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European Journal of Protistology 67 (2019) 1–14

European Journal of PROTISTOLOGY

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Morphology and SSU rDNA-based phylogeny of two *Euplotes* species from China: *E. wuhanensis* sp. n. and *E. muscicola* Kahl, 1932 (Ciliophora, Euplotida)

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Received 13 May 2018; received in revised form 3 October 2018; accepted 5 October 2018
Available online 13 October 2018

Abstract

The living morphology, infraciliature and silverline system of two small *Euplotes* species, *E. wuhanensis* sp. n. and *E. muscicola* Kahl, 1932, isolated from Wuhan, central China, were investigated. *Euplotes wuhanensis* sp. n. is characterized by a combination of features including small size (40–50 × 25–30 μm), two conspicuously small and eight normal-sized frontoventral cirri, five transverse cirri in two groups, two marginal and two caudal cirri, seven dorsal kineties with about 12 dikinetids in the mid-dorsal row and a double-*eurystomus* type of dorsal silverline pattern. The Wuhan population of *E. muscicola* closely resembles previously described populations. The establishments of three subspecies of *E. muscicola* are not supported. The small subunit ribosomal RNA gene sequences were determined for both species. We propose that the two sequences under the name of *E. muscicola* (No. AJ305254, DQ917684 deposited in GenBank) are very likely from misidentified material. Phylogenetic analyses based on these data support the validity of both *E. muscicola* and *E. wuhanensis* as distinct species.

Keywords: Ciliates; Protist biodiversity; rRNA gene sequences; Silverline system; Soil microbiology; Taxonomy

Introduction

The ciliate genus *Euplotes* is one of the most complicated and confused taxa, with a huge variety of species, worldwide distribution in marine, freshwater and terrestrial biotopes (Augustin and Foissner 1992; Bitencourt et al. 2015; Borror and Hill 1995; Chen et al. 2014; Di Giuseppe et al. 2015,

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https://doi.org/10.1016/j.ejop.2018.10.001
0932-4739/

2014; La Terza et al. 2001; Lian et al. 2018; Petz et al. 1995; Shao et al. 2010; Yan et al. 2018; Zhao et al. 2018). To date, over 160 nominal species have been established in this genus, mainly based on morphological and morphogenetic characters (Berger 2001; Borror 1972; Curds 1975; Kahl 1932; Song et al. 1998, 2009; Tuffrau 1960). In recent decades, many new *Euplotes* species have been found and studied in various habitats (Borror and Hill 1995; Chen et al. 2013; Coppellotti and Cisotto 1996; Jiang et al. 2010; Liu et al. 2017; Lobban et al. 2005; Pan et al. 2012; Schwarz and Stoeck 2007; Valbonesi et al. 1997; Wilbert and Song 2008).

Species of this genus are identified by a combination of morphological features including body shape and size, the number of dorsal and ventral ridges, the shape of the adoral zone, the numbers of adoral membranelles, frontoventral, marginal and caudal cirri, the number of dorsal kineties, the type of the silverline system, nuclear apparatus, as well as their habitats (Agamaliev 1966; Borror 1972; Carter 1972; Tuffrau 1954). However, most species share similarities in certain diagnostic features, e.g. body shape and size, numbers of cirri and dorsal kineties (Curds 1975; Kahl 1932). Thus, small subunit ribosomal RNA (SSU rRNA) gene sequences have also been widely referred to when dealing with new or ambiguous species (Dai et al. 2013; Fan et al. 2010; Fotedar et al. 2016; Yan et al. 2018).

Borror and Hill (1995) split *Euplotes* into four genera, i.e. *Euplotes*, *Euplotopsis*, *Euplotoides*, and *Moneuplotes*, based on characteristics of cortical structure, endosymbionts, morphometric data, morphogenetic patterns and ecology. Jankowski (1979) divided *Euplotes* into four subgenera: *Euplotes* (*Euplotes*), *E.* (*Moneuplotes*), *E.* (*Mideuplotes*) and *E.* (*Neteouplotes*). However, these classifications have been questioned by recent molecular phylogenetic studies (Schwarz et al. 2007; Yi et al. 2009). Thus, we follow Liu et al. (2015) and merge these taxa in the genus *Euplotes*.

According to the original description from Kahl (1932), *E. muscicola* has an oval body shape (aspect ratio about 5:4) and ribs on the dorsal side. More morphological data were revealed in the several redescriptions (Fauré-Fremiet et al. 1954; Foissner 1982; Gelei 1936, 1938; Shin and Kim 1995; Tirjakovã et al. 2015; Wang and Nie 1935). However, there are still no detailed illustrations and photomicrographs available relating to the morphology of specimens in vivo and after protargol preparation, and no SSU rRNA gene sequence data from specimens are linked with detailed morphological data.

In the present paper, we describe a new *Euplotes* species and provide a description of a Chinese population of *E. muscicola*. For the latter, no detailed illustrations and photomicrographs are available in spite of its numerous descriptions following the original one by Kahl (1932). SSU rRNA gene sequence data are also supplied for both species and their phylogenetic positions in the SSU rRNA gene tree are estimated.

Material and Methods

Sampling and morphological methods (Fig. 1A–D)

Euplotes wuhanensis was collected on 8 April 2016 from a soil sample on Luojia Hill (30°32′06″N, 114°21′19″E), Wuhan, China (Fig. 1D). *Euplotes muscicola* was isolated on 8 April 2016 from a moss sample on the wall of a concrete pond at the Institute of Hydrobiology (30°32′512″N, 114°21′01″E), Wuhan (Fig. 1C). Ciliates were made to excyst by employing the non-flooded Petri dish method as described by Foissner (1987). Raw cultures were established at room temperature (about 25 °C) in Petri dishes containing samples, mineral water (Nongfu Spring) and rice (enriching bacteria). Although clonal cultures were not set up, no other *Euplotes* morphotypes were present in the protargol preparations, 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 100–1000×. The infraciliature and nuclear apparatus were revealed by protargol staining (Pan et al. 2014; Wilbert 1975), while dry silver nitrate staining was used to reveal the silverline systems (Foissner 2014). Counts and measurements were performed at magnifications of 100–1000×. Drawings of stained specimens were made with the help of a drawing attachment and photomicrographs. Terminology is mainly according to Curds (1975).

DNA extraction, PCR amplification, and sequencing

Cells of *E. wuhanensis* and *E. muscicola* were washed to exclude potential contamination. Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, but modified by using 1/4 of the suggested volume for each solution (Luo et al. 2017; Yan et al. 2016a). The SSU rRNA gene was amplified by two primers 82F (5′-GAAACTGCGAATGGCTC-3′) or 18SF (5′-AACCTGGTTGATCCTGCCAGT-3′) and 18SR (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Jerome et al. 1996; Medlin et al. 1988). The PCR amplifications were performed using Q5® Hot Start High-Fidelity 2x Master Mix DNA Polymerase (NEB, Ipswich, MA) with the following protocol: one cycle of initial denaturation at 98 °C for 30 s, followed by 18 cycles of amplification (98 °C, 10 s; 69 °C–52 °C touch down, 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 bidirectionally in five reactions by the Tsingke Biological Technology Com-

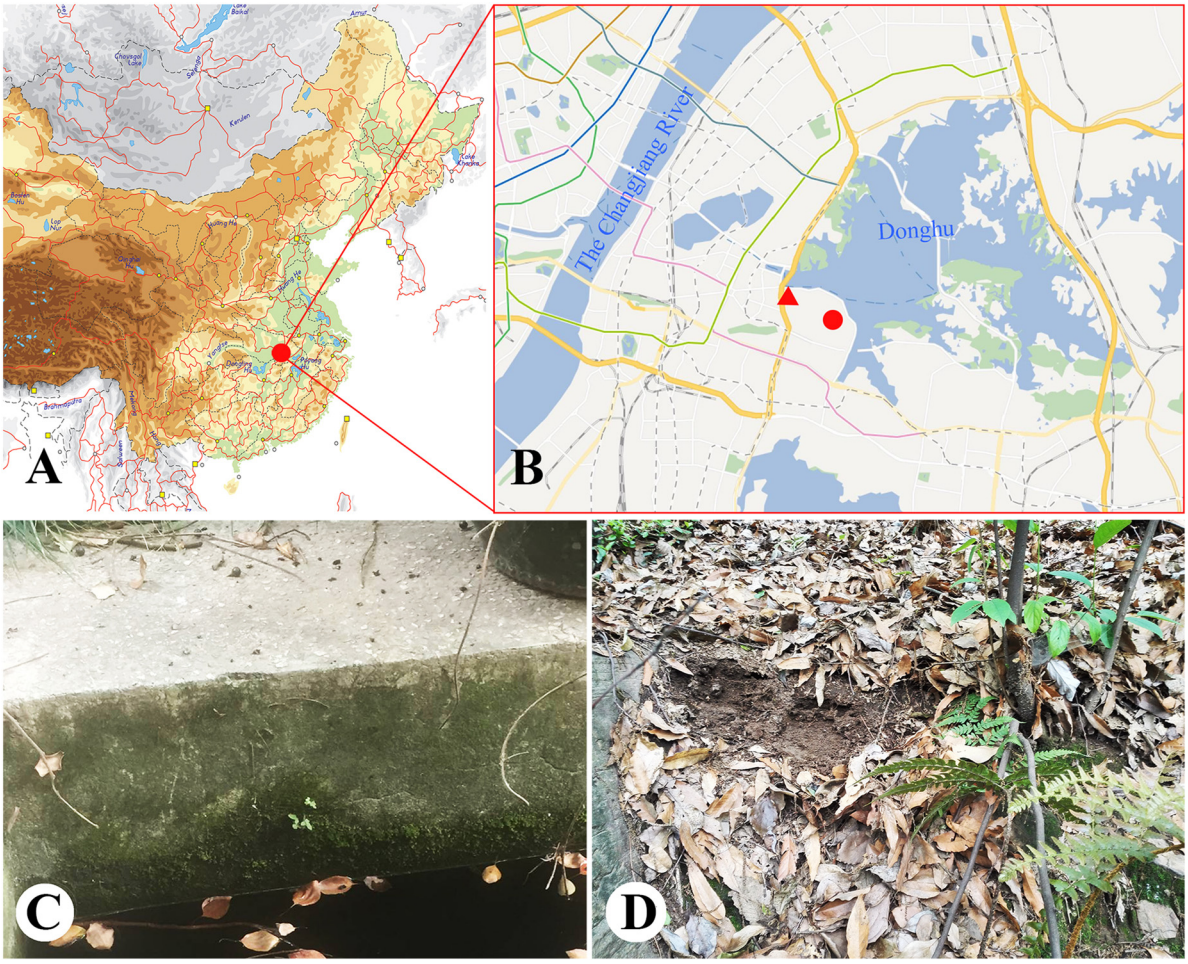

Fig. 1. **(A–D)** Locations of the sample sites. **(A, B)** Maps of sampling sites. **(C)** The moss on the wall of a concrete pond at the Institute of Hydrobiology, Chinese Academy of Sciences, corresponding to the dot in B. **(D)** The soil from Luojia Hill, Wuhan, Hubei Province, China, corresponding to the triangle in B.

pany (Qingdao, China). Contigs were assembled by Seqman (DNAStar).

Phylogenetic analyses

The SSU rRNA gene sequences of *E. wuhanensis* and *E. muscicola* were aligned with the sequences of 80 other ciliates downloaded from GenBank database (see Fig. 4 for accession numbers) using the GUIDANCE2 algorithm (http://guidance.tau.ac.il/ver2/). Representative species of Discocephalida were chosen as outgroup taxa. The final alignment used for phylogenetic analyses included 2091 sites. Maximum likelihood (ML) analysis with 1000 bootstrap replicates was performed on the CIPRES Science Gateway applying the GTRGAMMA model (Miller et al. 2010) using RAxML-HPC2 on XSEDE 8.2.9 (Stamatakis 2014). Bayesian inference (BI) analysis was carried out with MrBayes 3.2.6 on XSEDE (Ronquist et al. 2012) on the CIPRES Science Gateway using the model GTR + I + G selected by AIC in MrModeltest 2.2 (Nylander 2004). Markov chain Monte Carlo (MCMC) simulations were run for 1,000,000 generations with sampling every 100 generations and a burn-in of 1000 trees (Pan et al. 2017; Yan et al. 2016b). MEGA 5 (Tamura et al. 2011) was used to visualize the tree topologies.

ZooBank registration

The ZooBank registration number of the present work is: urn:lsid:zoobank.org: pub:6E6B563B- BDD3-460F −8874-21B6062A1613.

Results

Euplotida Small and Lynn, 1985
Euplotes Ehrenberg, 1830
Euploteswuhanensis sp. n. (Fig. 2A–L, Table 1)
Diagnosis: Cell size in vivo 40–50 × 25–30 μm; buccal field about 75% of body length with approximately 20 adoral membranelles; ten frontoventral cirri, among which cirri V/2 and VI/2 are highly reduced (having obviously smaller basal

Table 1. Morphometric data of *Euplotes wuhanensis* sp. n.

Characteristic	Min	Max	Mean	Med	SD	CV	n
Body length in μm	38.0	44.0	41.3	42.0	1.9	4.6	15
Body width in μm	25.0	32.0	28.9	29.0	2.4	8.2	15
Length of adoral zone	25.0	33.0	28.5	28.0	2.4	8.5	15
Number of adoral membranelles	18	24	20.7	21	1.9	9.0	15
Length of paroral membrane	5.0	7.0	5.9	6.0	0.8	13.5	15
Number of frontoventral cirri	10	10	10.0	10	0.0	0.0	15
Number of marginal cirri	2	2	2.0	2	0.0	0.0	15
Number of caudal cirri	2	3	2.1	2	0.4	16.5	15
Number of dorsal kineties	7	7	7.0	7	0.0	0.0	15
Number of dikinetids in middle kinety	9	13	11.8	12	1.3	10.7	15
Number of dikinetids in leftmost dorsal kinety	2	3	2.1	2	0.3	12.5	15
Number of dikinetids in rightmost dorsal kinety	7	12	9.9	10	1.4	14.3	15
Number of transverse cirri	5	5	5.0	5	0.0	0.0	15

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

plaques); two marginal and two caudal cirri; an indistinct gap separating transverse cirri into two groups, two on left and three on right; consistently seven dorsolateral kineties with about 12 dikinetids in middle row; macronucleus C-shaped; double-*eurystomus* type silverline system.

Type locality: Soil sample from Luojia Hill (30°32′6″N, 114°21′19″E), Wuhan, China.

Type material: The slide (registration number: LCY2016041201-1) with the protargol stained holotype specimen (Fig. 2K) and a paratype slide (registration number: LCY2016041201-2) with the dry silver nitrate stained specimens are deposited in the Laboratory of Protozoology, Ocean University of China (OUC). One paratype slide with protargol stained specimens (registeration number: NHMUK 2018.10.22.1) is deposited in the Natural History Museum, London, UK.

Etymology: The species-group name *wuhanensis* refers to the area (Wuhan) from where the sample was collected.

ZooBank registration of *E. wuhanensis*: urn:lsid:zoobank.org:act: 80D9AD14-DCAC-479A-9FEA-4997DBE35A32

Morphological description(Fig. 2A–L, Table 1)

Cells in vivo about 40–50 × 25–30 μm, generally oval in outline. Both left and right margins convex; anterior end narrowly rounded with a small projection at right side (Fig. 2A, B, F, G). Buccal field approximately 75% of body length (Fig. 2A, B, D, K). Several conspicuous ventral ridges extending posteriorly to transverse cirri with some shorter ridges between them (Fig. 2A, B, H, I). Dorsoventrally somewhat flattened, ventral side somewhat concave, dorsal side convex, with four or five ridges on dorsal side (Fig. 2G). No distinct cortical granules observed on dorsal or ventral side. Cytoplasm colourless, highly transparent at marginal area, and containing several to many different-sized lipid droplets and several food vacuoles in central part (Fig. 2A, B, I). Contractile vacuole about 10 μm in diameter, adjacent to rightmost transverse cirrus; contracting at intervals of 1 min (Fig. 2I). Macronucleus mirror-inverted C-shaped, with many small spherical nucleoli; one micronucleus, round to oval, located in anterior part of body near left side, generally located in a distinct depression of macronucleus (Fig. 2E). Locomotion typically by moderately fast crawling or slight jerking, occasionally remaining stationary for short periods.

Adoral zone prominent, composed of 18–24 membranelles, bases up to 10 μm in length (Fig. 2A, B, D). Paroral membrane long and thin, lying to the right of posterior portion of adoral zone (Fig. 2D, K). Invariably 10 frontoventral cirri (cilia about 18 μm long), cirri V/2 and VI/2 having obviously smaller basal plaques (Fig. 2H, K); five transverse cirri of about same size as most frontoventral cirri, cilia all about 20 μm long, all transverse cirri arranged sparsely, an indistinct gap between left two and the other three tansverse cirri; two marginal and two caudal cirri (cilia all about 15 μm long) (Fig. 2A, B, D, K). Seven dorsal kineties, with 9–13 dikinetids in mid-dorsal kinety and two or three basal bodies in dorsal kinety1 (Fig. 2D, E, L). A double-*eurystomus* type of dorsal silverline system (Fig. 2C, Table 1).

Euplotes muscicola Kahl, 1932 (Fig. 3A–O, Table 2)

Remarks: Although *E. muscicola* had been redescribed several times using silver staining methods (Fauré-Fremiet et al. 1954; Foissner 1982; Klein 1956; Shin and Kim 1995; Tuffrau 1960), no population has been defined according to both morphological and molecular data. Based on the Chinese population and all data available, an improved diagnosis is provided.

Improved diagnosis: *Euplotes* measuring 40–80 × 35–55 μm in vivo; body oval, buccal field about 75% of cell length with 25–33 adoral membranelles; usually nine frontoventral, five transverse, two marginal and two caudal cirri; nine or ten dorsal kineties with about 18–35 dikinetids in mid-dorsal row; macronucleus typically

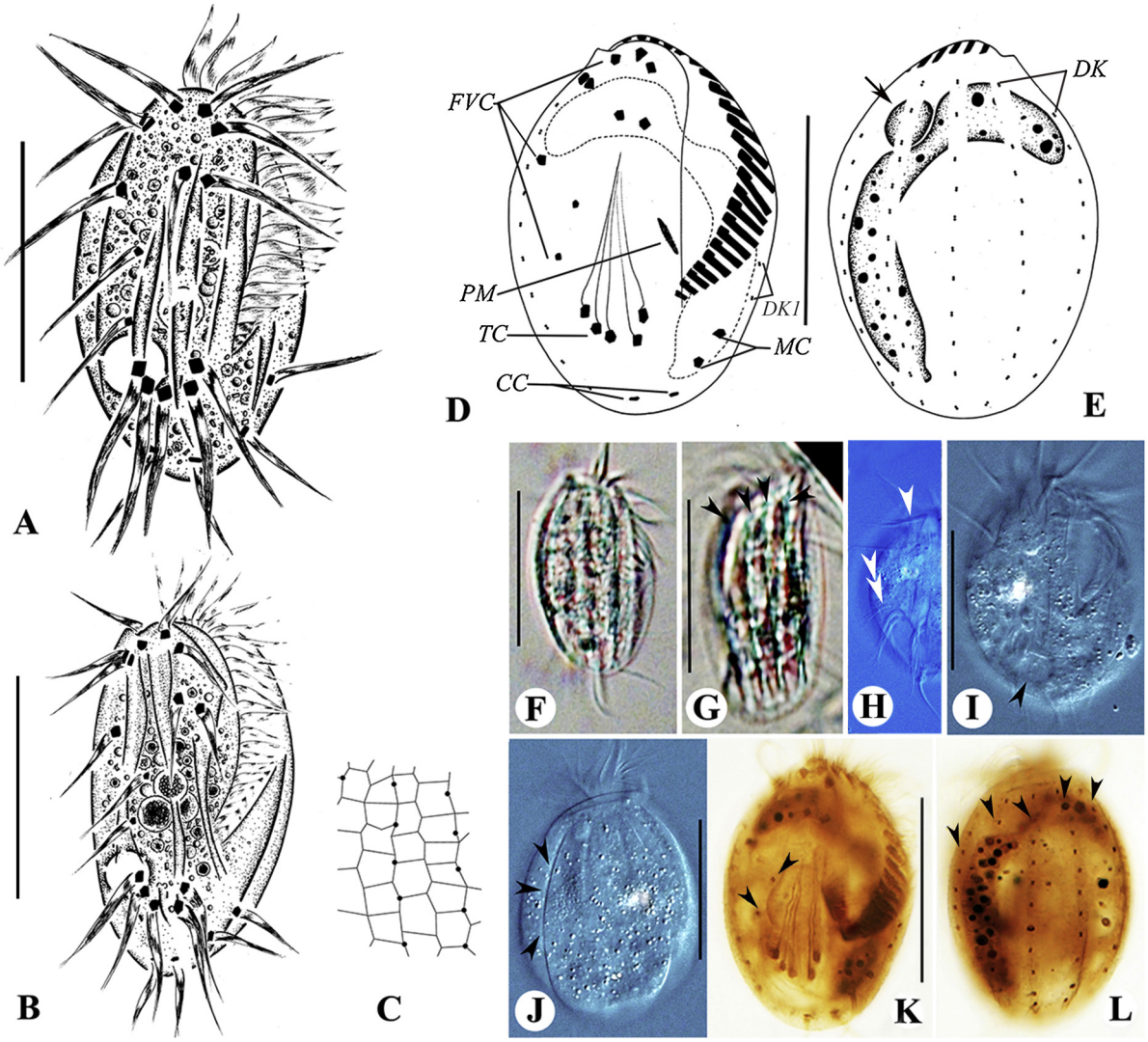

Fig. 2. **(A–L)** *Euplotes wuhanensis* sp. n. in vivo (A, B, F–J), after protargol (holotype specimen) (D, E, K, L) and silver nitrate (C) staining. **(A, B, F)** Ventral views, to show different body shapes. **(C)** Silverline system on dorsal side. **(D, E, K, L)** Ventral (D, K) and dorsal (E, L) views, showing the infraciliature and nuclear apparatus, arrow in E shows the micronucleus, arrowheads in K show the two smaller basal plaques, arrowheads in L show the dorsal kineties. **(G)** dorsal view, arrowheads show the ridges. **(H)** Ventral view of the right section of a squeezed cell, arrowhead shows the normal sized frontoventral cirrus, double-arrowhead shows a smaller cirrus. **(I)** To show the endoplasm of a highly squeezed cell, arrowhead indicates the contractile vacuole. **(J)** Dorsal view, arrowheads point to a groove. CC, caudal cirri; DK, dorsal kinety; DK1, dorsal kinety 1; FVC, frontoventral cirri; MC, marginal cirri; PM, paroral membrane; TC: transverse cirri. Scale bars: 30 μm.

C-shaped; a multiple type of dorsal silverline system. Soil and freshwater.

Morphological description of Wuhan population (Fig. 3**A–O**, Table 2)

Cell size about 65–80 μm × 40–55 μm in vivo. Body asymmetrically oval with anterior portion slightly wider than posterior; buccal field extending approximately 75% of cell length, with a visible protrusion at anterior end (Fig. 3A, B, G, H). Several ridges on dorsal side and conspicuous ridges on ventral side extending to transverse cirri, with some shorter ridges between them (Fig. 3A, B). Each dorsal cilium surrounded by five or six ellipsoid granules forming a rosette beneath pellicle (Fig. 3C, I). Cytoplasm colourless, usually with numerous crystals and food vacuoles of different sizes making cell somewhat opaque in vivo (Fig. 3G, L, M). Contractile vacuole located posteriorly near right body margin (Fig. 3A, B). Macronucleus generally C-shaped, somewhat variable among individuals, micronucleus not recognizable (Fig. 3F, O). Locomotion typically by moderately fast crawling or incessant jerking on substrate.

Adoral zone approximately 75% of cell length, composed of 28–33 membranelles, bases of which up to 17 μm long (Fig. 3A, B, E, N). Paroral membrane lying under buccal lip, thin and long, parallel to main body axis (Fig. 3E, N). Con-

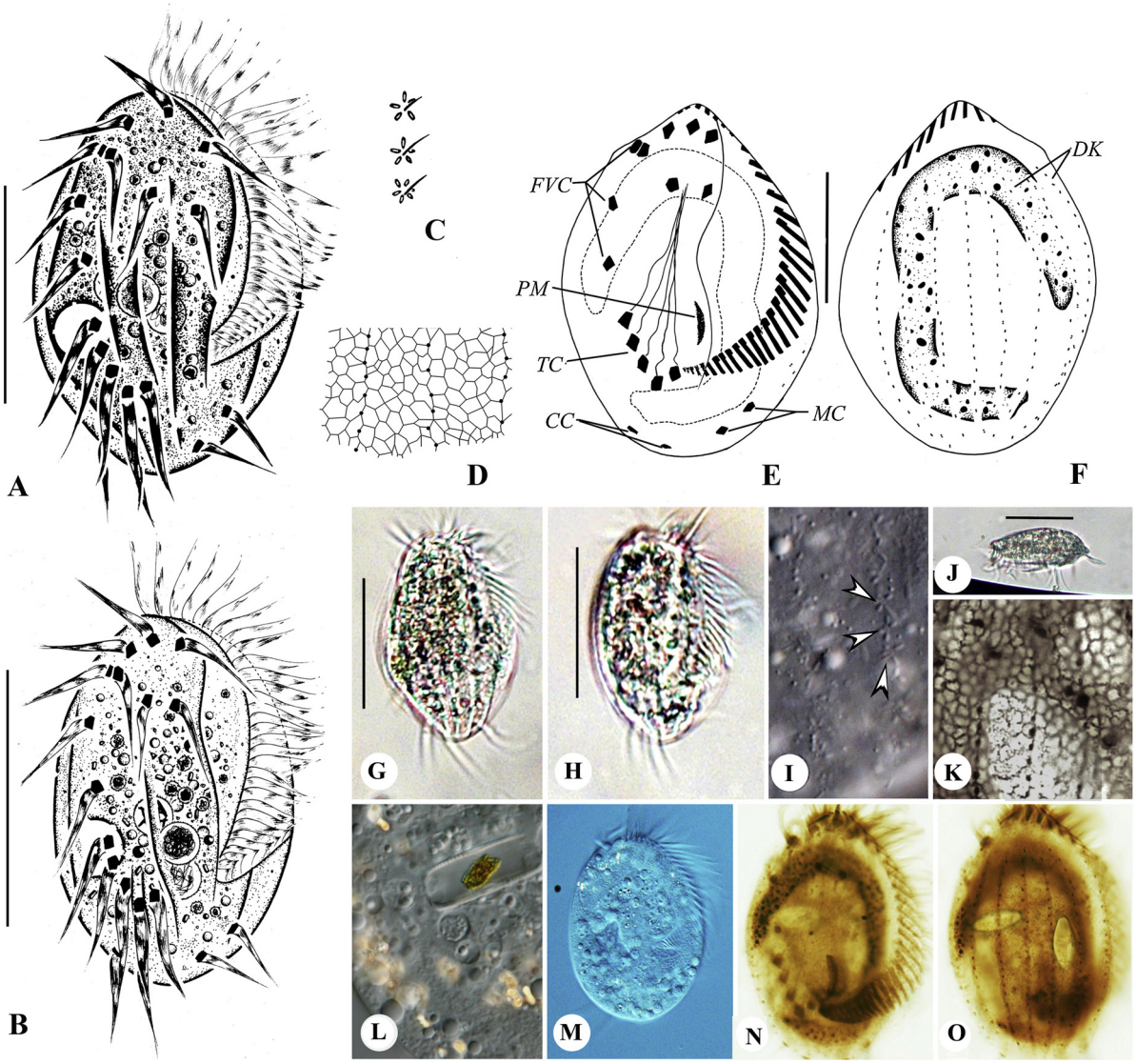

Fig. 3. **(A–O)** Photomicrographs of *E. muscicola* in vivo (A, B, G–J, L, M) and after protargol (E, F, N, O) and silver nitrate (D, K) staining. **(A, G)** Ventral views of the same representative individual. **(B, H)** Ventral views, to show different body shapes. **(C, I)** Sub-pellicular rosettes-like structures around dorsal cilia (arrowheads in I). **(D, K)** Silverline systems on dorsal side. **(E, F, N, O)** Ventral (E, N) and dorsal (F, O) views of the same specimen, showing the infraciliature and nuclear apparatus. **(J)** Lateral view. **(L, M)** Showing the endoplasm. CC, caudal cirri; DK, dorsal kinety; FVC, frontoventral cirri; MC, marginal cirri; PM, paroral membrane; TC, transverse cirri. Scale bars: 30 μm.

stantly nine frontoventral cirri (cilia about 20 μm long); five transverse cirri, cilia of which about 25 μm long; two caudal cirri at right posterior margin and two marginal cirri near posterior end, cilia all about 18 μm long (Fig. 3A, B, E). Nine or ten (usually nine) dorsal kineties with dikinetids extending almost entire length of body (Fig. 3F, O). Mid-dorsal kinety with 18–23 dikinetids (Fig. 3F, O). Dorsal silverline system of multiple type, consisting of 4–6 polygons between each kinety (Fig. 3D, K, Table 2).

Phylogenetic analyses (Fig. 4)

Phylogenetic trees were constructed based on the SSU rRNA gene sequence using both Bayesian inference (BI) and maximum-likelihood (ML) methods, with a broad selection of species in the order Euplotida. The topologies of the BI and ML trees are almost identical except for the topology of *E. wuhanensis*, and therefore only the ML tree is presented here with bootstraps and posterior probabilities from both algorithms. At the same time, we also show the different topology of the BI tree branch including *E. wuhanensis*.

The newly obtained sequence of *E. muscicola* clusters with an Italian population of *E. muscicola* (AJ305254, sampled from moss, bearing 9 frontoventral cirri, scanning electron microscopy performed, limited morphological data available) with maximum support (100% ML, 1.00 BI), and then forms a fully-supported sister relationship to a large clade containing two sequences of *E. muscorum* (HM140407,

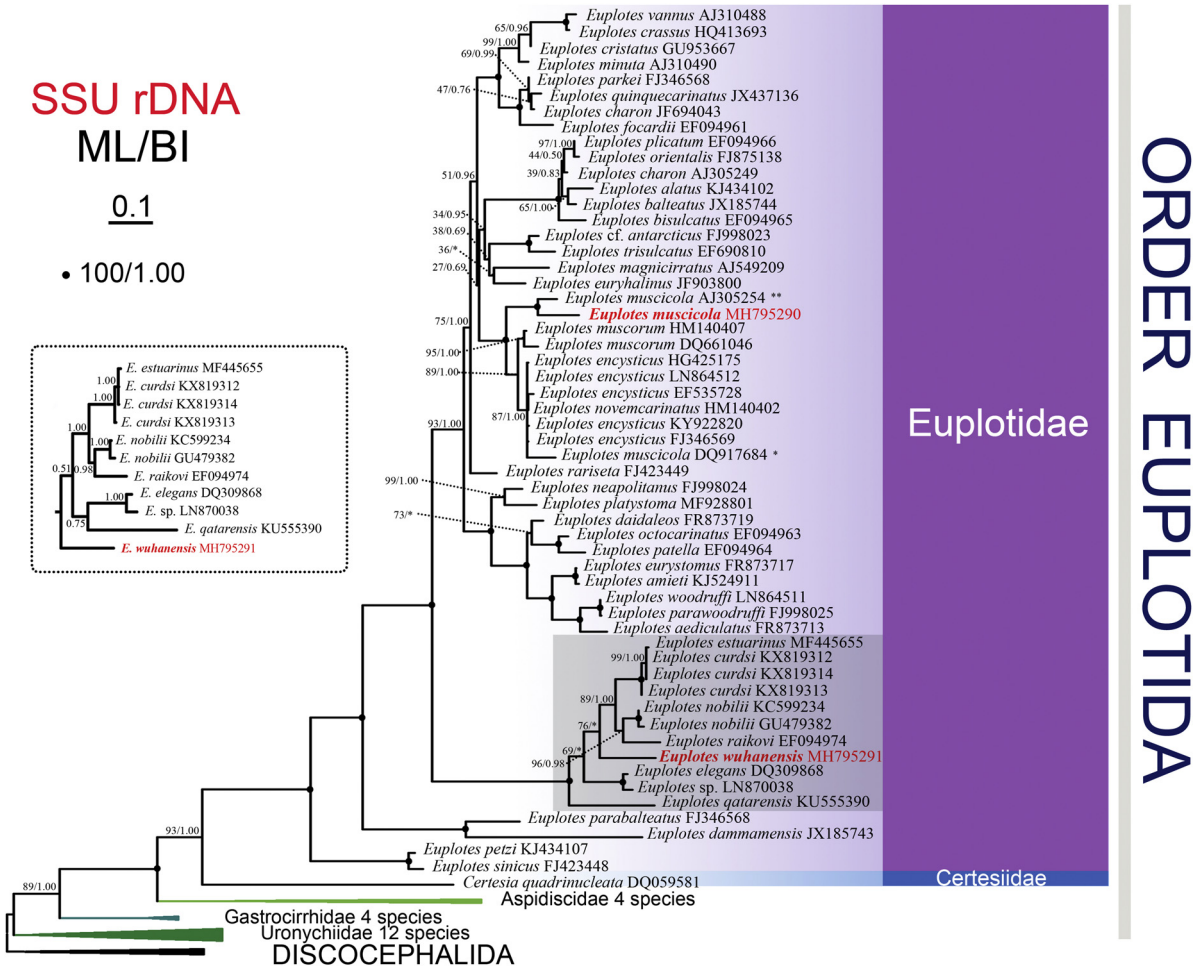

Fig. 4. Phylogenetic tree based on SSU rDNA sequences, showing the position of *Euplotes wuhanensis* sp. n. and *E. muscicola* Kahl, 1932 by Maximum Likelihood (ML) and Bayesian inference (BI). Numbers near branches denote ML bootstrap value/BI posterior probability value. '*' indicates topologies that differ between the ML and BI phylogenies. '**' to indicate that the sequences might be misidentified, DQ917687 is from the subspecies *E. muscicola lahorensi*. Fully supported (100%/1.00) branches are marked with solid circles. The dotted box represents the different topology of BI tree for *E. wuhanensis*. All branches are drawn to scale. The scale bar corresponds to 10 substitutions per 100 nucleotide positions.

Table 2. Morphometric data of *Euplotes muscicola* Kahl, 1932.

Characteristic	Min	Max	Mean	Med	SD	CV	n
Body length in μm	60.0	80.0	70.2	71.0	5.8	8.2	15
Body width in μm	43.0	56.0	49.0	49.0	4.1	8.3	15
Length of adoral zone	45.0	58.0	51.5	52.0	3.3	6.3	15
Number of adoral membranelles	28	33	30.7	31	1.2	4.0	15
Length of paroral membrane	8.0	12.0	9.9	10.0	1.0	9.7	15
Number of frontoventral cirri	9	9	9.0	9	0.0	0.0	15
Number of marginal cirri	2	2	2.0	2	0.0	0.0	15
Number of caudal cirri	2	2	2.0	2	0.0	0.0	15
Number of dorsal kineties	9	10	9.4	9	0.5	5.4	15
Number of dikinetids in middle kinety	18	23	19.5	19	1.8	9.5	15
Number of dikinetids in leftmost dorsal kinety	2	5	3.2	3	3	1.0	15
Number of dikinetids in rightmost dorsal kinety	10	18	14.3	14	2.4	16.6	15
Number of transverse cirri	5	5	5.0	5	0.0	0.0	15

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

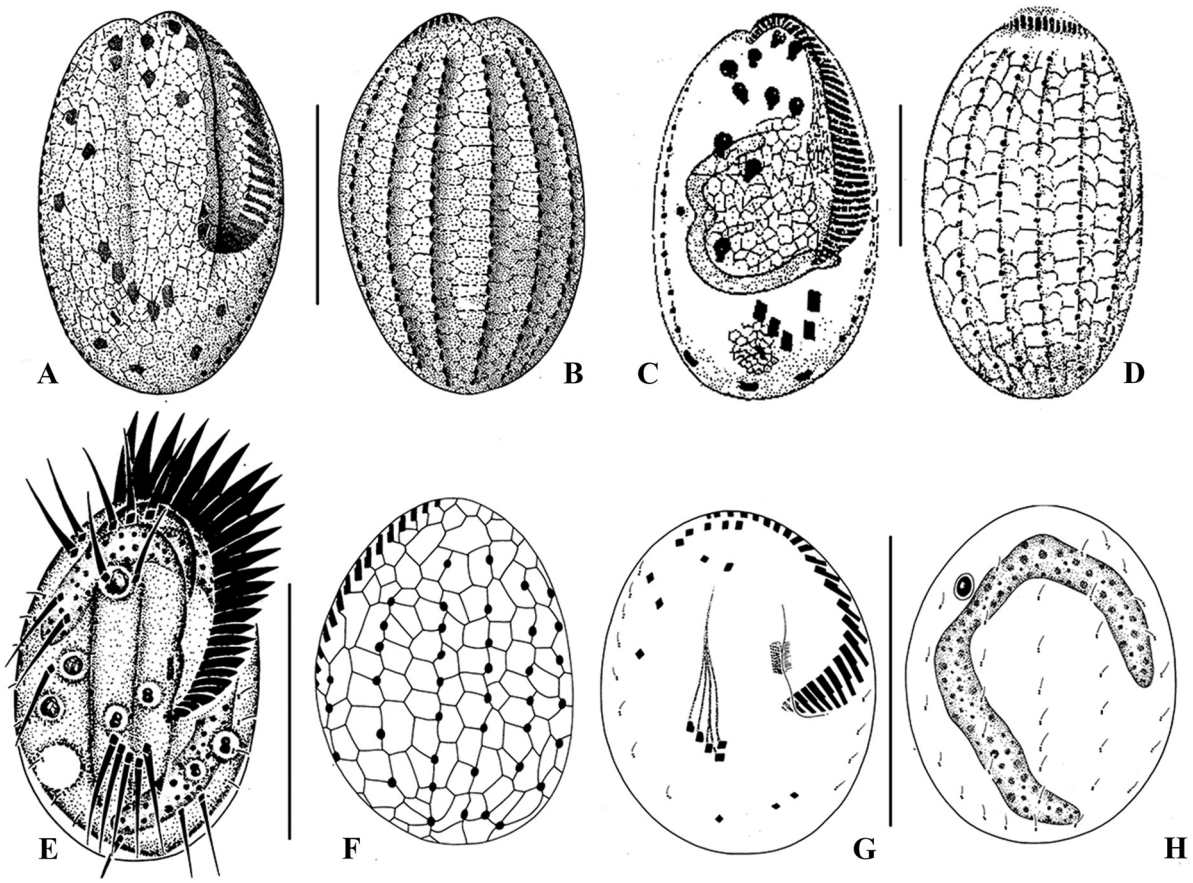

Fig. 5. **(A–H)** Related congeners of *E. wuhanensis* n. sp. **(A, B)** *E. crenosus* Tuffrau, 1960 (from Tuffrau, 1960). **(C, D)** *E. ogusi* Aliev, 1987 (from Aliev 1987). **(E, F)** *E. corsica* Berger and Foissner, 1989, (from Berger and Foissner 1989). Scale bars: 30 μm.

DQ661046), five sequences of *E. encysticus* (HG425175, LN864512, EF535728, KY922820, FJ346569), *E. novemcarinatus* (HM140402) and a Pakistani population of *E. muscicola* (DQ917684, no morphological information). The new species, *E. wuhanensis*, is closely related in the ML tree with a well-supported assemblage including *E. nobilii* (KC599234, GU479382), *E. raikovi* (EF094974), *E. curdsi* (KX819312, KX8190312, KX8190314) and *E. estuarinus* (MF445655), with moderate support (bootstrap values: 76%). In the BI tree, however, *E. wuhanensis* is placed outside a larger group comprising *E. estuarinus*, *E. curdsi*, *E. nobilii*, *E. raikovi*, *E.* sp., *E. elegans* and *E. qatarensis* (not registered in ZooBank, thus not valid yet).

Discussion

Euplotes wuhanensis sp. n.

Comparison with related congeners (Fig. 5A–H, Table 3)

This new species can be identified by the characteristics of its cells in both living and stained specimens, and it can be unambiguously identified by the combination of the following features: its small body size, its shape, the presence of dorsal ridges, the C-shaped macronucleus, and the basic ciliature (ten frontoventral cirri with the small basal plaque in V/2 and VI/2, five transverse cirri separated into two groups, two marginal cirri, two caudal cirri and seven dorsolateral kineties).

Only two small, freshwater or soil species of *Euplotes* possessing 10 frontoventral cirri and a double-*eurystomus* silverline pattern have been reported, namely *E. crenosus* Tuffrau, 1960 and *E. ogusi* Aliev, 1987 (Table 3).

Euplotes crenosus was first discovered by Tuffrau (1960). It differs from *E. wuhanensis* in the body size (50–75 μm vs. 40–50 × 25–30 μm), the size of the frontoventral cirri (normal sized vs. cirri V/2 and VI/2 obviously smaller) and the pattern of the transverse cirri (as a single group vs.as two groups) (Tuffrau 1960) (Fig. 5A, B).

Aliev (1987) described *E. ogusi* from silt-sandy soil in Azerbaijan. Compared with the new species studied here, *E. ogusi* can be distinguished by the appearance and number of adoral membranelles (adoral zone relatively straight but curved posteriorly, 35–40 membranelles vs. curved in a C-shaped, 18–24), the number of basal bodies in the mid-dorsal kinety (13–15 vs. 9–13) and the normal-sized frontoventral cirri (vs. cirri V/2 and VI/2 smaller). In addition, *E. ogusi* has three caudal cirri but no marginal cirri (maybe one cau-

Table 3. Comparison of *Euplotes wuhanensis* sp. n. with those related congeners having ten frontoventral and five transverse cirri.

Characteristic	*E. crenosus*	*E. ogusi*	*E. corsica*	*E. wuhanensis*
Cell size in vivo (μm)	50–70 × ?	50–55 × 30–32	40 × 20	40–50 × 25–30
Number of adoral membranelles	25–30	35–40	20–25	18–24
Number of caudal cirri	2	3	1–2	2
Number of marginal cirri	2	0	2	2–3
Number of dorsal kineties	8	7	7–8	7
Number of dikinetids in mid-dorsal kinety	ca. 22[a]	13–15	6–9	9–13
Biotope	Freshwater	Silt-sandy soil	Soil from a saline pool	Soil
Macronucleus	C-shaped	3-shaped	C-shaped	C-shaped
References	Tuffrau (1960)	Aliev (1987)	Berger and Foissner (1989)	Present

[a]Counted based on the illustration.

dally positioned marginal cirri and two caudal cirri), which is extremely unusual. Thus, this characteristic and the validity of this species await confirmation (Aliev 1987) (Fig. 5C, D).

Berger and Foissner (1989) reported *E. corsica* from the sediment of a saline pool in France (salinity not mentioned). *Euplotes corsica* is similar to *E. wuhanensis* in terms of its body size (40–50 × 25–30 μm) and shape, and in having ten frontoventral cirri and a double-*eurystomus* silverline pattern. However, conspecificity can be excluded, both because its frontoventral cirri are all normal sized (vs. cirri V/2 and VI/2 with small basal plaque), and also because *E. corsica* has fewer dikinetids in the mid-dorsal kinety (6–9 vs. 9–13). *Euplotes corsica* has two marginal and one or two caudal cirri, according to the original drawing and statistical data, while *E. wuhanensis* invariably has two caudal cirri (Fig. 5E–H).

Euplotes muscicola Kahl, 1932

Remarks on dargyrome of *Euplotes*

Curds (1975) divided the dargyrome of *Euplotes* into five type, i.e. single-vannus (single cross-connectives), double-eurystomus (two equal rows of polygons between ciliary rows), double-patella-I, II (two unequal rows of polygons), multiple (three or four polygons between ciliary rows), and complex (irregular network of polygons between ciliary rows). Subsequently, Gates and Curds (1979) reduced the number of types to three, namely single, double and multiple, although Borror and Hill (1995) still suggested that this revised scheme continued to recognize a distinction between the 'multiple' and 'complex' types. The 3-pattern scheme was accepted by Valbonesi and Luporini (1995) whose work showed that the type of dargyrome can, to a large extent, be attributed to cell cortical ridges. However, many other researchers, continued to use the original scheme (Agatha et al. 1990; Jiang et al. 2010; Liu et al. 2015; Shin and Kim 1995; Song and Bradbury 1997). In the present study, we tend to support to merge the 'complex' and 'multiple' types, because sometimes these two types can not be clearly distinguished, e.g. in *E. muscicola* from Korea and in *E. illifei* (Achilles-Day et al. 2008; Hill et al. 1986). However, considering the classification in Curds (1975) is widely used in recent decades, we still adapt the original 5-pattern scheme.

Identification of *Euplotes muscicola* and comparison with other populations (Fig. 6A–M)

This organism has been found repeatedly in Germany (Kahl 1932; Klein 1956), Hungary (Gelei 1938), China (Wang and Nie 1935), France (Fauré-Fremiet et al. 1954), Austria (Foissner 1982), Korea (Shin and Kim 1995) and Slovakia (Tirjakovã et al. 2015). We identify our population by (1) the basic ciliature on the ventral (constantly nine frontoventral, five transverse, two caudal and two marginal cirri) and dorsal surface (9–10 dorsal kineties and 18–35 dikinetids in the mid-dorsal kinety), (2) biotope (freshwater and soil, especially moss) and (3) the silverline system pattern. Nonetheless, one or several dorsal ribs formed a pterygoid or flap-like process in the German, Hungary, and Austria populations (Foissner 1982; Gelei 1938; Kahl 1932; Klein 1956), the silverline grids of the Austria, German, and Chinese population are not as regular as that of the Korean population (Foissner 1982; Klein 1956; Shin and Kim 1995). These, however, are regarded as population-level differences. It should also be noted that Tuffrau (1960) reported a population of *E. muscicola* with about 35 dikinetids in the mid-dorsal kinety, which is much more than those of the other populations (no more than 30). Thus, we propose that his identification requires confirmation.

Subspecies of *Euplotes muscicola*

Kahl (1932) established three subspecies, *E. muscicola muscicola* (nominotypical subspecies), *E. muscicola alatus*, and *E. muscicola bialatus*, based on the number of the conspicuously high dorsal ridges (wings) in vivo. Considering the development of wings could be influenced by the nutrition condition and the presence of the predator (Kuhlmann and Heckmann 1994), it is not an appropriate characteristic to distinguish subspecies. Chaudhry and Shakoori (2012) established a further subspecies *E. muscicola lahorensis* mainly

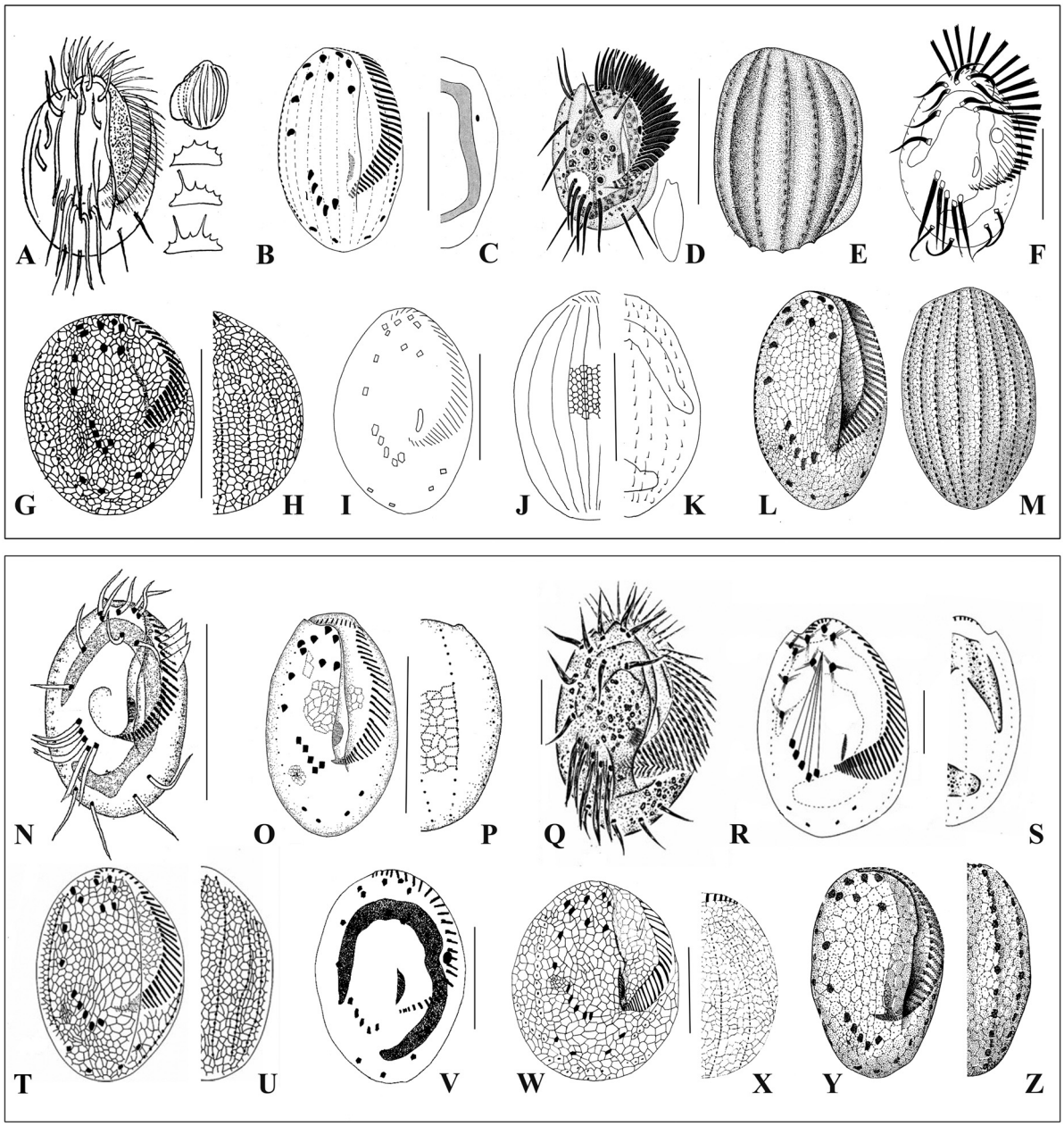

Fig. 6. **(A–Z)** *Euplotes muscicola* (A–M) and its related congeners (N–Z). **(A)** from Kahl (1932). **(B, C)** from Fauré-Fremiet et al. (1954). **(D–H)** from Foissner (1982). **(I–K)** from Shin and Kim (1995). **(L, M)** from Tuffrau (1960), probably misidentified. **(N–P)** *E. elegans* Kahl, 1932 (from Dragesco and Dragesco-Kernéis, 1986). **(Q–U)** *E. encysticus* Yonezawa, 1985 (from Fan et al. 2010). **(V–X)** *E. muscorum* Dragesco, 1970 (from Jo and Shin 2003). **(Y, Z)** *E. gracilis* Tuffrau, 1960 (from Tuffrau 1960). Scale bars: 30 μm.

according to the SSU rRNA gene sequence which clustered with other *Euplotes* species in our tree (Fig. 4). Also, few morphological data of this form is available, so we think their identification is questionable.

Comparison with related congeners (Fig. 6N–Z; Table 4)

Considering the ambiguousness of multiple and complex-type silverline systems. In the present paper, we compared *E. muscicola* with similar species with both silverline system types. Four small to medium sized species with nine frontoventral, two caudal cirri, two marginal cirri, and a multiple or complex silverline system have to be compared: *E. elegans* Kahl, 1932; *E. encysticus* Yonezawa, 1985; *E. muscorum* Dragesco, 1970 and *E. gracilis* Kahl, 1932.

Euplotes muscorum, a small to medium-sized freshwater species discovered in Cameroon and also found in Korea, appears to resemble *E. muscicola* in respect to the cell size, biotope and general ventral infraciliature. It deviates from the latter, however, by possessing fewer dorsal kineties (8 vs.

Table 4. Comparison of *Euplotes muscicola* Kahl, 1932 with those related congeners having nine frontoventral, five transverse, two marginal, and two caudal cirri.

Characteristics	*E. elegans*	*E. encysticus*	*E. muscorum*	*E. gracilis*	*E. muscicola*
Cell size in vivo (μm)	32–88 × ?	60–90 × 40–65	63–78 × 40–52	37–50 × ?	45–80 × 33–55
Number of adoral membranelles	20–39	30–43	32–36	ca. 30	25–33
Number of dorsal kineties	7–8	7–8	8	7	9–10
Number of dikinetids in mid-dorsal kinety	ca. 20	21–30	22–28	10–13	18–30
Biotope	Brackish/ freshwater	Brackish/ freshwater	Freshwater	Freshwater	Freshwater
Macronucleus	C-shaped	C-shaped	C-shaped or 3-shaped	C-shaped	C-shaped
References	Dragesco and Dragesco-Kernéis (1986) and Kahl (1932)	Fan et al. (2010) and Yonezawa (1985)	Dragesco (1970) and Jo and Shin (2003)	Kahl (1932) and Tuffrau (1960)	Kahl (1932), Foissner (1982), Fauré-Fremiet et al. (1954), Shin and Kim (1995); present

9–10) and a complex silverline system (vs. multiple, which is more regular) (Dragesco 1970; Dragesco and Dragesco-Kernéis 1986; Jo and Shin 2003) (Fig. 6V–X).

Euplotes elegans was originally discovered in Germany (Kahl 1932), and then reported in detail in USA (Carter 1972), France (Dragesco 1960) and Benin (Dragesco and Dragesco-Kernéis 1986). The identification of this medium-sized euryhaline species is still unclear due to the confusion as to the type of the dorsal silverline system (complex type or double-*eurystomus* type). *Euplotes elegans* is similar to *E. muscicola* in respect to its body size, ventral infraciliature and the number of dorsal kineties, but it is euryhaline (vs. freshwater and soil habitat), has more adoral membranelles (40–45 vs. 25–35), fewer dorsal kineties (8 vs. 9–10), and more dikinetids in the mid-dorsal kinety (30–46 vs. 18–35), and is thus distinctly different from *E. muscicola* (Carter 1972; Dragesco 1960; Dragesco and Dragesco-Kernéis 1986; Kahl 1932; Schwarz et al. 2007; Tuffrau 1960) (Fig. 6N–P).

Euplotes gracilis, a small freshwater species, was originally briefly described by Kahl (1932) but its description now relies largely upon that of Tuffrau (1960). Although *E. gracilis* and *E. muscicola* share similarities in having nine frontoventral, two caudal and two marginal cirri, the former is distinguishable by its smaller size (37–50 μm vs. 44–78 μm), fewer dorsal kineties (7 vs. 9–10) and fewer dikinetids in the mid-dorsal kinety (10–13 vs. 18–35) (Kahl 1932; Tuffrau 1960) (Fig. 6Y, Z).

Euplotes encysticus was first reported from Japan by Yonezawa (1985). This description was very basic and later, the general cortical morphogenesis and detailed morphology were added by Matsusaka et al. (1989) and Fan et al. (2010), respectively. *Euplotes encysticus* and *E. muscicola* show great similarity in their general ventral infraciliature and the appearance of the adoral membranelles. The latter, however, has fewer adoral membranelles (25–33 vs. 30–43) and more dorsal kineties (9–10 vs. 7–8) (Fan et al. 2010; Matsusaka et al. 1989; Yonezawa 1985) (Fig. 6Q–U).

SSU rRNA gene sequences and phylogenetic analyses

As shown in Fig. 4, Euplotida and its five families, namely Aspidiscidae, Certesiidae, Euplotidae, Gastrocirrhidae and Uronychiidae, are all monophyletic groups. This is consistent with previous studies (Achilles-Day et al. 2008; Fan et al. 2013; Gao et al. 2016; Huang et al. 2012; Liu et al. 2015; Petroni et al. 2002; Yi et al. 2012).

To date, the SSU rDNA sequences of *E. muscicola* are available from two nominal isolates deposited under the same name in GenBank (Italian population, AJ305254; Pakistani population, DQ91768) which differ from each other considerably at 62 nucleotide sites, indicating that they should not be conspecific. Although our isolate clusters with the Italian population in the present molecular tree, it differs at 56 nucleotide sites from that population, and 84 nucleotide sites from the Pakistani population. Considering either detailed descriptions of living organisms nor morphometric data was reported with these two sequences under the name of *E. muscicola* (AJ305254, DQ917684), we propose that these two sequences could be from misidentified populations.

The newly obtained *E. muscicola* is closely related with *E. muscorum*, *E. encysticus* and *E. novemcarinatus* in both ML and BI trees, which corresponds well with the shared morphological characters of a similar silverline system (multiple type in *E. muscicola*, complex type in the other three species).

According to Syberg-Olsen et al. (2016), *E. curdsi*, *E. nobilii*, *E. raikovi*, *E. elegans* and *E. qatarensis* with similar dargyrome type are closely related. Likewise, our new isolate *E. wuhanensis* fell within a fully-supported group including *E. estuarinus*, *E. curdsi*, *E. nobilii*, *E. raikovi*, *E. elegans*

and *E. qatarensis* in the updated phylogeny of *Euplotes*. This is consistent with their morphological similarities. However, the phylogenetic position of *E. wuhanensis* is not yet stable as it is placed in different positions in the ML and BI trees. Therefore, more molecular and morphological data are needed to infer a more robust phylogeny of *Euplotes*.

Acknowledgments

This work was supported by the Nature Science Foundation of China (project Nos. 31772477, 31572247), Fundamental Research Funds for the Central Universities (project No. 201762017) and the, Biodiversity survey of aquatic organisms in Three Gorges Reservoir area and the illustrated handbook compilation (No. 2014FY120200), King Saud University Deanship for Scientific Research, International Research Group Program (IRG14-22). We thank Professor Weibo Song, Ocean University of China for helping to improve our manuscript.

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