulsan, South Korea and with registration numbers NIBRPR0000102719 and LLQ-20091123-05-02, respectively.

Etymology. The species-group name *pseudomonilata* is a composite of the Greek adjective pseudo- (wrong, lying) and the name of the most similar congener *Anteholosticha monilata* which resembles our form in having the

macronucleus-nodules forming a chain (see details about etymology of *monilata* in Berger 2006). The ending of the species-group name is defined (fixed) via the genus.

Description. Size mostly 110–140 µm × 40–60 µm *in vivo*. Body shape slightly variable and elliptic-like, anterior portion conspicuously narrowed and forming a slightly cephalized appearance, while rear end broadly rounded; left margin more convex than right part (Figs. 1A; 2A, 2B). Body flexible, only slightly contractile. Dorsoventrally flattened about 2:1. Pellicle thin and soft. Cortical granules colourless and spherical in shape, about 0.5 µm in diameter, basically arranged in more or less longitudinal short rows on whole cortex except along cirral rows and dorsal kineties with some single ones sparsely distributed throughout the cell surface (Figs. 1C; 2C). Food vacuoles difficult to recognize except for many yellowish to brownish light-reflecting crystals scattered within transparent cytoplasm. Two groups of inclusions remarkably recognized, which are globular and concave like, respectively, 2–5 µm in diameter, consistently situated and give rise to darker colour in both ends of the cell (Figs. 1A, 1B; 2E). 8–12 colourless macronuclear nodules globular to ellipsoidal shaped, serially distributed in mid-portion of body left of midline which can be easily observed under middle to high magnification *in vivo*; individual nodules, in impregnated specimens, 8–20 µm× 8–18 µm, containing small nucleoli (Figs. 1E; 2D, 2F). Usually two micronuclei ovoid in shape located separately among the macronuclei, 5–10 µm × 3–8 µm across after fixation (Figs. 1E; 2D, G). Single contractile vacuole positioned in mid-body near left margin with two long collecting canals (Fig. 1A).

Locomotion relatively slow, but crawling without pause. Body apparently flexible, folded or twisted when crawling on bottom of Petri dish or drilling through debris.

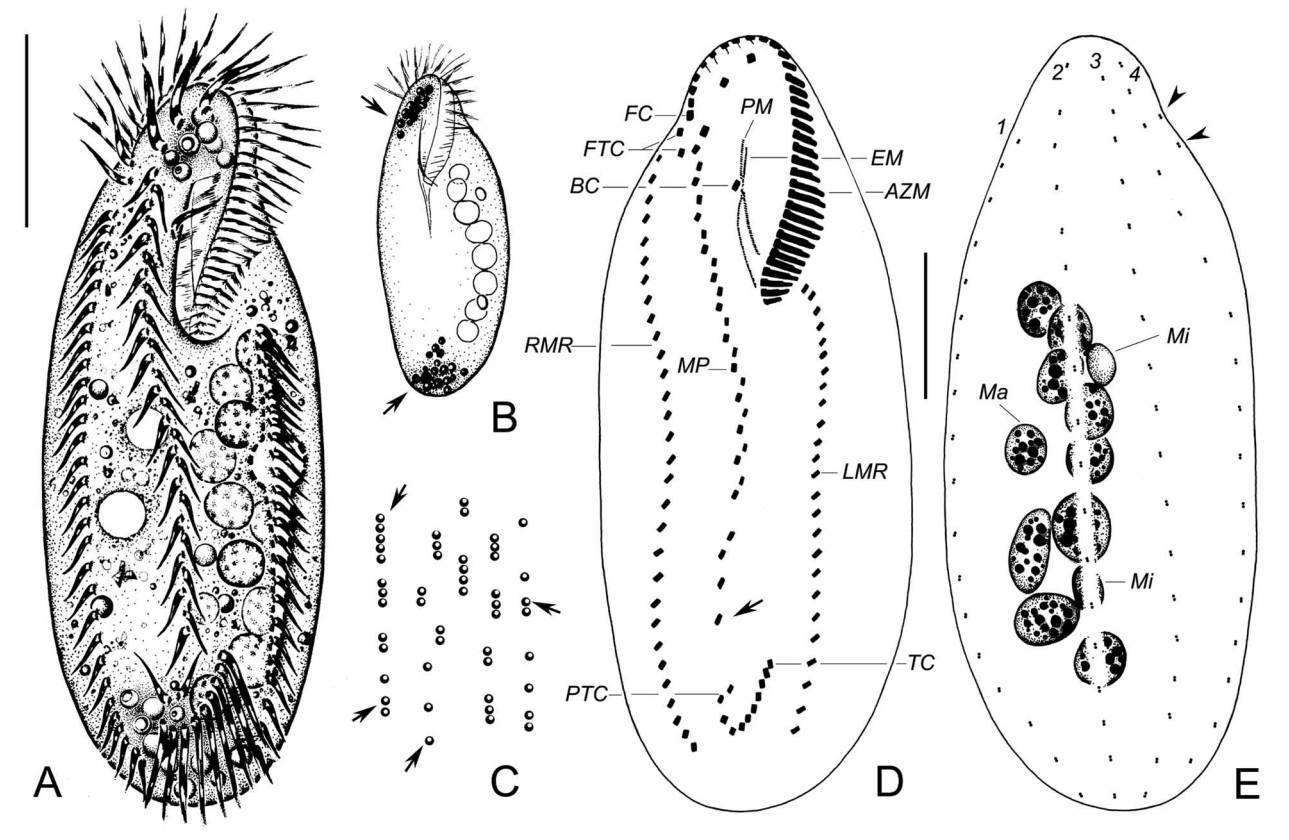

FIGURE 1. *Anteholosticha pseudomonilata* **n. sp.** from live cells (**A**–**C**), and after protargol impregnation (**D, E**). (**A**) Ventral view of a typical individual. (**B**) Ventral view, arrows indicate the inclusions within cytoplasm at both cell ends. (**C**) Noting arrangement of cortical granules (arrows). (**D, E**) Ventral and dorsal views of the holotype specimen, showing the general infraciliature. Arrow in **D** marks the posterior end of the midventral complex. Arrowheads in **E** depict the "extra" dikinetids ahead of 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** = macronuclei; **Mi** = micronuclei; **MP** = midventral pairs; **PM** = paroral membrane; **PTC** = pretransverse ventral cirri; **RMR** = right marginal row; **TC** = transverse cirri; **1-4** = dorsal kineties. Scale bars in (A) = 40 µm; in (D, E) = 30 µm.

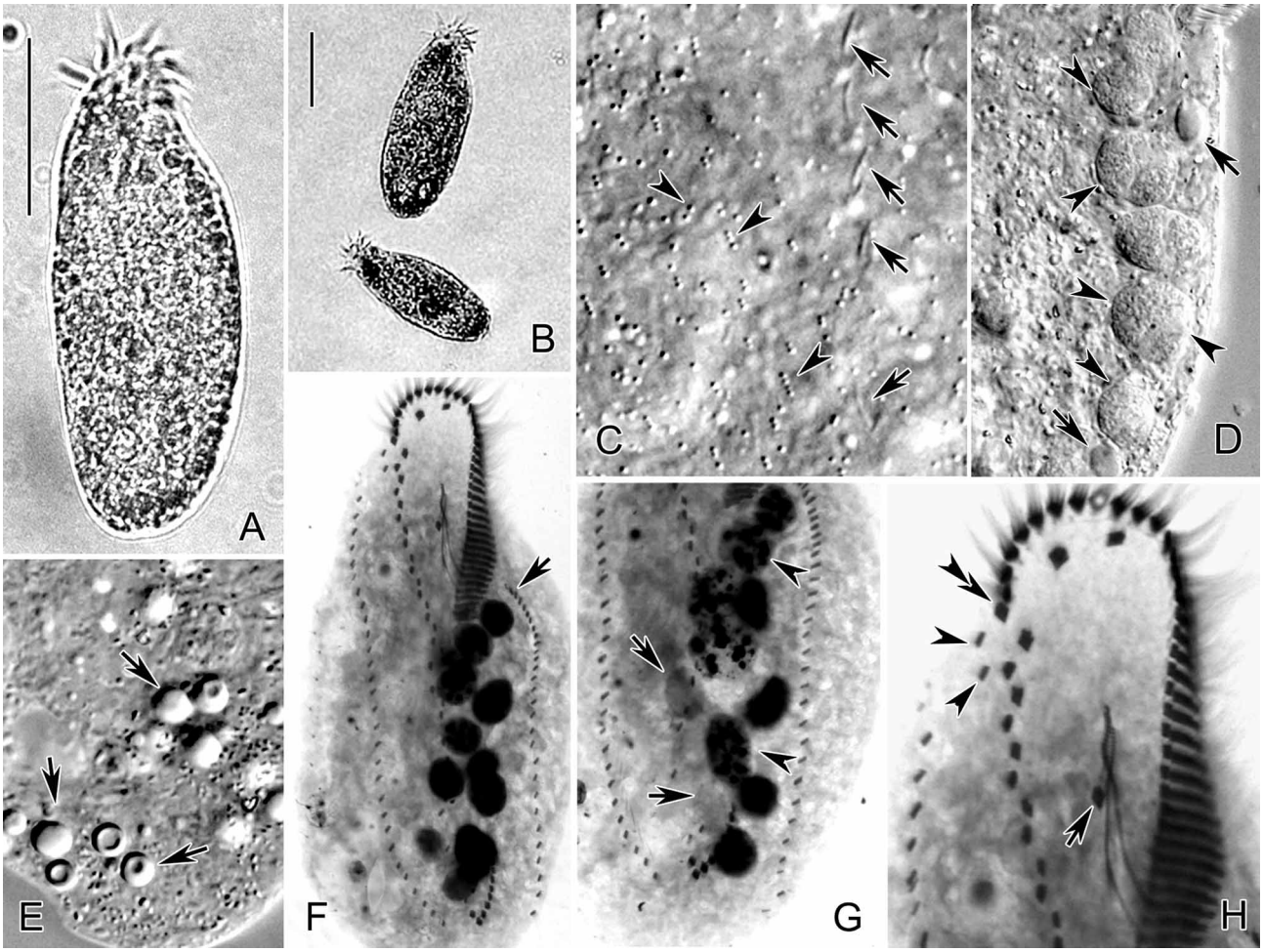

FIGURE 2. Photomicrographs of *Anteholosticha pseudomonilata* **n. sp.** from live cells (**A**–**E**), and after protargol staining (**F**–**H**). (**A, B**) Ventral view of typical individuals. (**C**) Partial of dorsal view, to show the distribution of cortical granules (arrowheads) and dorsal bristles (arrows). (**D**) Ventral view showing macronuclear nodules (arrows) and micronuclei (arrowheads). (**E**) Posterior body, arrows mark the inclusions within cytoplasm. (**F**) Ventral view of the holotype specimen (the same individual as illustrated in 1**D, E** and 2**G, H**), arrow denotes anterior end of left marginal row. (**G**) Ventral view of mid-posterior portion, arrows and arrowheads show micronuclei and macronuclear nodules, respectively. (**H**) Anterior portion of ventral side, showing the buccal cirrus (arrow), frontoterminal cirri (arrowheads), and the right frontal cirrus (double-arrowheads). Scale bars = 50 μm.

Adoral zone of membranelles occupied 30.3–41.7% of body length in fixed specimens (Table 1), base of longest membranelles about 9 μm long. Distal end of AZM terminated at right margin of cell and bending posteriad at about anterior 1/3 of buccal field (Figs. 1D; 2F). Paroral and endoral membrane almost the same length, likely both composed of monokinetids, distinctly intersecting each other and terminating anteriorly at about 2/5 of buccal field (Figs. 1D; 2H). Single buccal cirrus situated near the intersection of undulating membranes (Figs. 1D; 2H). Three slightly enlarged frontal cirri lying in anterior frontal area with right one very close to the distal end of adoral zone of membranelles (Fig. 2H). Consistently two frontoterminal cirri near and right to the distal end of adoral zone of membranelles (Fig. 2H). 8–11 relatively undeveloped transverse cirri arranged in J-shaped row, *ca.* 15 μm long *in vivo*, slightly protruding beyond rear end of cell (Figs. 1A, 1D; 2G). Always two pretransverse ventral cirri located close to the right transverse cirrus (Fig. 1D). Midventral complex composed of 10–16 pairs of midventral cirri arranged in a typical zig-zag pattern, continuing with frontal cirri and terminated ahead of the level of the left-most transverse cirrus (Figs. 1D; 2F, 2G). The last midventral cirri often dispersedly situated other than in a zigzagging pattern (Fig. 1D). Two marginal rows distinctly separated posteriorly ahead of cell end, the cirri within which about 10 μm long and never protruding the margins in life; 23–33 cirri in left row, while 24–34 in right one (Figs. 1A, 1D; 2A, 2F). Invariably four complete dorsal kineties with dorsal cilia about 3 μm long. Usually two “extra” dorsal bristles present on the anterior right margin of the body (Figs. 1E; 2C).

TABLE 1. Morphometrical characterization of *Antheholosticha pseudomonilata* **n. sp.** All data based on protargol-impregnated specimens. Measurements in micrometers.

Character	Min	Max	Median	Mean	SD	SE	CV	n
Body, length	113	185	150.0	147.7	15.4	2.8	10.4	30
Body, width	45	80	56.0	59.2	9.5	1.7	16.0	30
Body length:width, ratio	2.1	3.2	2.5	2.5	0.3	0.1	12.0	30
Length of adoral zone	42	61	52.5	53.5	5.1	0.9	9.5	30
Body length:length of adoral zone, ratio	2.4	3.3	2.7	2.8	0.2	0.0	7.1	30
Number of membranelles	29	41	37.0	36.4	2.8	0.5	7.7	30
Number of frontal cirri	3	3	3.0	3.0	0.0	0.0	0.0	30
Number of buccal cirri	1	1	1.0	1.0	0.0	0.0	0.0	30
Number of frontoterminal cirri	2	2	2.0	2.0	0.0	0.0	0.0	20
Number of midventral pairs	10	16	13.0	13.2	1.3	0.3	9.8	27
Number of cirri in right marginal row	24	34	29.0	29.4	2.2	0.4	7.5	29
Number of cirri in left marginal row	23	33	28.0	27.7	2.7	0.6	9.7	22
Number of pretransverse cirri	2	2	2.0	2.0	0.0	0.0	0.0	27
Number of transverse cirri	8	11	9.0	9.5	0.8	0.2	8.4	27
Number of dorsal kineties	4	4	4.0	4.0	0.0	0.0	0.0	21
Number of macronuclei	8	12	10.0	9.4	1.0	0.2	10.6	30
Number of micronuclei	1	3	2.0	2.0	0.4	0.1	20.0	26

Abbreviations: CV = coefficient of variation in %, Max = maximum, Mean = arithmetic mean, Median = median value, Min = minimum, n = number of individuals examined, SD = standard deviation, SE = standard error of arithmetic mean.

Comparison. Currently, over 40 morphotypes have been included in the genus *Antheholosticha*, most of which need further studies and redescription (Berger 2006). As one of the most significant criteria for species circumscription, nuclear apparatus (number, arrangement) usually can be easily detected and described even in the cursory data, furthermore, the diversity of which is quite high in *Antheholosticha*, therefore the congeners resemble our form in having a series of macronuclear nodules should be compared here.

Morphologically, the most similar congener *Antheholosticha monilata* (Kahl, 1928) Berger, 2003 (type species) resembles *A. pseudomonilata* **n. sp.** in several features (e.g. body shape, contractile vacuole, ciliary arrangement, the number of membranelles and transverse cirri), however, differs from the latter in having 6 or more dorsal kineties (vs. 4), 19–27 midventral pairs (vs. 10–16), 4–23 macronuclear nodules (vs. 8–12), extrusomes (vs. absent), and no cortical granules (vs. present) thus both forms can be distinctly separated (e. g. Augustin & Foissner 1992; Berger 2006).

Likewise, other similar *Antheholosticha* spp., namely, *A. distyla* (Buitkamp, 1977) Berger, 2003, *A. xanthichroma* (Wirnsberger & Foissner, 1987) Berger, 2003, *A. australis* (Blatterer & Foissner, 1988) Berger, 2003, *A. mancoidea* (Hemberger, 1985) Berger, 2003, *A. randani* (Grolière, 1975) Berger, 2003, *A. sphagni* (Grolière, 1975) Berger, 2003, *A. sigmoidea* (Foissner, 1982) Berger, 2003, and *A. extensa* (Kahl, 1932) Berger, 2003 can be easily distinguished from *A. pseudomonilata* **n. sp.** by a number of features including habitat, body shape, size, the position of the contractile vacuole, cortical granules, and morphometric data (details see Table 2) (Berger 2003, 2006; Blatterer & Foissner 1988; Borror & Wicklow 1983; Buitkamp 1977; Foissner 1982; Grolière 1975; Hemberger 1985; Kahl 1932; Wirnsberger & Foissner 1987).

SSU rRNA gene sequence analysis: The length of the complete SSU rRNA gene sequence of *Antheholosticha pseudomonilata* **n. sp.** is 1772 bp; the nucleotide sequence has been given the accession number HM568416. The GC content is 44.75%, which is within the range of other ciliates. Among the dataset of 1795 total positions, a total of 244 mismatched nucleotides are revealed from the alignment of six *Antheholosticha* species (Fig. 3). Of these, 24 positions are unique to *A. pseudomonilata*. SSU rRNA gene sequence similarity among *A. pseudomonilata* and the other five species are listed in Table 3.

TABLE 2. Comparison of *Anteholosticha pseudomonilata* n. sp. with nine most similar congeners.

Character	*A. pseudomonilata*	*A. distyla*	*A. monilata*	*A. mancoidea*	*A. randani*	*A. xanthichroma*	*A. australis*	*A. sphagni*	*A. sigmoidea*	*A. extensa*
Body length *in vivo*	110–190 µm	150–180 µm	90–160 µm	*ca.* 120 µm	95–125 µm	110–220 µm	130–190 µm	60–90 µm	90–130 µm	140–240 µm
Body shape	elliptical, anterior end narrowed	ribbon-shaped, both ends rounded	elongated and both ends narrowly rounded	elongate, both margins parallel	elongate elliptical	long belt-like, both ends rounded	oblong, both ends rounded	elongate elliptical	slightly sigmoidal	long belt-like
Adoral zone:body length, ratio after fixation	30–42%	33%	33%	25%	34%	27%	27%	26%	23–25%	20%
Position of CV	mid-body	anterior 40%	mid-body	-	anterior 1/3	above mid-body	above mid-body	anterior 1/3	ahead of mid-body	-
Cortical granules	globular, colourless, 0.5 µm across, several attached forming short longitudinal rows	-	rod-shaped extrusomes	-	-	-	ellipsoidal colourless 2.5×1.5 µm	absent	globular, colourless, 0.5–1 µm across in more or less distinct longitudinal rows	absent
Number of AM	29–41	30–33	30–44	17–20	22–33	21–46	*ca.* 30	24–25	16–24	-
Number of FC	3	4–5	3–4	4	3	3	3	2	3	2–3
Number of MP	10–16	15–16	19–27	*ca.* 7	20–27	20–65	*ca.* 17	17–24	9–12	(19)
Position of last cirri in MC	slightly ahead of TC	slightly ahead of TC	near TC	at 38% of body length	near TC	near TC	near TC	at 63% of body length	at 50–58% of body length	near TC
Number of TC	8–11	2	7–10	*ca.* 5	3–5	0–6	*ca.* 4	~5	3–6	6–7
Number of DK	4	4	6	3	4	4	4	4	4	-
Number of Ma	8–12	*ca.* 16	9–23	*ca.* 8	16–20	11–42	*ca.* 12	8–11	(6–8)	6–8
Habitat	Brackish	freshwater	freshwater	freshwater	freshwater	freshwater	freshwater	freshwater	freshwater	marine
Data source	present work	Buitkamp (1977)	Augustin & Foissner (1992)	Hemberger (1985)	Grolière (1975)	Wirnsberger & Foissner (1987)	Blatterer & Foissner (1988)	Borror & Wicklow (1983)	Foissner (1982)	Kahl (1932)

AM = adoral membranelles; BC = buccal cirri; CV = contractile vacuole; DK = dorsal kinety; FC = frontal cirri; Ma = macronuclear nodules; MC = midventral complex; MP = midventral pairs; TC = transverse cirri. - = Data unavailable. ( ) = data counted from drawing.

The SSU rRNA gene sequence comparison study clearly exhibits a considerable inconsistency of *Anteholosticha pseudomonilata* from other five congeners. Pairwise sequence similarities between *A. pseudomonilata* and its congeners range from 92.38% to 96.56% (Table 3). The comparatively high sequence discrepancy (3.44%) with the most similar morphotype *A. monilata* strongly suggests our form as a distinct species. *Anteholosticha monilata* is the type species indicating that the new species is a true member of the genus. By contrast, some other species seem to be not congeneric as indicated by the high differences in the structural similarity (Table 3). This supports the assumption by Berger (2003, 2006) that members of the genus *Anteholosticha* are likely not to be a monophyletic group. These higher ranges of molecular divergence also coincide with the statement of morphological analysis to establish *A. pseudomonilata* as an individual species of the genus *Anteholosticha*.

TABLE 3. The structural similarities (%) of the SSU rRNA gene sequences of six *Anteholosticha* species determined according to Elwood *et al*. (1985).

	A. pseudomonilata	*A. monilata*	*A. parawarreni*	*A. scutellum*	*A. multistilata*
A. monilata	96.56				
A. parawarreni	93.07	93.12			
A. scutellum	93.11	92.88	97.37		
A. multistilata	94.67	94.55	94.09	94.58	
A. manca	92.38	93.15	91.32	91.52	93.45

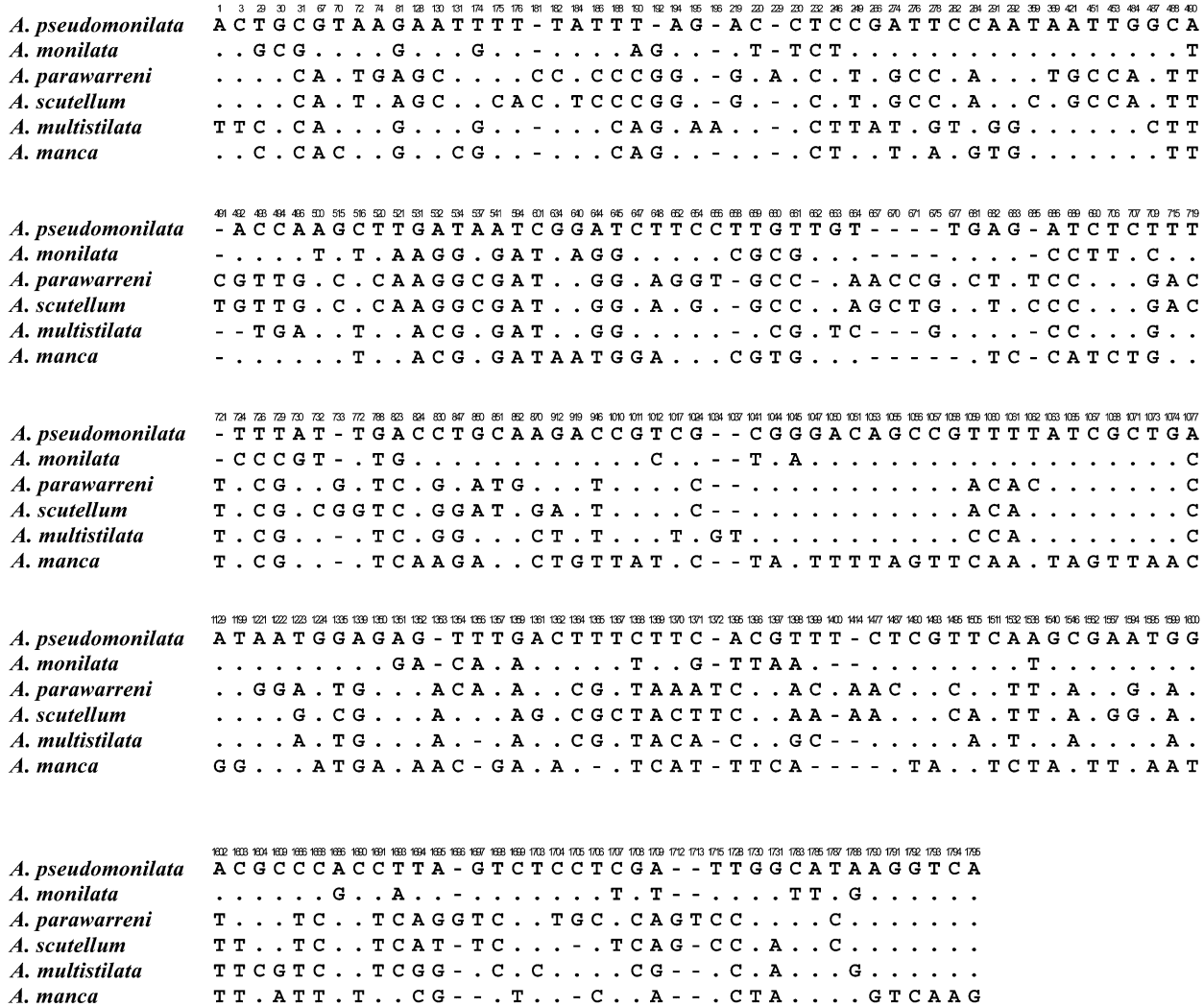

FIGURE 3. The alignment of variable sites for SSU rRNA gene sequences of *Anteholosticha pseudomonilata* **n. sp.** and five congeners. Nucleotide position number is marked at the top of each aligned line. Missing sites are indicate by gaps (–) and the sites matched with *A. pseudomonilata* are represented with dots.

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