International Journal of Systematic and Evolutionary Microbiology

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--Manuscript Draft--

Manuscript Number:	IJSEM-D-16-00828R1
Full Title:	One freshwater Cyclidium species, C. sinicum spec. nov. (Protozoa; Ciliophora), with an improved diagnosis of the genus Cyclidium
Article Type:	Note
Section/Category:	New taxa - Eukaryotic micro-organisms
Keywords:	Cyclidium, Cyclidium sinicum spec. nov.,SSU rDNA sequence,new species
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Manuscript Region of Origin:	CHINA
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One freshwater *Cyclidium* species, *C. sinicum* spec. nov. (Protozoa; Ciliophora), with an improved diagnosis of the genus *Cyclidium*

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Running title: Pan *et al*., one freshwater scuticociliates

The GenBank accession number for *Cyclidium sinicum* spec. nov.: KX853100

Abstract

The morphology and infraciliature of one freshwater ciliate, *Cyclidium sinicum* spec. nov., isolated from a farmland pond in Harbin, northeastern China, was investigated using living observation and silver staining methods. *Cyclidium sinicum* spec. nov. is distinguished by the following features: body approximately 20–25 × 10–15 μm *in vivo*; buccal field about 45–50 % of body length; 11 somatic kineties; somatic kinety n terminating sub-caudally; two macronuclei and one micronucleus; M1 almost as long as M2; M2 triangle-shaped. The genus *Cyclidium* is re-defined as follows: body outline usually oval or elliptical, ventral side concave, dorsal side convex; single caudal cilium; contractile vacuole posterior terminal; adoral membranelles usually not separated; paroral membrane "L"-shaped, with anterior end terminating at the level of anterior end of M1; somatic kineties longitudinally arranged and continuous. Phylogenetic trees based on the SSU rDNA sequences show that *C. sinicum* spec. nov. clusters with the type species, *C. glaucoma*, with full support. *Cyclidium* is not monophyletic with members of the clade of *Cyclidium* + *Protocyclidium* + *Ancistrum* + *Boveria*.

INTRODUCTION

Scuticociliates are common inhabitants of freshwater, brackish, and marine habitats (Budiño *et al.*, 2011; Castro *et al.*, 2014; Fan *et al.*, 2011a, b; Foissner *et al.*, 2014; Foissner & Wilbert, 1981; Lynn & Strüder-Kypke, 2005; Pan H. *et al.*, 2015; Pan X. *et al.*, 2013a, b, 2015a, b, 2016; Pan & Bullard, 2016; Song *et al.*, 2002, 2003, 2009; Zhan *et al.*, 2014). Investigations over the past ca. 20 years have demonstrated that the sub-class Scuticociliatia Small, 1967 is much more diverse than was previously assumed (Mallo *et al.*, 2014; Ofelio *et al*., 2014; Perez-Uz & Song, 1995; Song, 2000; Song & Wilbert, 2000, 2002). Recent investigations in China have shown a high diversity of marine scuticociliates, but few reports about freshwater species (Fan *et al.*, 2009, 2010; Gao *et al.*, 2010, 2012a, b, 2013, 2014; Pan H. *et al.*, 2010, 2015; Pan X. *et al.*, 2011, 2015a, 2016).

The well-known scuticociliate genus *Cyclidium* comprises more than 40 species isolated from terrestrial, marine and freshwater habitats (Agamaliev, 1983; Alekperov, 2005; Borror, 1972; Didier & Wilbert, 1981; Grolière, 1980; Song & Wei, 1998). Three species have been recorded from China, all from the northern China seas, namely *Cyclidium citrullus* Cohn, 1865, *C. glaucoma* Müller, 1786 and *C. varibonneti* Song, 2000 (Song, 2000; Song *et al*., 2003; Song & Wei, 1998; Song & Wilbert, 2002). *Cyclidium* and *Cylidium*-like species are generally recognized by having a conspicuously truncated apical end, non-separated membranelles and a prominent, wing-like paroral membrane (Borror, 1972; Song *et al.*, 2009). All members of *Cyclidium* are small and have high degree of morphological similarity *in vivo* therefore their infraciliature as revealed by silver staining is of great importance for species identification (Foissner *et al.*, 1994; Song, 2000; Song & Wilbert, 2002). Many nominal species, however, are insufficiently described and/or lack gene sequence data (Foissner *et al.*, 1994; Song & Wilbert, 2002).

During a survey of the freshwater ciliate fauna in northeastern China, one scuticociliates was isolated and observed *in vivo* and after silver staining. In addition, the molecular phylogeny of *C. sinicum* spec. nov., was investigated based small subunit ribosomal DNA (SSU rDNA) sequence data. The diagnosis of the genus *Cyclidium* is emended based on current observations.

METHODS

Cyclidium sinicum spec. nov. was collected on 26 Oct 2015 from a farmland pond (44° 87’ 14.7’’ N; 127° 09’ 12.0’’ E) in Harbin, Heilongjiang province, northeastern China (water temperature 14 ºC, pH 7.3; Fig. 1). About 0.4 L water was collected from 0.1–0.5 m below the surface using a sampling bottle. Ciliates were maintained in habitat water in Petri dishes as raw cultures at room temperature (ca. 25 ℃) with rice grains added to enrich the growth of bacteria as food.

Isolated cells were observed and photographed *in vivo* using differential interference contrast microscopy. The protargol method used to reveal the infraciliature follows Wilbert (1975). The protargol was made according to Pan X. *et al.* (2013a). Silver carbonate (Ma *et al.*, 2003) and Chatton-Lwoff silver nitrate (Wilbert & Song, 2008) stains were also were used to reveal the infraciliature and argyrome, respectively. Counts and measurements of stained specimens were performed at magnifications of 100–1250×. Drawings were made with the help of a drawing device. Systematics and terminology are mainly according to Lynn (2008).

Genomic DNA of *Cyclidium sinicum* spec. nov. was extracted, using the DNeasy Tissue kit (Qiagen, Valencia, CA), from about five to seven cells that had been starved overnight. The amplification of SSU rDNA was performed with universal primers, Euk A and Euk B (Medlin *et al.*, 1988). Purified PCR products of the appropriate size were inserted into the pMD™18-T vector (Takara Biotechnology, Dalian Co., Ltd.), transformed into *E. coli* competent cells and products from transformed clones were sequenced on an ABI-PRISM 3730 automatic sequencer (Applied Biosystems) using M13 forward and reverse primers. The SSU rDNA sequence has been deposited in the GenBank database with accession number KX853100. Other sequences used in the study were obtained from the GenBank database (accession numbers shown in Fig. 4). Sequences were aligned using Clustal W implemented in BioEdit 7.0 (Hall, 1999) enabling pairwise analysis. *Uronema marinum* and *Paranophrys magna* were used as the outgroup taxa. Maximum likelihood (ML) analysis was conducted using RAxML-HPC2 on XSEDE (8.1.11) (Stamatakis, 2006; Stamatakis *et al*., 2008) via the CIPRES Science Gateway website (http://www.phylo.org/sub_sections/portal), using the GTR + I + G model as

selected by Modeltest v.3.4 (Posada & Crandall, 1998). The reliability of internal branches was estimated by bootstrapping with 1,000 replicates. Bayesian inference (BI) analysis was performed with MrBayes v3.2.3 (Ronquist & Huelsenbeck, 2003) via the CIPRES Science Gateway using the GTR + I + G model selected by MrModeltest v.2.0 (Nylander, 2004). The chain length of Markov chain Monte Carlo simulations was 1,000,000 generations with a sampling frequency of 100 generations. The first 25% of sampled trees was discarded as burn-in. Phylogenetic trees were visualized with TreeView v.1.6.6 (Page, 1996) and MEGA v.4 (Tamura *et al.*, 2007).

RESULTS AND DISCUSSION

Subclass Scuticociliatia Small, 1967

Family Cyclidiidae Ehrenberg, 1838

Genus *Cyclidium* O. F. Müller, 1773

***Cyclidium sinicum* spec. nov. (Fig. 2; Table1)**

Diagnosis. Body about 20–25 × 10–15 μm *in vivo*; buccal field about 45–50 % of body length; 11 somatic kineties; somatic kinety n (SK_n) extending to posterior sub-terminally; two macronuclei and one micronucleus; M1 almost as long as M2; M2 triangle-shaped; paroral membrane consisting of rows of basal bodies forming a zig-zag pattern; scutica composed of two pairs of kinetosomes positioned posterior of cytostome; freshwater habitat.

Type locality. A farmland pond near Harbin (44° 87’ 14.7’’ N; 127° 09’ 12.0’’ E), northeastern China.

Type slides. The slide with protargol-stained holotype specimen is deposited in the Natural History Museum, London, UK with registration NHMUK.2016.10.15.1. A paratype specimen in slide is deposited in the Laboratory of Protozoology, Ocean University of China with registration number PXM-2015102602.

Etymology. The name '*sinicum*' recalls the fact that this species was first found in China, si'ni.cum. N.L. neut. adj. sinicum, pertaining to China.

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Description. Body size 20–25 × 10–15 μm *in vivo*, ellipsoidal with an apical plate about 1/4 body width (Fig. 2a, h, n, l). Laterally flattened about 3:1 with right side concave and left side convex (Fig. 2d). Pellicle smooth, extrusomes not observed (Fig. 2a, h). Buccal field about 45–50 % of body length, with prominent paroral membrane on ventral side (Fig. 2i–o). Somatic cilia and cilia of paroral membrane about 7 μm long, caudal cilium about 12 μm long (Fig. 2i–o). Cytoplasm colourless, containing several to many large (approximately 3 μm in diameter) bacteria-filled food vacuoles and variable-sized (0.5–1 μm) refringent granules (Fig. 2a, h, o). Two globular macronuclei approximately 5 μm in diameter; one micronucleus approximately 2 μm across (Fig. 2c, e, p–r). Contractile vacuole located near posterior end of cell, approximately 4 μm in diameter (Fig. 2n). Movement moderately fast, rotating clockwisely about main body axis, sometimes motionless for short periods.

Invariably 11 bipolar somatic kineties extending from near anterior apical plate to posterior contractile vacuole. All somatic kineties (SK_{1-n}) about equal length, comprising loosely spaced monokinetids; somatic kinety 1(SK_1) comprises 11 or 12 monokinetids (Fig. 2b, c, p–r). Membranelle 1 composed of two longitudinal rows of monokinetids; M2 triangle-shaped, almost as long as M1 and composed of about six horizontally oriented rows; membranelle 3 small, two-rowed (Fig. 2g, r). Paroral membrane "L"-shaped, extending to about 45% of body length (Fig. 2g, r). Scutica comprises four kinetosomes arranged in two groups, located near posterior end of paroral membrane (Fig. 2g, r). No silver nitrate preparations of sufficient quality were obtained to allow observation of the entire argyrome or contractile vacuolar pore (Fig. 2f).

SSU rDNA sequence data. The SSU rDNA sequence of *Cyclidium sinicum* spec. nov. has been deposited in the GenBank database with the accession number, length and G+C content as follows: KX853100, 1679 bp (not including Euk A and Euk B primer sites), 45.44%.

Remarks and comparison. It is widely accepted that the most important criteria for species

identification and separation in *Cyclidium* are the structure of membranelles 1–3, body size and shape, the length of the buccal field relative to the body length, the presence of extrusomes, the number of somatic kineties, the number of macronuclei, the termination position of somatic kineties in posterior end of SK_n and SK_{n-1} and the habitat (Agamaliev, 1983; Alekperov, 2005; Borror, 1972; Didier & Wilbert, 1981; Foissner *et al.*, 1994; Grolière, 1980; Fig. 4).

With respect to its body shape and size, prominent paroral membrane and freshwater habitat, *Cyclidium sinicum* spec. nov. most closely resembles *C. glaucoma* Müller, 1786, *C. varibonneti* Song, 2000 and *C. bonneti* Grolière, 1980. It can be easily separated from other congeners (Fig. 3).

Although *C. sinicum* spec. nov. and *C. glaucoma* share a number of features such as small body size, ellipsoidal body shape and two pairs of kinetosomes in the scutica, the former can be separated from the latter by the following combination of characters: the number of macronuclei (two vs. one in *C. glaucoma*); the number of somatic kineties (invariably 11 vs. 8–10 in *C. glaucoma*); the length of the buccal field relative to the body length (45–50% vs. 55–65% in *C. glaucoma*) and the relative lengths of membranelles 1 and 2 (M2 and M1about equal length vs. M2 longer than M1 in *C. glaucoma*) (Song & Wilbert, 2002; Song & Wei, 1998; Fig. 3a–d, h–i).

Cyclidium sinicum spec. nov. can be clearly distinguished from *C. varibonneti* Song, 2000 by the length of the buccal field relative to the body length (45–50% vs. 75% in *C. varibonneti*), the arrangement of kinetids in the somatic kineties (each kinety composed of monokinetids only vs. each kinety composed of dikinetids in anterior half of body and monokinetids in posterior half in *C. varibonneti*), and habitat (freshwater vs. marine in *C. varibonneti*) (Song, 2000; Fig. 3h–i, k, l).

Cyclidium sinicum spec. nov. can be distinguished from *C. bonneti* Grolière, 1980 by the number of somatic kineties (invariably 11 vs. 14–16 in *C. bonneti*) and the length of the buccal field relative to the body length (45-50% vs. >60% in *C. bonneti*) (Grolière, 1980).

Phylogenetic analyses. The topologies of the SSU rDNA trees constructed using BI and ML analyses were almost identical, therefore only the ML tree is presented here with support values from both

algorithms (Fig. 4). The phylogenetic relationships of pleuronematids have previously been examined using SSU rDNA, ITS1-5.8SITS2, and LSU rDNA gene sequences (Gao *et al.*, 2014; Pan H. *et al.*, 2015). The present study reveals similar relationships among families within the order Pleuronematida: i.e., Pleuronematidae, Histiobalantiidae, Eurystomateliidae and Ctedoctematidae are all monophyletic, whereas Cyclidiidae is non-monophyletic because several of its members group with thigmotrichids (represented by *Ancistrum* spp. and *Boveria subcylindrica*). These include *Cyclidium marinum* and *C. varibonneti* which cluster with full support with the clade comprising *Protocyclidium citrullus* and the thigmotrichids *Ancistrum* sp., *A. crassum* and *Boveria subcylindrica*. With the addition of one new sequence, *Cyclidium* is still non-monophyletic with other pleuronematids and thigmotrichids grouped within it. *Cyclidium sinicum* spec. nov. and *C. glaucoma* form a fully supported clade that is sister to the clade comprising *C. marinum* and *C. varibonneti* with full support. This finding supports the placement of the new species in the genus *Cyclidium*.

Cyclidium is a well-known genus to which many forms have been assigned (Agamaliev, 1983; Alekperov, 2005; Borror, 1972; Didier & Wilbert, 1981; Grolière, 1980; Song *et al.*, 2003; Song & Wei, 1998; Song & Wilbert, 2002). Morphological data based on observations of specimens both in vivo and after silver staining are, however, available for relatively few species so many might have been misidentified. Moreover, with the application of molecular techniques in taxonomy, further descriptions and comparisons based on combinations of morphological and molecular data of *Cyclidium* spp. are needed. Hence, an improved diagnosis of the genus *Cyclidium* is supplied here, based both on previous studies and the present study.

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Improved diagnosis of genus *Cyclidium*

Body outline usually oval or elliptical, truncated to form an apical plate, ventral side concave, dorsal side convex; single caudal cilium; contractile vacuole posterior terminal or sub-terminal; membranelles usually non-separated; paroral membrane “L”-shaped, with the anterior end terminating at or before anterior end of M1; somatic kineties longitudinally arranged and continuous.

ACKNOWLEDGEMENTS

This work was supported by the Natural Science Foundation of China (project number: 31501844).
We thank Dr. Xinglong Cai, a PHD student of the Harbin Normal University for sample collection.

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Fig. 1. Map and sampling site. (a) Map showing collecting sites. (b–d) Three photographs showing the same farmland pond in Harbin, Heilongjiang province, northeastern China (44° 87’ 14.7’’ N; 127° 09’ 12.0’’ E).

Fig. 2. Morphology and infraciliature of *Cylidium sinicum* spec. nov. from life (a, d, h–o) and after silver nitrate- (f) and protargol-staining (b, c, e, g, p–r). (a, h) Ventral views of a representative individual, arrowhead in (a) marks caudal cilium and in (h) depicts paroral membrane, arrow in (a) marks paroral membrane, in (h) shows caudal cilium. (b, c) Infraciliature in ventral (b) and dorsal view (c) of the holotype specimen. (d) Lateral view, to show contractile vacuole. (e) Different shapes of macronuclei, also showing nucleoli. (f) Detail of argyrome. (g, r) Oral ciliature of the holotype specimen. (i–o) Different individuals, arrowheads in (l, n) mark the apical plate, in (m) shows caudal cilium and in (o) exhibit somatic cilia, arrow in (n) marks contractile vacuole, in (m) shows paroral membrane. (p, q) Anterior parts, to show macronuclei (arrowheads in p) and micronucleus. CV, contractile vacuole; M1–3, membranelles 1, 2 and 3; Ma, macronucleus; Mi, micronucleus; PM, paroral membrane; Sc, scutica; SK1, somatic kinety 1. Bar, 8 μm (g, p, q), 10 μm (a–d, h–o).

Fig. 3. Various *Cyclidium* species *in vivo* (h, k, n) and after silver nitrate- (a, b, e, g) and protargol staining (c, d, f, i, j, l, m). (a–d) *Cyclidium glaucoma* Müller, 1786 (a, b from Berger & Thompson, 1960; c, d from Song & Wilbert, 2002). (e) *C. plouneouri* Dragesco, 1963 (from Dragesco, 1963). (f) *C. plouneouri* sensu Wilbert, 1986 (from Wilbert, 1986). (g) *C. borrori* Small & Lynn, 1985 (from Small & Lynn, 1985). (h–j) *C. sinicum* spec. nov. (present work). (k, l) *C. varibonneti* Song, 2000 (from

Song, 2000). (m, n) *C. citrullus* Cohn, 1865 (from Song & Wilbert, 2002). Bar, 5μm (d), 10 μm (a–c, e, h–n), 20 μm (f, g).

Fig. 4. Phylogenetic tree inferred from SSU rDNA sequences, showing the position of *Cyclidium sinicum* spec. nov. (in bold). Numbers at nodes represent the bootstrap values of ML out of 1,000 replicates and the posterior probability of BI. Fully supported (100%/1.00) branches are marked with solid circles. The scale bar corresponds to five substitutions per 100 nucleotide positions.

Table 1. Morphometric data based on silver staining specimens of *Cyclidium sinicum* spec. nov.

Character	Min	Max	Mean	M	SD	CV	n
Body, length	21	32	28.3	27	5.7	21.4	15
Body, width	12	16	13.2	13	1.2	9.1	15
Buccal field, length	10	15	12.2	12	1.1	9.1	13
Buccal field, width	3	5	3.6	4	0.3	7.5	13
Somatic kineties, number	11	11	11	11	0	0	13
Macronucleus, number	2	2	2	2	0	0	13
Basal bodies in somatic kinety 1, number	11	12	11.4	11	0.4	3.8	14
Basal bodies in somatic kinety n, number	12	13	12.6	13	0.5	4.6	14

All measurements in μm. Abbreviations: CV, coefficient of variation (%); M, Median; Max, maximum; Mean, arithmetic mean; Min, minimum; n, number of specimens; SD, standard deviation.

Figure 1

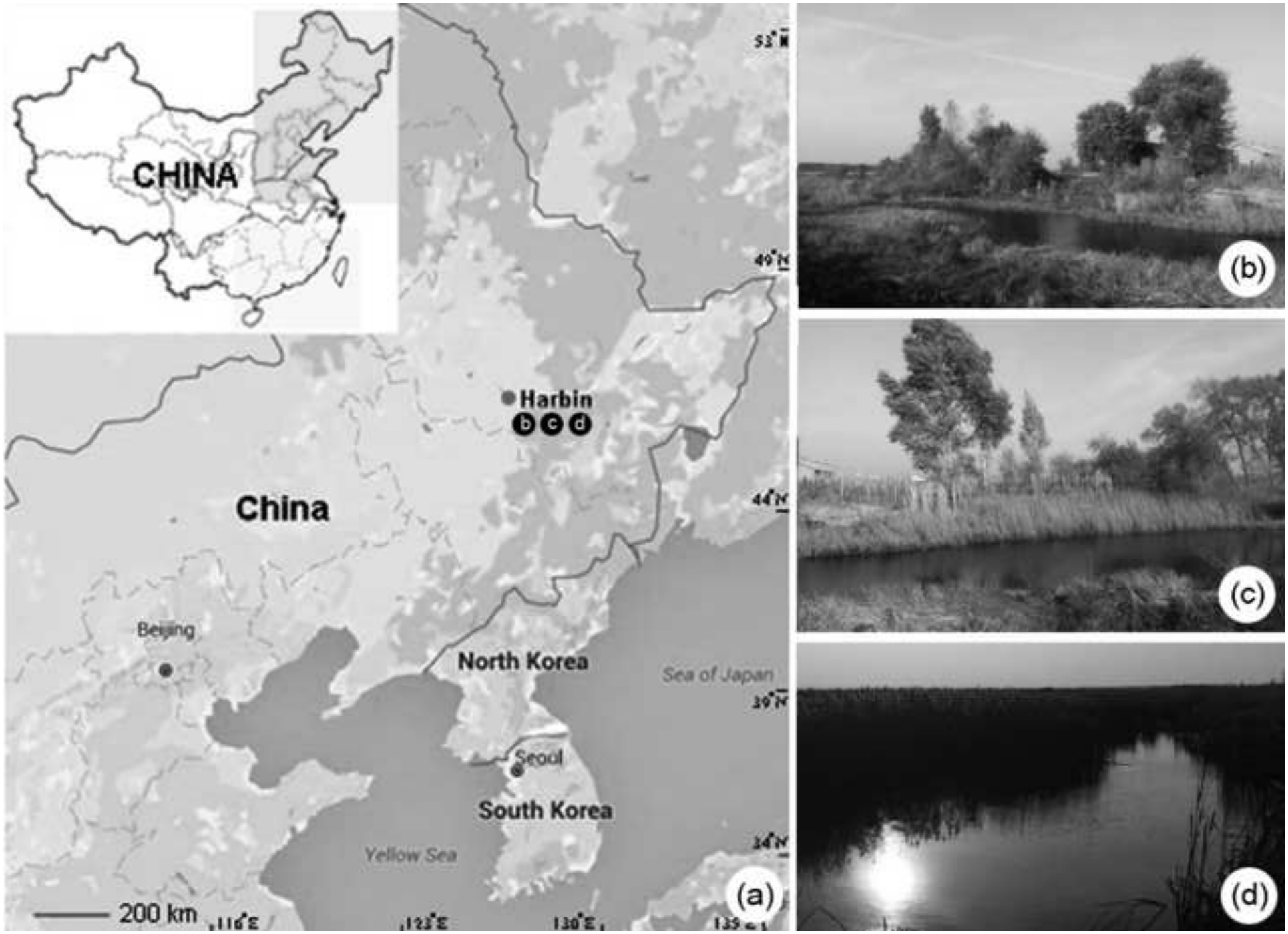

Figure 2

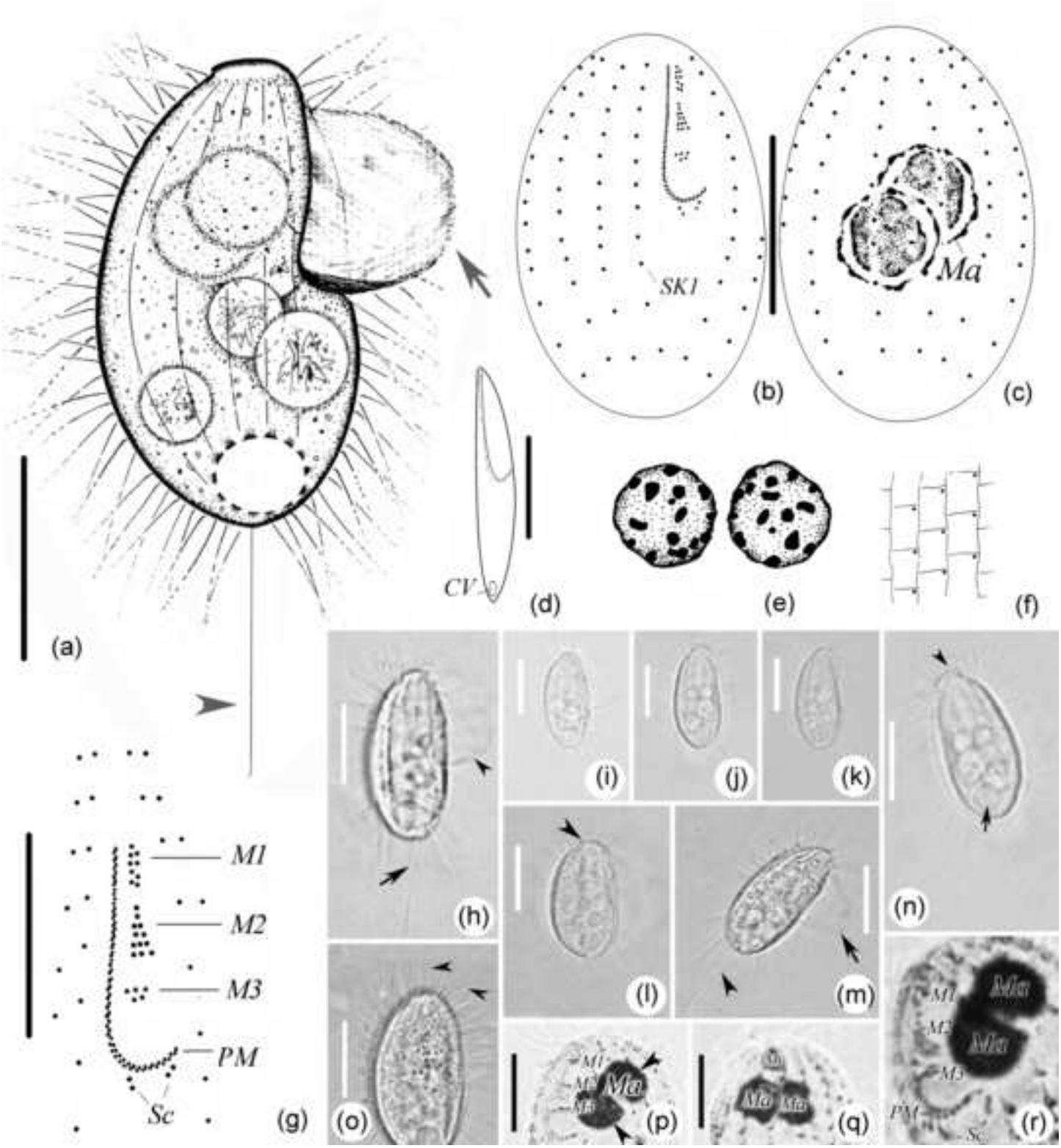

Figure 3

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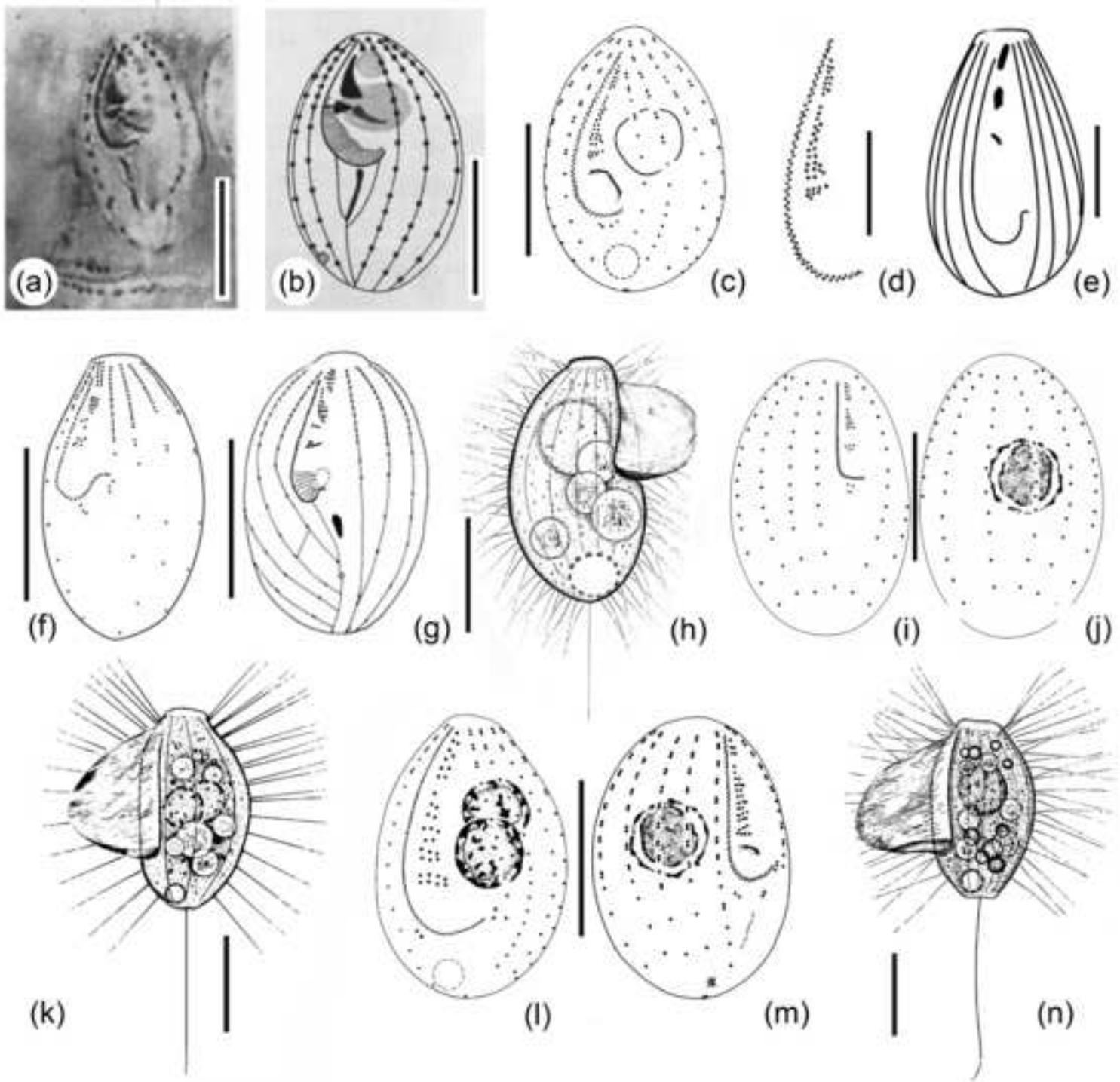

Figure 4