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ORIGINAL ARTICLE

Morphological and Molecular Redefinition of *Euplotes platystoma* Dragesco & Dragesco-Kernéis, 1986 and *Aspidisca lynceus* (Müller, 1773) Ehrenberg, 1830, with Reconsideration of a "Well-known" *Euplotes* Ciliate, *Euplotes harpa* Stein, 1859 (Ciliophora, Euplotida)

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ABSTRACT

We documented the morphology, infraciliature, silverline system, and molecular data of two euplotid species isolated from China, including two populations of the poorly known *Euplotes platystoma* Dragesco & Dragesco-Kernéis, 1986 and the previously well described *Aspidisca lynceus* (Müller, 1773) Ehrenberg, 1830. Based on the information available, an improved diagnosis of *Euplotes platystoma* is given, including: a narrow adoral zone with 44–68 membranelles, 10 frontoventral, 5 transverse, 2 left marginal and 2 caudal cirri, 11–13 dorsal kineties with 17–25 dikinetids in the mid-dorsal row, and dorsal silverline system of the double-*eurystomus* type. The Chinese population of *Aspidisca lynceus* closely resembles previously described populations. Phylogenetic analyses inferred from SSU rDNA sequences show that *E. platystoma* is closely related with *E. neapolitanus*, and the internal position of *A. lynceus* within this genus is still not robust. A reconsideration of the "well-known" *Euplotes harpa* and a comparison of all SSU rDNA sequences of *E. harpa* in GenBank are provided. We speculate that the sequences available from GenBank under the name of *E. harpa* are very likely from misidentified materials, that is, the identity of the species currently associated with the SSU rDNA of this "well-known" form in molecular databases requires further confirmation.

EUPLOTID ciliates, comprising numerous species with a wide geographical distribution and high adaptive potential, have been of great interest to protozoologists in recent decades (Bitencourt et al. 2014; Di Giuseppe et al. 2015; Gao et al. 2017; La Terza et al. 2001; Liu et al. 2017; Modeo et al. 2013; Yi et al. 2012). The most species-rich genus of this group, *Euplotes* Ehrenberg 1830; is now known to be much more divergent and more widely distributed than previously believed (Chen et al. 2013, 2014; Dai et al. 2013; Di Giuseppe et al. 2014; Fotedar et al. 2016; Wang et al. 2017a; Yi et al. 2009). *Euplotes platystoma*, a poorly known species, was originally described by Dragesco and Dragesco-Kernéis (1986), and no detailed illustrations or photomicrographs are available.

Another species-rich genus of the euplotid ciliates with about 60 species, *Aspidisca* Ehrenberg, 1830, has been investigated for over a century (Borror 1972; Dragesco 1960; Kahl 1932; Li et al. 2008; Plough 1916; Song et al. 2009; Tuffrau 1964; Wu and Curds 1979). However, it remains one of the most confused groups, and SSU rDNA

sequences of only eight species are available. The type species, *Aspidisca lynceus* (Müller 1773) Ehrenberg, 1830, is probably the most widespread species of the genus and has been described in detail for many times (Augustin and Foissner 1992; Bick 1972; Claparède and Lachmann 1858; Gelei 1939; Kahl 1932; Stein 1859), however, no molecular data have been available for *A. lynceus*.

During the last 10 years, more than 30 euplotid ciliates found in China have been described, including new taxa and little-known species (e.g. Fan et al. 2013; Gao et al. 2016; Jiang et al. 2010; Li et al. 2010; Liu et al. 2015; Pan et al. 2012; Shen et al. 2011). As part of an on-going faunistic study of free-living euplotid ciliates in China, two populations of *Euplotes platystoma* and one population of *Aspidisca lynceus*, were investigated based on detailed morphological data. We also sequenced the SSU rDNA of *Euplotes platystoma* and *Aspidisca lynceus* for the first time and analyzed the phylogenetic relationships of this group.

MATERIALS AND METHODS

Sampling and morphological methods

The Shenzhen population of *Euplotes platystoma* was isolated on 23 November 2015 from a fresh water puddle (salinity 0‰) near Dameisha Beach (22°35′19″N; 114°18′2″E), Shenzhen, China, when the water temperature was about 25 °C. The Shanghai population of *Euplotes platystoma* was collected on 9 June 2014 from brackish water on Chongming Island (31°43′43″N; 121°13′37″E), Shanghai, China, when the water temperature was about 21 °C, and the salinity was about 6‰, and cultivated in fresh water. The cells of Shanghai and Shenzhen populations of *Euplotes platystoma* used for morphological and phylogenetic studies were from pure culture (culture only contains one species, but not from a single cell) and monoclonal culture, respectively. Both populations were maintained in Petri dishes at room temperature (20–24 °C), using mineral water with rice grains to enrich the growth of bacteria as food for the ciliates.

Aspidisca lynceus was isolated on 16 October 2015 from a fresh water pond (salinity 0‰) in Wuhan Botanical Garden (30°32′43″N; 114°25′4″E), Wuhan, China, when the water temperature was 24 °C. Raw culture (ciliates are cultured in Petri dishes with site water by only picking out the metazoan) of *Aspidisca lynceus* was maintained in Petri dishes at room temperature (20–24 °C), using filtered (0.22 μm) habitat water or Eau de Volvic mineral water with rice grains to enrich bacteria as food resource. We failed to establish clonal cultures for *Aspidisca lynceus*, however, no other *Aspidisca* morphotypes were present in the protargol preparation, indicating that our morphological and molecular studies deal with the same species.

Cells were observed in vivo using bright field and Nomarski differential interference contrast microscopy at 100–1,000 ×. The infraciliature and nuclear apparatus were revealed by the protargol impregnation (Wilbert 1975), and protargol was made according to Pan et al. (2013). The Chatton–Lwoff method and dry silver nitrate impregnation were used to reveal the silverline systems (Foissner 2014). Counts and measurements were performed at magnification of 100–1,000X. Drawings of impregnated specimens were made with the help of a drawing attachment and photomicrographs. Terminology is mainly according to Curds (1975) and Wu and Curds (1979).

Scanning electron microscopy (SEM)

Specimens for SEM were prepared according to the method described by Gu and Ni (1993). Ciliates were fixed in a 1:6 mixture of 1% OSO_4 and saturated solution of $HgCl_2$ at 4 °C for 10 min and rinsed with 0.1 M phosphate buffer. The fixed cells were then dehydrated in a graded series of ethanol, dried with a critical point dryer (Hitachi HCP-2), and sputter-coated with gold (Cressington 108auto). Observation was performed using a scanning electron microscope (Hitachi S-4800) at an accelerating voltage of 10 kV.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions, with the modification that 25% of the volume suggested for each reagent solution was used (Luo et al. 2017). Q5® Hot Start High-Fidelity DNA Polymerase (NEB, Ipswich, MA) was used to amplify the SSU rDNA using universal eukaryotic primers 82F (5′-GAAA CTGCGAATGGCTC-3′) (Jerome et al. 1996; Yan et al. 2016a) or 18SF (5′-AACCTGGTTGATCCTGCCAGT-3′) and 18SR (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Medlin et al. 1988). Cycling parameters of touchdown PCR were as follows: 1 cycle of initial denaturation at 98 °C for 30 s, followed by 18 cycles of amplification (98 °C, 10 s; 69–52 °C touchdown, 30 s; 72 °C, 1 min), and another 18 cycles (98 °C, 10 s; 51 °C, 30 s; 72 °C, 1 min), with a final extension of 72 °C for 5 min. The PCR products were detected using agarose gel and then sequenced directly in both directions using primers 82F, 18SR, and internal primers (Wang et al. 2016, 2017b) in TSINGKE (Qingdao, China). Contigs were assembled by Seqman (DNAStar).

Phylogenetic analyses

Newly obtained SSU rDNA sequences of *Aspidisca lynceus* and *Euplotes platystoma*, together with 81 sequences of euplotids obtained from GenBank database were used in the final phylogenetic analyses. *Discocephalus ehrenbergi* (FJ196397), *Leptoamphisiella vermis* (FJ865203), *Paradiscocephalus elongatus* (EU684746), *Prodiscocephalus borrori* (DQ646880), and *Pseudoamphisiella quadrinucleata* (EU518416) were selected as the outgroup species. Sequences were aligned using the CIPRES Science Gateway (The CIPRES Portals. URL: http://www.phylo.org/sub_sections/portal) with Muscle version 3.7 (Edgar 2004; Miller et al. 2010). Further

modifications were made manually using BioEdit 7.0 (Hall 1999). Ambiguously aligned regions and gaps were excluded manually by using BioEdit 7.0 prior to phylogenetic analysis, resulting in a matrix of 1,832 characters. Maximum likelihood (ML) analysis was performed with RAxML-HPC2 version 8.2.9 on XSEDE (Stamatakis 2014) on the CIPRES Science Gateway. Support for the best ML tree came from 1,000 bootstrap replicates with the GTR+CAT model. Bayesian inference (BI) analysis was performed with MrBayes version 3.2.6 on XSEDE (Ronquist et al. 2012) on the CIPRES Science Gateway, using the GTR + I (0.2554) + G (0.4907) model as selected by MrModeltest version 2.0 (Nylander 2004) according to the Akaike Information Criterion (AIC) for Bayesian inference (BI). Markov chain Monte Carlo (MCMC) simulations were then run with two sets of four chains using the default settings: that is, a chain length of 1,000,000 generations, with a sample frequency of 100. After discarding the first 25% of trees as burn-in, all the remaining trees were used to calculate the posterior probabilities using a majority rule consensus. TreeView v.1.6.6 (Page 1996) and MEGA 4.0 (Tamura et al. 2007) were used to visualize tree topology. Systematic classification is mainly according to Lynn (2008).

The statistical possibility of alternative phylogenetic hypotheses was evaluated using the approximately unbiased (AU) test (Shimodaira 2002), following the protocol of Huang et al. (2016). Constrained ML trees of *Aspidisca lynceus* and all congeners for which the SSU rDNA sequence data are available were generated using the same toolkit as the unconstrained ML trees (Yan et al. 2016b; Zhao et al. 2016). The resulting constrained topologies were then compared to the unconstrained ML topologies using the AU test implemented in CONSEL v0.1 (Shimodaira and Hasegawa 2001).

RESULTS

Redescription of *Euplotes platystoma* Dragesco & Dragesco-Kernéis, 1986 based on Shanghai and Shenzhen populations

Remarks

Although the two populations of *Euplotes platystoma* were collected from different habitats (brackish water vs. fresh water) and have different numbers of dorsal kineties (13 vs. 11), they do agree with regard to the living morphology, cirral pattern on ventral side, similar number of dikinetids in mid-dorsal row, pattern of silverline system, and the identical SSU rDNA sequences (Fig. 1A–H, 2A–H, 3A–K and Table 1). Hence, they are treated as two populations of the same species, and the description is based on both populations.

Morphological description

Body size 85–130 μm × 45–90 μm in vivo. Body generally broadly oval in outline, dorsoventrally flattened about 2:1 with dorsal side a little arched and ventral side almost flat (Fig. 1A, F, 2A, B, E and 3A, C, F). Ventral side with four conspicuous ridges between transverse cirri and several longitudinal ridges between frontoventral cirri, about 10 inconspicuous ridges on the arched dorsal side, with dorsal kineties located in the grooves beside them (Fig. 2A, B, E and 3B, E). The bases of dorsal cilium surrounded by a rosette of subpellicular ampullae (Fig. 1D, 2F and 3D). Cytoplasm colorless, often filled with numerous granules and a few food vacuoles (Fig. 1A, F, 2A, E and 3A, C). Ellipsoidal granules about 1.5–2 μm across on dorsal aspect, possibly mitochondria (Fig. 2E, F and 3E). One contractile vacuole pore positioned to right of the transverse cirri on ventral side (Fig. 1A, F, G, 2C and 3C, K). Macronucleus C-shaped (Fig. 1C, E and 3H, I), micronucleus located in anterior part of body near left side in Shenzhen population, micronucleus not being observed in Shanghai population. Locomotion typically by slowly intermittent crawling on substrate.

Buccal field long and narrow, extending nearly 75% of cell length, buccal lip with distinct hyaline protrusion anteriorly (Fig. 1A, B, F, G, 2A, B, E, and 3A, C, H, K). Adoral zone composed of 45–65 membranelles. Paroral narrow and long, lying to the right of the posterior portion of adoral zone of membranelles, parallel to main body axis (Fig. 1B, 2C and 3H). Consistently 10 large frontoventral cirri, about 30 μm in length, transverse cirri thick, about 40-μm long, two caudal cirri at right posterior margin, two left marginal cirri near posterior end, about 25 μm long, fimbriation of caudal cirri distinct (Fig. 1B, 2A, B, D and 3G, H). Thirteen dorsal kineties in Shanghai population and 11 dorsal kineties in Shenzhen population with dikinetids extending almost entire length of body (Table 1). Mid-dorsal kinety with 18–25 dikinetids (Fig. 1C, H, 2G and 3I, J). Silverline system on dorsal side of the double-*eurystomus* type (Fig. 1H, 2G and 3J).

Aspidisca lynceus (Müller, 1773) Ehrenberg, 1830 based on Wuhan population

Size in vivo 35–55 μm × 25–40 μm. Body broadly oval, broader posterior half, both margins slightly convex, body outline smooth, without any spurs, spines or dorsal ridges, dorsoventrally flattened about 3:1 (Fig. 4A, F, G). Contractile vacuole subterminal near the right margin of cell on dorsal side, about 8 μm in diameter in diastole (Fig. 4A, F, G). Macronucleus horseshoe shaped; one spherical micronucleus, closely associated with macronucleus (Fig. 4C, H, I).

Locomotion characterized by fast circular crawling on substrate, attaching firmly to substrate when disturbed.

Cirral pattern stable (Table 2). Seven large frontoventral cirri, about 8 μm long, approximately arranged in two oblique arching rows, the right row consisting of four cirri positioned along right anterior margin of ventral surface, other three cirri posterior to this row and more centrally located. Five relatively large transverse cirri (about 10 μm long) in two groups, left two cirri always visibly separated, located immediately posterior to peristome, the other three slightly apart on right, tightly arranged in an oblique row (Fig. 4B, H). Adoral zone bipartite, anterior part

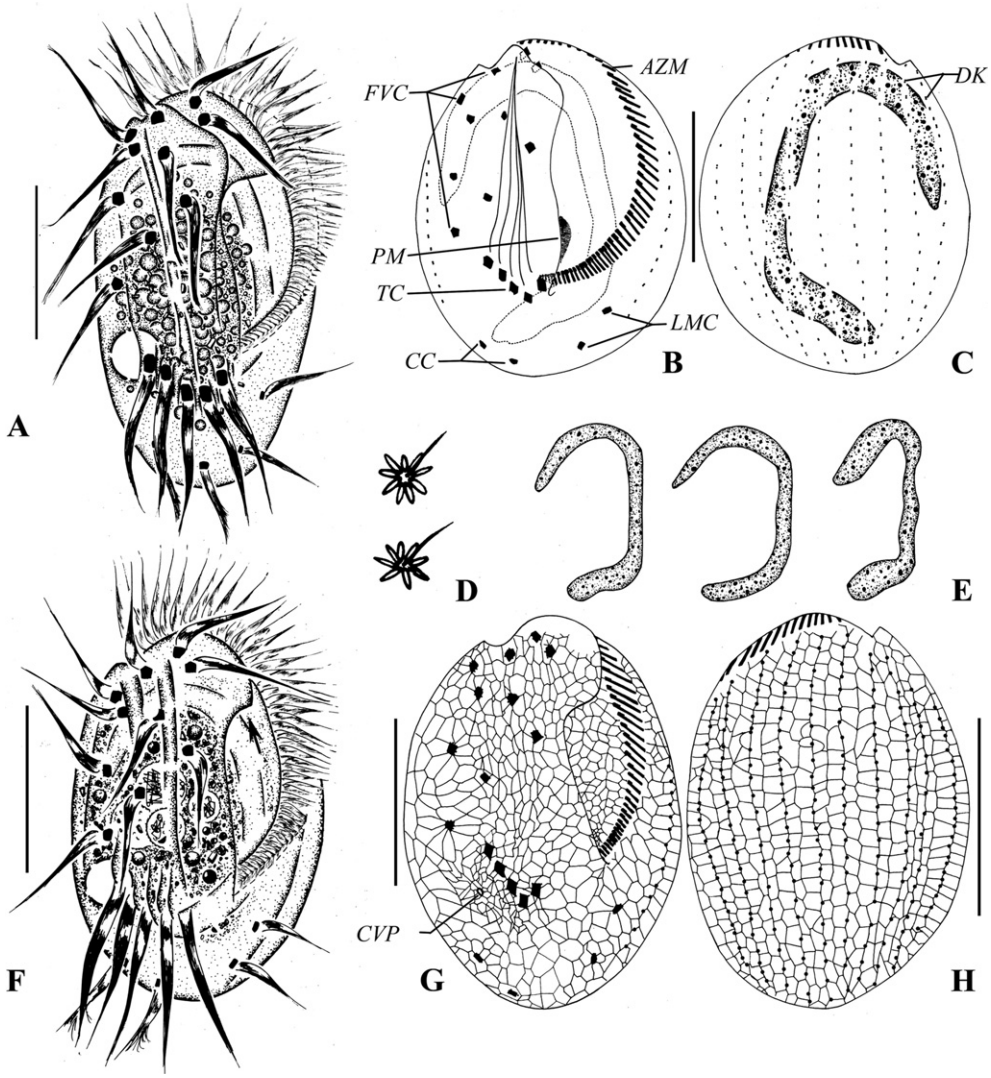

Figure 1 The Shanghai population of *E. platystoma* in vivo (**A**) and the Shenzhen population of *Euplotes platystoma* in vivo (**D, F**), after protargol (**B, C, E**), after silver nitrate impregnation (**G, H**). (A, F) Ventral view of a representative cell. (B, C) Ventral (B) and dorsal (C) view, showing the infraciliature and nuclear apparatus. (D) Subpellicular rosette-like structures around dorsal cilia. (E) Different shapes of macronucleus. (G, H) Silverline system on ventral (G), and dorsal (H) side. AZM, adoral zone of membranelles; CC, caudal cirri; CVP, contractile vacuole pore; FVC, frontoventral cirri; LMC, left marginal cirri; PM, paroral membrane; TC, transverse cirri. Scale bars: 50 μm.

(AZM1) invariably composed of three membranelles, located in shallow concavity at anterior end of body, just left of anterior-most frontal cirris, posterior part (AZM2) containing 10–14 smaller membranelles (Fig. 4A, B, H). Paroral small, adjacent to posterior end of AZM2 (Fig. 4B). Constantly five dorsal kineties, comprising, from left to right, about 4, 8, 9, 2, 6 dikinetids, respectively. Dorsal kinety 4, with only one to three dikinetids, positioned in the posterior half of cell along the right margin, dorsal kinety 5 located along anterior one-third of the right margin of the cell, other dorsal kineties running along the main body axis, generally bipolar with dikinetids irregularly arranged (Fig. 4C, E, I). Dorsal silverline system consisting of five longitudinal primary meridians connecting dikinetids of dorsal kineties, silverlines of dorsal kinety 5 and 3 converged anteriorly, silverline of kinety 4 shortened anteriorly, oblique secondary silverline connected anterior end of dorsal kineties 1, 2, 3, and 5 (Fig. 4D, E).

SSU rDNA sequences and phylogenetic analyses

All new SSU rDNA sequences have been deposited in GenBank with accession numbers, lengths and GC contents as follows: *Euplotes platystoma* (Shenzhen population: MF928801, 1802 bp, 42.73%; Shanghai population: MF928800, 1802 bp, 42.73%, these two SSU rDNA sequences are identical) and *Aspidisca lynceus* (MF928799, 1575 bp, 45.27%).

Phylogenetic trees based on the SSU rDNA sequences were constructed using Bayesian inference (BI) and maximum likelihood (ML). The topologies of the BI and ML trees were almost identical, thus only the ML tree was presented here with nodal support (bootstrap values and posterior probabilities) from both methods (Fig. 5). A broad selection of species of the order Euplotida was included in the phylogenetic analyses. As shown in Fig. 5, Euplotida and its five families, namely Aspidiscidae, Certesiidae, Euplotidae, Gastrocirrhidae, and Uronychiidae, are all monophyletic groups.

The monophyly of the family Euplotidae is fully supported (100% bootstrap support in ML, 1.00 posterior probabilities in BI) in both ML and BI trees, as consistent with the previous studies (Chen et al. 2013; Fotedar et al. 2016; Liu et al. 2015). Our new isolates of *Euplotes platystoma* formed a well-supported polytomy (90% bootstrap support in ML, 1.00 posterior probabilities in BI) with seven sequences of *E. harpa* and one isolate of *E. neapolitanus* (HM635774), another isolate of *E. neapolitanus* (FJ998024) is sister to this clade.

Nine known *Aspidisca* species formed a monophyletic clade with full support (100% bootstrap support in ML, 1.00 posterior probabilities in BI). Within *Aspidisca*, two subclades were recovered in both BI and ML trees, however, the phylogenetic positions of *A. lynceus* and *A. orthopogon* within this genus were not robust. The grouping of *A. lynceus* with the clade comprising of *A. orthopogon*, *A. hexeris*, *A. magna*, *A. leptaspis*, and *A. hongkongensis* was only poorly supported in the ML tree (bootstrap values: 39%), while *A. lynceus* formed a polytomy with *A. orthopogon* and a cluster of four congeners in the BI tree. The remaining species, *A. aculeata*, *A. fusca*, and *A. steini*, formed a moderately supported group (64% bootstrap support in ML, 0.91 posterior probabilities in BI).

DISCUSSION

Identification of the Shanghai and Shenzhen populations of *Euplotes platystoma*

Euplotes platystoma, originally described by Dragesco and Dragesco-Kernéis (1986), was found in brackish puddles bordering along the lagoon in Benin, West Africa. The description was based only on fixed or impregnated cells. It has not been redescribed since. We identified our two populations as *E. platystoma* because both of them share all the following features with the type population: (1) similar body size after impregnation (Shanghai population: 112 μm × 82 μm, Shenzhen population: 111 μm × 83 μm, type population: 99 μm × 69 μm); (2) similar number of adoral membranes; (3) the number of dikinetids in mid-dorsal kinety overlaps (Shanghai population: 45–60, Shenzhen population: 54–65, type population: 44–68); (4) 10 frontoventral cirri, 2 left marginal cirri and 2 caudal cirri; (5) similar number of dikinetids in mid-dorsal kinety (Shanghai population: 18–25, Shenzhen population: 17–23, type population: 18–21); (6) dorsal silverline system double-*eurystomus* type. The original isolate is from brackish water while one of our two populations, the

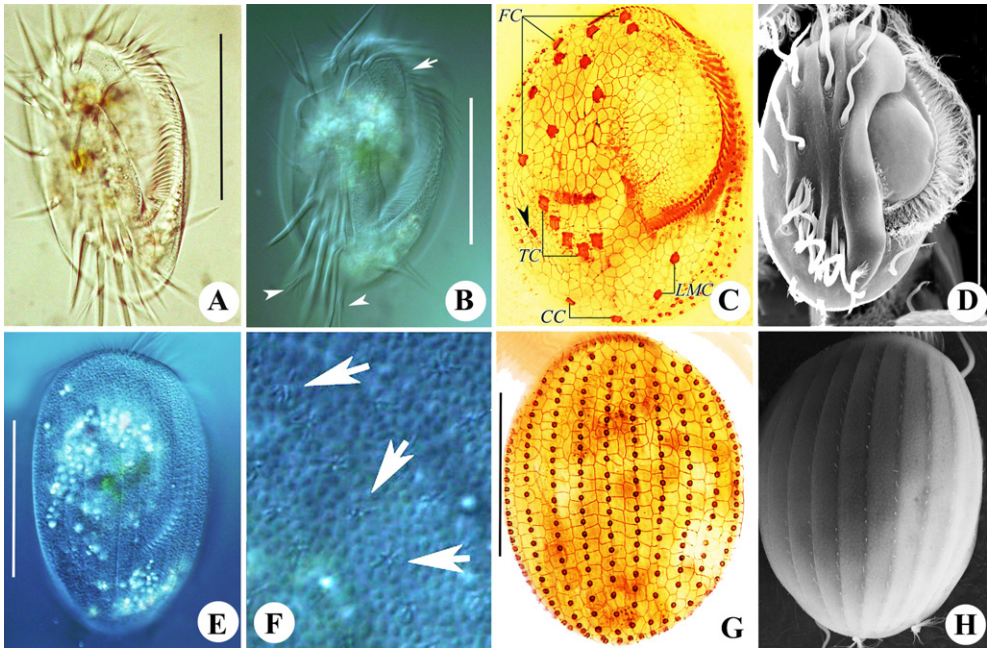

Figure 2 The Shanghai population of *Euplotes platystoma* from life (**A, B, E, F**), after silver nitrate impregnation (**C, G**) and scanning electron microscopy (**D, H**). (A–B, E–F) Ventral (A, B) and dorsal (E, F) views in life, arrowheads indicate the caudal cirri, arrow in (B) shows the sigmoidal adoral zone, arrows in (F) show the rosette-like granules on dorsal side. (C, G) Ventral (C) and dorsal (G) view of the same specimen, arrow indicates the position of contractile vacuole pore. (D, H) Ventral and dorsal surface. CC, caudal cirri; FC, frontoventral cirri; LMC, left marginal cirri; TC, transverse cirri. Scale bars: 50 μm.

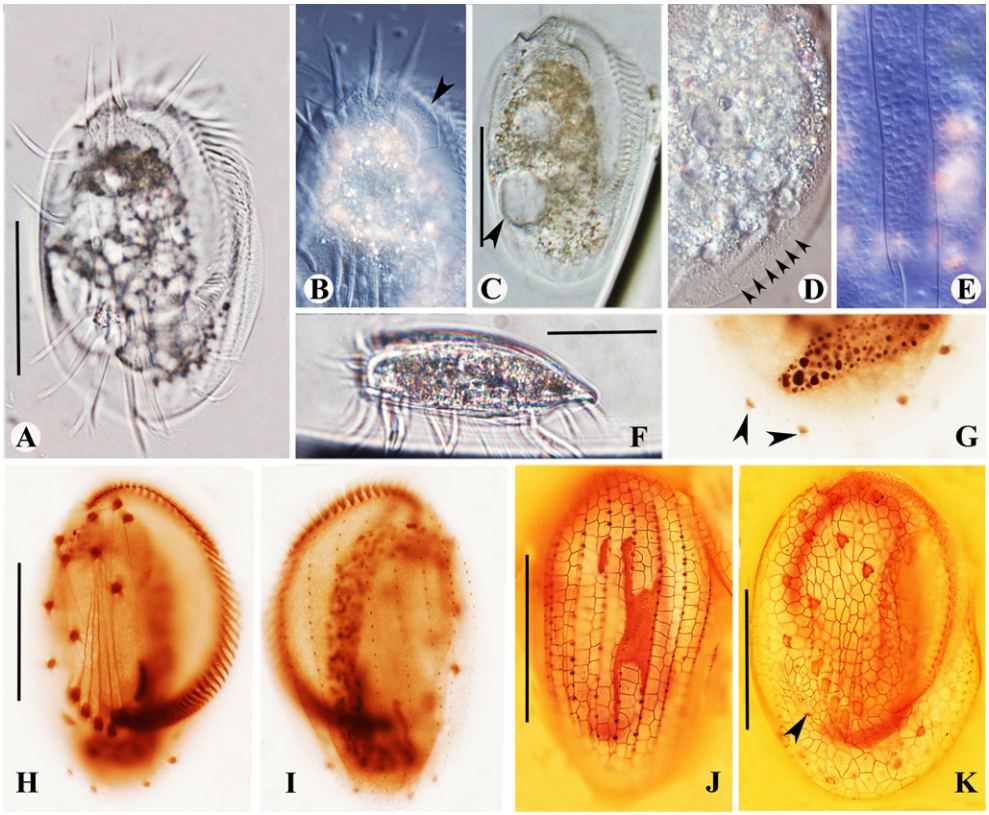

Figure 3 Photomicrographs of the Shenzhen population of *Euplotes platystoma* in vivo (**A–F**), after protargol (**G–I**) and silver nitrate (**J, K**) impregnation. (A) Ventral view of a representative individual. (B) Ventral view showing the prominent anterior lip, arrow points to the lip. (C) Different body shape, arrow points to the contractile vacuole. (D) Rosette-like granules on dorsal side (arrows). (E) Numerous irregular ellipsoid to oval granules (possibly mitochondria) extremely densely packed beneath dorsal and ventral pellicle. (F) Lateral view showing dorsoventrally flattened body. (G) Individual with two caudal cirri (arrowheads). (H, I) Ventral (H) and dorsal (I) view of the same specimen, showing the infraciliature and nuclear apparatus. (J, K) Silverline system on dorsal (J) and ventral (K) side. Arrow indicates the position of contractile vacuole pore. Scale bars: 50 μm.

Shenzhen population was collected from freshwater (salinity 0 ‰). Nevertheless, all information available, that is, the meristic of the ciliature, pattern of the silverline system, living morphology indicates that they belong to the same taxon. Moreover, the SSU rDNA sequences of the two populations of *Euplotes platystoma* are identical, which confirms the morphological identification.

However, the differing number of dorsal kineties (13 vs. 11) and the differing habitats (fresh vs. brackish water) of the two populations raise doubt about whether these two populations represent different subspecies, especially, considering the specificity of the number of dorsal kineties in *Euplotes*. Due to the similar morphological characters, the identical SSU rDNA sequences, and the lack of other gene sequences (we failed in the amplification of mitochondrial SSU rDNA), this question needs further information to confirm.

Comparison of *Euplotes platystoma* with its congeners

Euplotes species with ten frontoventral cirri, two left marginal and two caudal cirri, 11–13 dorsal kineties and dorsal silverline system double-*eurystomus* type are easily confused with *Euplotes platystoma* (especially *Euplotes harpa*). Here, we provided a comparison with similar species, which share the features mentioned above: *Euplotes antarcticus* Fenchel & Lee, 1972, *Euplotes neapolitanus* Wichterman, 1964, *Euplotes harpa* Stein, 1859, and *Euplotes shanghaiensis* Song et al., 1998.

Different from our forms, the "well-known" species *Euplotes harpa* Stein, 1859 can be recognized by the combination of the following features: very large in size (120–260 μm vs. 85–130 μm), marine habitat (vs. fresh or brackish water), especially having more than 40 dikinetids in mid-dorsal row (vs. 17–25 in *E. platystoma*) (see discussion below).

Euplotes antarcticus differs from *E. platystoma* in the following points: 1) the body shape (elongate elliptical to sac-like, buccal field with cytoplasmic lip along right border, posterior portion of adoral zone bent about 90° vs. general oval) and 2) biotope (sea ice of the Weddell Sea vs. fresh or brackish water) (Petz et al. 1995).

In contrast to *Euplotes neapolitanus*, *E. platystoma* has a distinctly wider body shape and different shape of silverline grid (each mesh being conspicuously shortened

Table 1. Morphometric data of *Euplotes platystoma* Dragesco and Dragesco-Kernéis, 1986 (upper line, Shanghai population based on silver nitrate-stained specimens; lower line, Shenzhen population, based on protargol-stained specimens).

Character	Min	Max	Mean	SD	CV	*n*
Body length in μm	98	124	111.6	6.9	6.2	32
	76	131	111.4	13.1	11.8	25
Body width in μm	64	104	82.2	8.3	10.0	32
	62	120	83.2	15.2	18.3	25
Length of adoral membranelles	72	90	80.8	5.9	7.3	32
	65	95	82.8	7.9	9.6	25
Number of adoral membranelles	45	60	56.1	3.1	5.5	32
	54	65	58.3	3.1	5.4	25
Number of frontoventral cirri	10	10	10.0	0.0	0.0	32
	10	10	10.0	0.0	0.0	25
Number of transverse cirri	5	5	5.0	0.0	0.0	32
	5	5	5.0	0.0	0.0	25
Number of marginal cirri	2	2	2.0	0.0	0.0	32
	2	2	2.0	0.0	0.0	25
Number of caudal cirri	2	2	2.0	0.0	0.0	32
	2	2	2.0	0.0	0.0	25
Number of dorsal kineties (DK)	13	13	13.0	0.0	0.0	32
	11	11	11.0	0.0	0.0	25
Number of dikinetids in mid-DK	18	25	22.8	1.3	5.7	32
	17	23	19.8	2.1	10.8	24

All data are based on protargol-impregnated specimens. Abbreviations: CV, coefficient of variation in %; DK, dorsal kinety; Max, maximum; Mean, arithmetic mean; Min, minimum; *n*, number of cells measured; SD, standard deviation.

rectangular or polygonal in *Euplotes platystoma* vs. elongated in *Euplotes neapolitanus*). In addition, the latter is a marine form (Curds 1975).

In 1998, Song et al. described a fresh water species, *Euplotes shanghaiensis*, which was found from fresh water in Shanghai. Morphologically, it shows almost no differences in principle from *E. platystoma*. Unfortunately, no molecular data are available and thus, it is hard to separate it from the latter. That is, the validity of *Euplotes shanghaiensis* remains unconfirmed and conspecificity with *E. platystoma* cannot be excluded without additional molecular data.

Reconsideration of the "well-known" *Euplotes harpa* Stein, 1859

Considering the similarity in SSU rDNA sequences and morphological characteristics between *Euplotes harpa* and *Euplotes platystoma*, we here provide a separate discussion on *Euplotes harpa*. The well-known form, *Euplotes harpa* Stein, 1859 was first described based on a population isolated in Ostsee (Baltic Sea), Germany (Stein 1859). Kahl (1932) redescribed a German population with detailed live observations (Fig. 6A). Subsequently, numerous redescriptions have been conducted (Carter 1972; Curds 1975; Czapik and Jordan 1976; Dragesco and Dragesco-Kernéis 1986; Kahl 1932; Tuffrau 1960). Among those studies, Kahl depicted and described the living features for this organism found from the same water (Ostsee) (Fig. 6A), while Tuffrau (1960) documented the infraciliature and silverline system for the first time (Fig. 6A from Kahl; 6B–J from Tuffrau).

Thus, according to the original and "relatively reliable" redescriptions (Kahl 1932; Stein 1859; Tuffrau 1960), we may list the diagnostic characters as follows: *Euplotes harpa* should be a very large ("sehr grosse Art" as mentioned in original report, most massive *Euplotes* with a good diet, much smaller with starvation as mentioned by Kahl) marine form (120–260 μm in length) with a silverline pattern of the typical double-*eurystomus* type and characterized by 10 frontoventral cirri, 13 dorsal kineties with large number (about 40–45) of dikinetids in the mid-dorsal row.

In the last half century, this form has been reported by several authors from different parts of the world (Agamaliev 1968; Dragesco and Dragesco-Kernéis 1986; Tuffrau 1960). Nevertheless, many of them are very clearly misidentifications. For example, the Azerbaijan isolate (reported by Agamaliev 1968) with only about 16 dikinetids in mid-dorsal kinety (a stable diagnostic character) is definitely not conspecific with this species although other details about its living morphology were not given (Fig. 6D,E). Moreover, the Azerbaijan population corresponds well with our Shenzhen population of *Euplotes platystoma* in body shape and size, ventral cirri pattern, number of dorsal kineties and kineties in mid-dorsal kinety. Thus, we suspect that the Azerbaijan population is likely to be another population of *E. platystoma*. Similarly, the population described by Dragesco and Dragesco-Kernéis (1986) is likely also a misidentified form simply because of its smaller size and much lower number of dikinetids in mid-dorsal kinety compared to the original report of *E. harpa* (Fig. 6F–J).

There are at least seven sequences named as *E. harpa* in GenBank, however, six of them are identified by the same author/group (Italy), and no morphological information is available for the Korean strain. Since the very limited morphological characters of the six Italian strains provided correspond with our *Euplotes platystoma* populations, it is reasonable to question if these strains are misidentified. Moreover, all these "*E. harpa*" SSU rDNA sequence vary by < 0.2% from those of *Euplotes platystoma* obtained in this study, that is, their SSU rDNA sequences are either identical or only differs at one nucleotide position. Hence, the identification of the *Euplotes harpa* sequences so far available from GenBank should be considered questionable.

Comparison of *Aspidisca lynceus* (Müller 1773) Ehrenberg, 1830 with previous populations

Aspidisca lynceus was first described by Müller (1773) as *Trichoda lynceus* and was transferred to the genus *Aspidisca* by Ehrenberg (1830). Stein (1859) was the first person gave a detailed description. Our population resembles Stein's population except for the structure of the dorsal

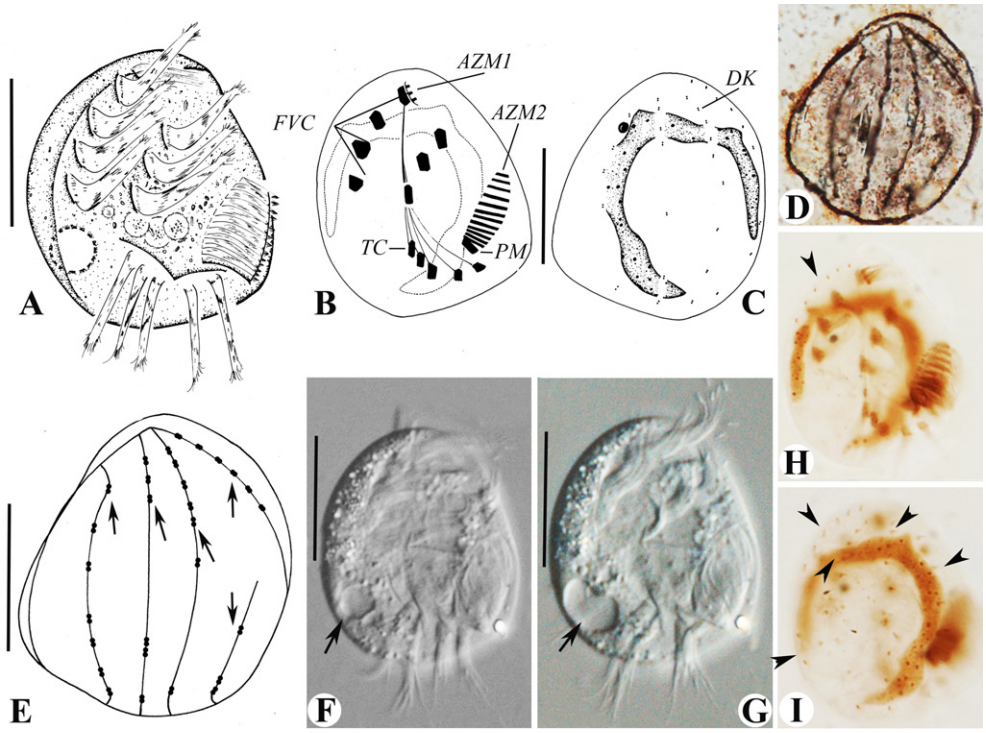

Figure 4 *Aspidisca lynceus* (Müller 1773) Ehrenberg, 1830 in life (**A, F, G**), after protargol staining (**B, C, H, I**), and dry silver (**D, E**). (A) Ventral view of a representative cell. (B, C) Ventral (B) and dorsal (C) view, showing the infraciliature and nuclear apparatus. (D, E) Dorsal views showing the silverline system on dorsal side, arrow shows the dorsal kineties 1–5. (F, G) Ventral view of a representative individual, arrows point to the contractile vacuole. (H, I) Ventral (H) and dorsal (I) view of the same specimen, showing the infraciliature and nuclear apparatus, arrowheads indicate five dorsal kineties. AZM1, 2, anterior and posterior portion of adoral zone of membranelles; DK, dorsal kineties; FVC, frontoventral cirri; PM, paroral membrane; TC, transverse cirri. Scale bars: 20 μm.

surface (smooth in Wuhan population vs. smooth or with three inconspicuous longitudinal ridges in Stein's description). As type species of the genus, *Aspidisca lynceus* has been reported several times (Augustin and Foissner 1992; Bick 1972; Claparède and Lachmann 1858; Foissner 1982; Gelei 1939; Kahl 1932; Wu and Curds 1979) (Fig. 6K–U).

The Wuhan population of *Aspidisca lynceus* matches closely with previously described populations, differing only in the absence or presence of dorsal ridges and spines, that is, no spines, and a smooth outline in the Wuhan population vs. a spine in Kahl's description, and three shallow dorsal ridges in Stein's description, which was not found in other populations (Kahl 1932; Stein 1859). Besides, according to Foissner (1982), there is an arcuate projection on the right side of the body and overlapping the dorsal side, which is not observed in our population. These features have long been considered as diagnosed characters, for example, the difference between *Aspidisca lynceus* and three *Aspidisca* species is the structure on dorsal surface (smooth in *A. lynceus* vs. six to eight longitudinal ridges in *A. cicada* vs. usually one, rarely two, reclined spines in *A. turrita* vs. four dorsal ribs and one bearing a thorn in *A. herbicola*) (Augustin and Foissner 1992; Wu and Curds 1979), but according to Li et al. (2008), some characters (e.g. furrows, spurs, dorsal

Table 2. Characterization of *Aspidisca lynceus* (Müller 1773) Ehrenberg, 1830.

Characteristics	Min	Max	Mean	SD	CV	*n*
Body length in μm	32	49	41.8	5.0	12.0	15
Body width in μm	28	40	35.0	4.1	11.6	15
Number of membranelles in AZM1	3	3	3.0	0.0	0.0	15
Number of membranelles in AZM2	10	14	12.4	1.2	9.5	15
Number of frontoventral cirri	7	7	7.0	0.0	0.0	15
Number of transverse cirri	5	5	5.0	0.0	0.0	15
Number of dorsal kineties (DK)	4	4	4.0	0.0	0.0	15
Number of dikinetids in DK1	3	7	4.3	1.2	27.3	15
Number of dikinetids in DK2	7	10	8.1	1.1	13.8	15
Number of dikinetids in DK3	7	10	8.7	0.8	9.4	15
Number of dikinetids in DK4	1	3	2.3	0.8	35.0	15
Number of dikinetids in DK5	5	8	6.3	0.7	11.4	15

All data are based on protargol-impregnated specimens. Abbreviations: AZM1, 2, anterior and posterior portion of adoral zone of membranelles, respectively; CV, coefficient of variation in %; DK, dorsal kineties; DK1–5, dorsal kineties 1–5; Max, maximum; Mean, arithmetic mean; Min, minimum; *n*, number of cells measured; SD, standard deviation.

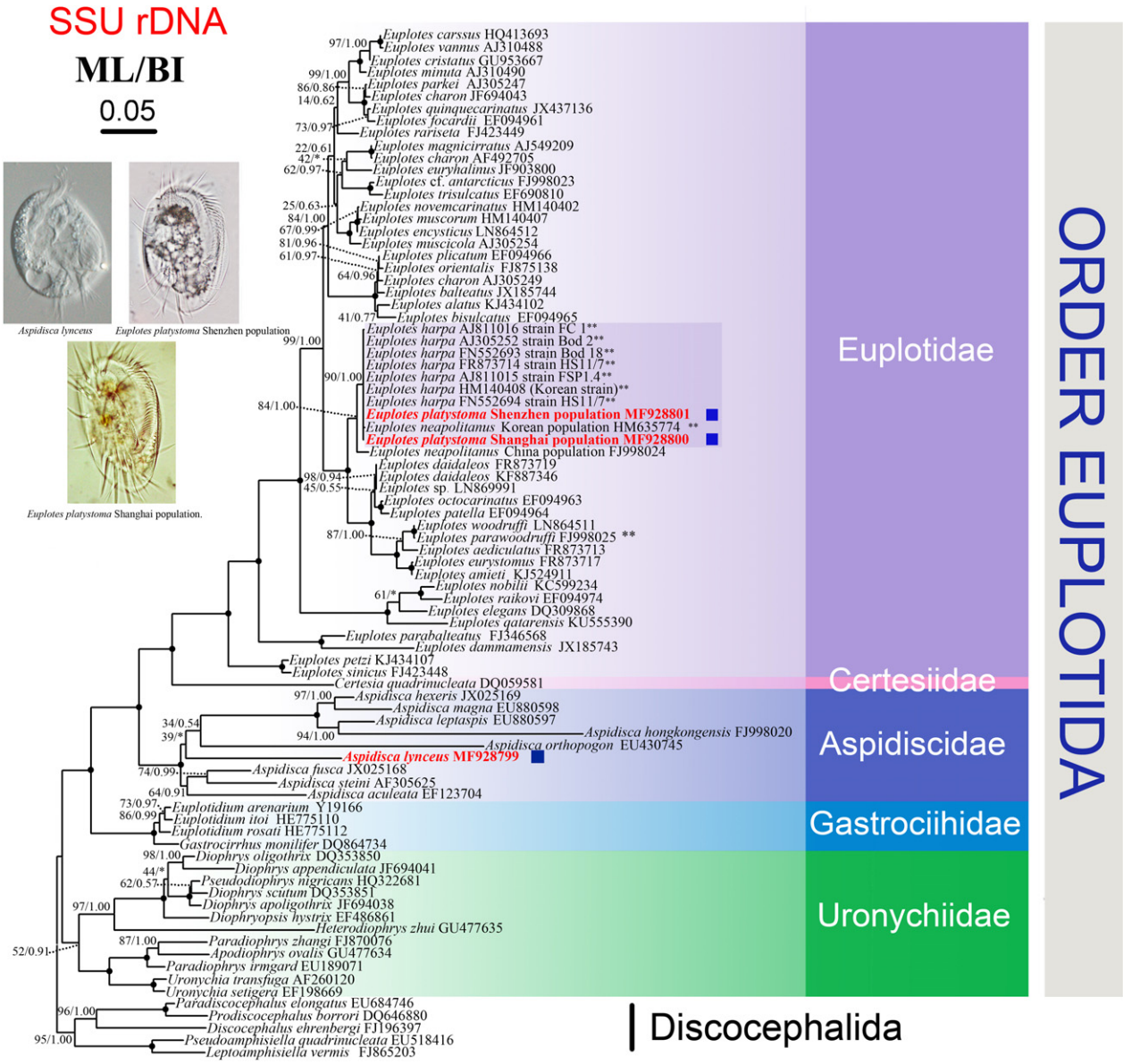

Figure 5 Phylogenetic tree based on SSU rDNA sequences, showing the position of *Euplotes platystoma* and *Aspidisca lynceus* by Maximum Likelihood (ML) and Bayesian inference (BI). Numbers near branches denote ML bootstrap value/BI posterior probability value. "*" indicates topologies that differ between the ML and BI phylogenies. Fully supported (100%/1.00) branches are marked with solid circles. "**" indicates sequences could be from misidentified material. All branches are drawn to scale. The scale bar corresponds to 5 substitutions per 100 nucleotide positions. Systematic classification is mainly according to Lynn (2008).

ribs, thorns) mentioned in Wu and Curds (1979) may vary in number or size under different growth conditions. Furthermore, it has been demonstrated that some of these features are formed in response to chemical cues produced by certain predators, but are lost or significantly reduced in the absence of these cues (Wicklow 1997). In terms of other key characteristics, that is, the cell size, body shape, pattern of infraciliature, and biotope, especially the transverse cirri separated in two groups and the silverline system, the Wuhan population corresponds very well with known populations of *Aspidisca lynceus*. Therefore, we consider that the Wuhan population is conspecific with previous populations.

Phylogenetic analyses

The phylogenetic grouping of two populations of *Euplotes platystoma* with seven isolates of "*Euplotes harpa*" and the Korean population of *E. neapolitanus* (HM635774), considering the highly similarity of their SSU rDNA sequences, offers one or both of two possibilities: (1) *Euplotes harpa* and the Korean population of *Euplotes*

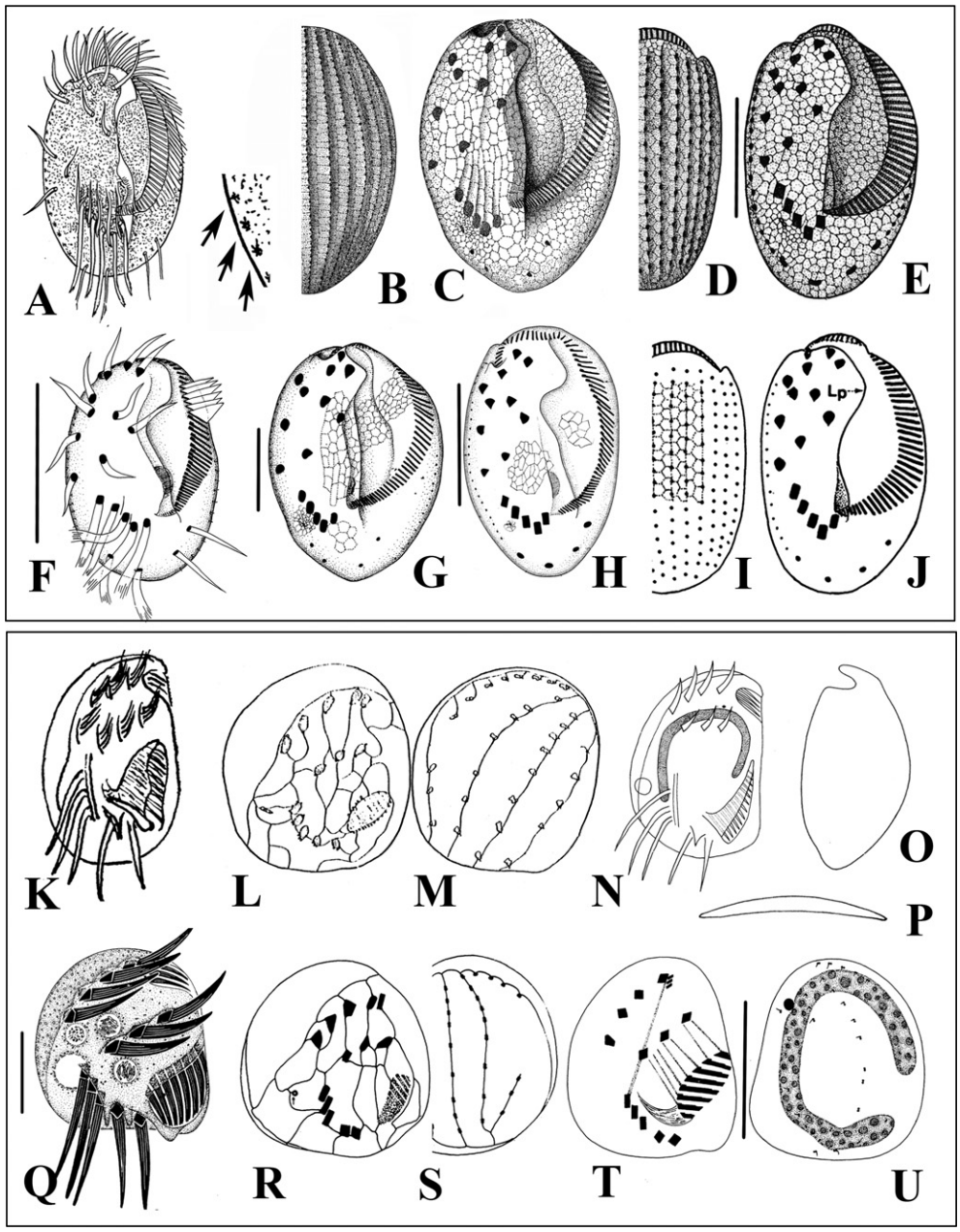

Figure 6 Isolates under the name of *Euplotes harpa* (**A**, from Kahl 1932; **B, C,** from Tuffrau 1960; **D, E,** misidentified, from Agamaliev 1968; **F–J**, misidentified, from Dragesco and Dragesco-Kernéis 1986) and *Aspidisca lynceus* (**K**, from Kahl 1932; **L, M**, from Gelei 1939; **N, P**, from Bick 1972; **O, Q–U**, from Foissner 1982) (A, K, N–Q, in vivo; B–J, L, M, R–U, after silver staining). (A) Ventral view of a representative individual, insight shows the Rosette-like granule (arrows). (B–J) Silverline system on ventral (C, E, G, H, J) and dorsal side (B, D, I). (K, N–Q) Ventral view (K, N, Q) and cross-section (O, P) of representative cells. (L, M, R, S) Silverline system on ventral (L, R) and dorsal side (M, S). (**T, U**) Ventral (T) and dorsal (U) view, showing the infraciliature and nuclear apparatus. Scale bars: A–J, 50 μm; K–U, 20 μm.

neapolitanus (HM635774) are not correctly identified; (2) the SSU rDNA is too conserved to separate these taxa. Both options are possible scenarios as there are no detailed morphological descriptions associated with the sequences of *E. harpa* and the *E. neapolitanus* (HM635774), thus, misidentification cannot be excluded. Moreover, the Korean population of *E. neapolitanus* (HM635774) differs at 57 nucleotide positions from the other population of *E. neapolitanus* (FJ998024), which is accompanied by detailed morphological data (from personal communication with Dr. Weiwei Liu, Voucher No. LWW07033116). Yet, the fact remains that *E. harpa*,

E. platystoma, and *E. neapolitanus* are very similar in the ciliary pattern of ventral side, number of adoral membranelles, and the body shape. Thus, detailed morphological information and additional genes from more populations of "true" *E. harpa* and *E. neapolitanus* are required to resolve this problem.

Kahl (1932) suggested a separation between freshwater species and marine species of *Aspidisca*. The genus was further divided into three groups based on the number and pattern of the frontoventral cirri by Wu and Curds (1979). However, these hypotheses cannot be tested previously as all available sequences of *Aspidisca* are from marine habitats and belong to "section B" (frontoventral cirri in "*polystyla*-arrangement") in the system of Wu and Curds (1979). The newly sequenced *Aspidisca lynceus* is the only freshwater species belonging to "section A" (frontoventral cirri in "*lynceus*-arrangement"), but *A. lynceus* did not separate from marine species in the present phylogenetic analyses. Yet, the nodal support was very low, and the hypothesis that *Aspidisca lynceus* could be separated from other congeners was not rejected by AU test ($p = 0.902$). Thus, increased sampling of freshwater taxa will be needed to assess whether marine and freshwater forms group separately. Although the addition of *Aspidisca lynceus* has helped to increase the resolution of the topology, the relationships within *Aspidisca* are still far from being settled because of the low bootstrap values and the limited taxon sampling of freshwater taxa.

TAXONOMIC SUMMARY

Euplotes platystoma Dragesco and Dragesco-Kernéis, 1986.

Improved diagnosis (based on original description and data on two Chinese populations). In vivo 85–130 × 45–90 μm; buccal field about three quarters of body length with 44–68 membranelles; 10 frontoventral, 5 transverse, 2 left marginal cirri, and 2 caudal cirri; 11–13 dorsal kineties with 17–25 dikinetids in mid-dorsal kinety. Dorsal silverline system of double-*eurystomus* type.

Voucher material locality. Shanghai population: Shanghai, China, 31°43′43″N; 121°13′37″E; Shenzhen population: Shenzhen, China, 22°35′19″N; 114°18′2″E.

Voucher material. Shanghai population: Slides with silver nitrate-impregnated specimens are deposited in the Biological History Museum of East China Normal University Biological Museum (registration number: XDM20140609).

Shenzhen population: The slides with protargol- and silver nitrate-impregnated specimens are deposited in the Laboratory of Protozoology, Ocean University of China. (OUC, registration number: LCY2015112301).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Sampling sites.