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Morphology and SSU rRNA Gene Sequences of Three Marine Ciliates from Yellow Sea, China, Including One New Species, *Uronema heteromarinum* nov. spec. (Ciliophora, Scuticociliatida)

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Summary. The morphology, infraciliature, and silverline system of three marine scuticociliates, *Uronema marinum* Dujardin, 1841, *U. heteromarinum* nov. spec. and *Pleuronema setigerum* Calkins, 1902, isolated from coastal waters off Qingdao, China, were investigated using living observation and silver impregnation methods. Due to the great confusion in the species definition of the well-known species *U. marinum*, we have documented a detailed discussion/comparison and believe that most of the confusion is due to the fact that at least 2 closely-related sibling morphotypes exist which are often not recognized. Based on the data available, *U. marinum* is strictly defined as follows: marine *Uronema* ca. 30 × 10 μm in size, with truncated apical frontal plate and smooth pellicle, extrusomes inconspicuous, cytostome located equatorially, 12–14 somatic kineties and one contractile vacuole pore near posterior end of kinety 2. *Uronema heteromarinum* nov. spec. resembles *U. marinum* but can be distinguished morphologically by its notched pellicle with conspicuous extrusomes and reticulate ridges, the 15–16 somatic kineties, widely separated membranelle 1 and membranelle 2, as well as the subequatorially positioned cytostome. Based on the Qingdao population, an improved diagnosis for the poorly known *Pleuronema setigerum* is: marine slender oval-shaped form, *in vivo* about 40–50 × 15–20 μm; 3–5 preoral kineties and 14–22 somatic kineties; membranelle 1 and 3 three-rowed, and posterior end of M2a ring-like. The small subunit (SSU) rRNA gene for all three organisms were sequenced and analyzed with standard methods.

Key words: Ciliate, Infraciliature, Oligohymenophorea, *Pleuronema*, SSU rDNA sequence, *Uronema*.

INTRODUCTION

Scuticociliated ciliates are ubiquitous in various habitats worldwide. However, owing to a small body size and great similarity in living aspects, the taxonomy of this group of organisms still remains difficult and confused despite of some interesting work (Borror 1963, Thompson 1964, Dragesco 1968, Grolière and Detcheva 1974, Dragesco and Dragesco-Kernéis 1986, Coppellotti 1990, Foissner *et al*. 2009). Furthermore, recent studies of our group demonstrated that morphospecies diversity of scuticociliates is much higher than expected and new forms are still awaiting discovery (Song 1995, 2000; Song *et al*. 2002; Wang *et al*. 2008a, b, 2009a, b). Even many "known" species are not adequately investigated with regards to current taxonomic criteria: some

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of them are poorly defined, or lack the type material, and need to be redescribed in order to evaluate inter-populational variation and to avoid misidentification. Moreover, with the application of molecular techniques in taxonomy, species need to be compared not only at the morphological but also at the molecular level (Chen *et al*. 2008, Li *et al*. 2008).

As part of a faunistic study on marine ciliates in northern China seas, this study presents the morphology and phylogeny of three scuticociliate species.

MATERIALS AND METHODS

Sample collection and identification: *Uronema marinum* was sampled from seawater along the coast of Yellow Sea at Qingdao (36°18′ N, 120°43′ E), China in 2006. After isolation, it was maintained in the laboratory as pure culture in petri dishes with a water salinity of 29‰, at a temperature of ca. 13°C.

Uronema heteromarinum was isolated from a similar occasion on August 29, 2008. The water temperature was ca. 25°C and salinity was 30‰.

Pleuronema setigerum was collected using the PFU method from seawater off a port at Qingdao on October 14, 2008 . The water temperature was about 16°C and salinity was about 29‰.

Observations on living cells were carried out under differential interference contrast microscopy. Protargol (Wilbert 1975) and Chatton-Lwoff (Song and Wilbert 1995) staining methods were used to reveal infraciliature and silverline system, respectively. Drawings of impregnated specimens were conducted with the help of a camera lucida; measurements were performed at 100–1250 × magnification; the taxonomy and terminology are mainly according to Lynn (2008).

SSU rRNA gene sequence and Phylogenetic analyses: Genomic DNA extraction, PCR amplification, and SSU rRNA gene cloning and sequencing were performed according to the method described by Yi *et al*. (2009). All new SSU rDNA sequences were deposited at GenBank database with the following accession numbers: *Uronema marinum* (1,758bp, GQ465466), *Uronema heteromarinum* (1,759bp, FJ870100), *Pleuronema setigerum* (1,754bp, FJ848874).

The new SSU rDNA sequences together with other 43 sequences download from GenBank database were aligned using Clustal W implemented in Bioedit 7.0 (Hall 1999) and refined by removing ambiguous gaps at both termini of the alignment as well as highly variable regions. The program MrModeltest v.2.0 (Nylander 2004) selected the GTR + I (= 0.3634) + G (= 0.5033) as the best model of substitution, based on the AIC criterion. Using these parameter values, a maximum likelihood (ML) tree was constructed with PhyML V2.4.4 (Guindon and Gascuel 2003). The reliability of internal branches was assessed using a nonparametric bootstrap method with 1,000 replicates. Bayesian (BI) analysis was conducted with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the Markov chain Monte Carlo algorithm. The program was run for 1,000,000 generations with a sample frequency of 100 and a burn-in of 2,500. Maximum parsimony (MP) tree was obtained via random addition and swapped using the tree-bisection-reconnection (TBR) algorithm. Gaps were treated as missing data. MP analysis was performed with the software package PAUP* 4.0b10 (Swofford 2002), and the support for the internal branches was estimated using the bootstrap method with 1,000 replicates.

RESULTS AND DISCUSSION

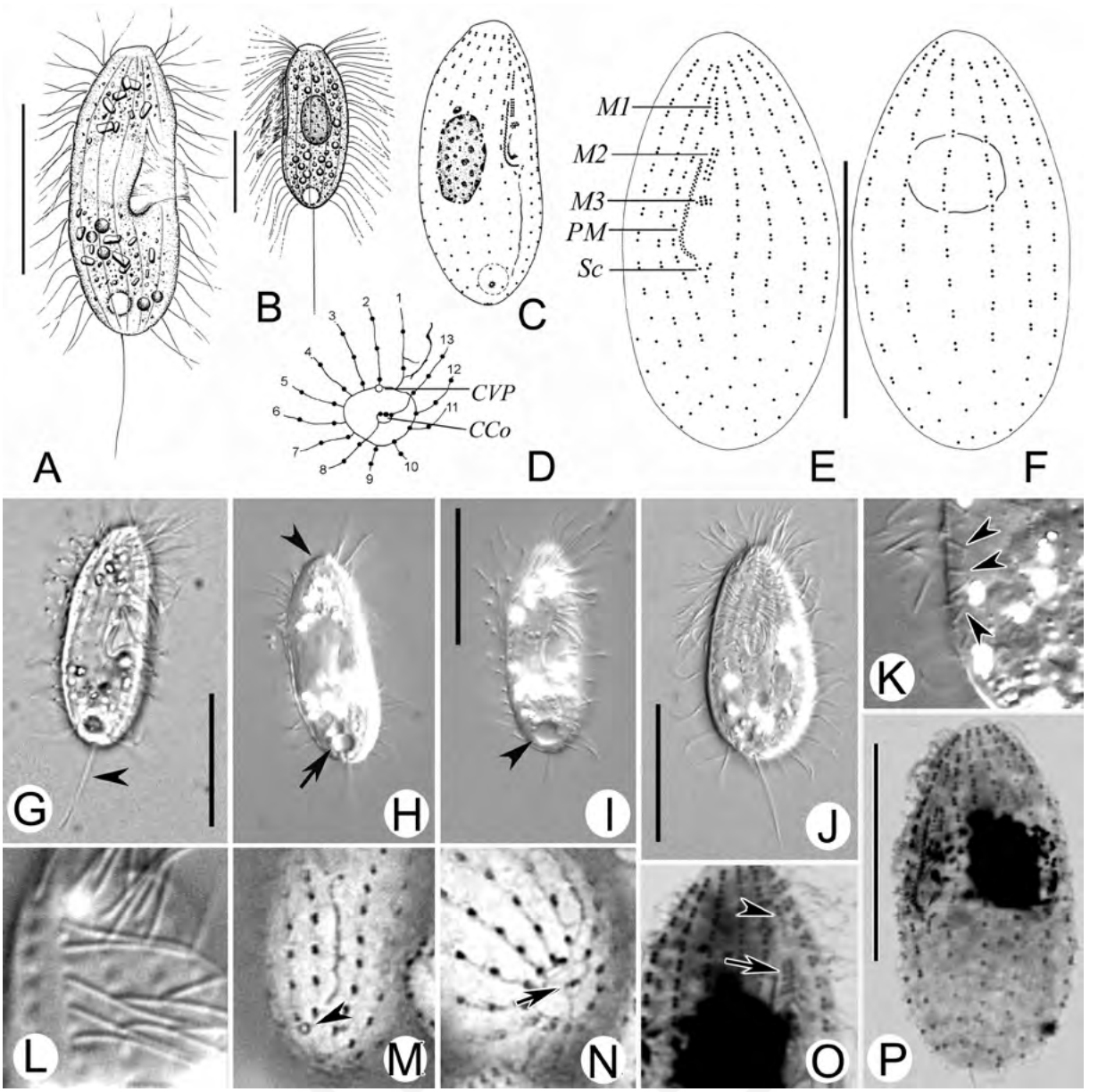
Uronema marinum Dujardin, 1841 (Figs 1, 2, 8; Tables 1, 2, 3)

Uronema marinum is a well-known species. Since it was first reported, many forms have been redescribed under this name by many researchers (Kahl 1931; Parducz 1939; Borror 1963; Czapik 1964; Jankowski 1964; Thompson 1964, 1972; Dragesco and Dragesco-Kernéis 1986; Coppellotti 1990; Song and Packroff 1997). However, among these works, the importance of the living observation was often ignored and species identification depended exclusively on stained specimens. Therefore, some forms might have been misidentified (details see below). As a result, variability exists in some key morphological features (e.g. cell size and number of somatic kineties) for this species. In order to accurately outline this species, we have collected more than ten isolates from various locations in China during the past 20 years. After careful investigation, we are sure that these isolates are "true" *U. marinum* because they agree well with the original population in living features, and the morphological characters (e.g. the number of somatic kineties) are very constant among these isolates.

Since details of living features and the buccal apparatus are not included in the original species diagnosis, we provide an amended diagnosis based on current observations on the Qingdao isolates.

Improved diagnosis of *Uronema marinum* s. str.: Marine *Uronema* with truncated apical frontal plate and smooth pellicle, body size *in vivo* ca. 30 × 10 μm; extrusomes inconspicuous; cytostome equatorially located, 12–14 somatic kineties, single contractile vacuole caudally positioned with pore near posterior end of somatic kinety 2.

Redescription based on Qingdao population: *In vivo* 25–35 × 7–13 μm; usually elongated and cylindrical, with ventral side straight to slightly concave while dorsal side more or less convex; anteriorly with distinct, almost truncated apical frontal plate; posterior end rounded (Figs 1A, B, G, H, I, J). Cilia densely arranged, 5 μm long; single caudal cilium ca. 13 μm in



Figs 1A–P. *Uronema marinum* from life (A, B, G–L), after protargol (C, E, F, O, P) and silver nitrate (D, M, N) impregnations. **A** – lateral-ventral view of typical cell; **B** – lateral view (from Song and Packroff 1997); **C** – lateral view of specimen (from Song and Packroff 1997); **D** – caudal view to show silverline system; **E**, **F** – ventral and dorsal view of the same specimen; **G** – right-ventral view of a typical cell, arrowhead refers to caudal cilium; **H**, **I**, **J** – to show different body shapes, arrow in (H) and arrowhead in (I) point to contractile vacuole, arrowhead in (H) marks apical plate; **K** – detailed view of cortex, arrowheads show extrusomes; **L** – detail view of cortex to show smooth pellicle; **M** – part of silverline system, arrowhead points to contractile vacuole pore; **N** – caudal view of a specimen, arrow shows caudal cilium complex; **O** – detailed view of oral apparatus, arrowhead marks membranelle 1, arrow refers to membranelle 2; **P** – left-ventral view to show infraciliature. M1–3 – membranelles 1–3, PM – paroral membrane, Sc – scutica, Cco – caudal cilium complex, CVP – contractile vacuole pore. Scale bars: 20 μm.

length. Buccal field occupying almost half of the body length, cytostome located at mid-body (Figs 1A, C, E, G). Pellicle thin, basically smooth with inconspicuous extrusomes underneath, which are short-bar-shaped, about 2 μm long (often overlooked even under 1250 × magnification) (Figs 1K, L).

Cytoplasm colorless to grayish, transparent, often filled with few to several 1–2 μm brick or dumb-bell

shaped refractive crystal inclusions, which are often concentrated in the anterior and posterior portions of the cell (Figs 1A, H). Single macronucleus oval to spherical, centrally located (Figs 1C, P). Contractile vacuole moderately large, 5 μm in diameter, caudally positioned (Figs 1A, B, H, I). Swimming behavior generally fast, without peculiarities. But mostly, when not disturbed, quiet on the bottom.

Twelve to fourteen longitudinally arranged somatic kineties, of which SK1 and SKn have about 20 and 23 basal bodies respectively (Figs 1C, E, F, P). Anteriorly one small, rounded cilia-free apical area is formed. Buccal apparatus consisting of 3 membranelles (M1–3) and paroral membrane (PM): membranelle 1 (M1) one row with 3–6 basal bodies; membranelle 2 (M2) longer than M1, constantly containing 2 rows of kineties, about 5–6 basal bodies each; membranelle 3 smaller, close to M2. PM composed of two rows of basal bodies in typical 'zig-zag' pattern, extending anteriorly to about middle of M2 (Figs 1C, E, O, P). Scutica (Sc) consisting of 3 pairs of basal bodies (Figs 1C, E).

Silverline system as shown in Figs 1D, M and N: Contractile vacuole pore at posterior end of somatic kinety 2; line from somatic kinety n (left-most one to buccal field) extending posteriorly through caudal cilium complex (CCo) and connecting dorsally with kinety 9. Cytopyge between SK1 and SKn, as narrow, irregularly shaped structure.

Morphological comparison and remark

We identified the Qingdao population based on the following features: oval body shape with truncated apical plate, smooth pellicles, number of ciliary rows, position of contractile vacuole pore and habitat. It agrees well with the original population by Dujardin (1841) in main living appearance, thus both can be regarded as conspecific.

Extensive interspecific comparison can be found in the section for its other congener.

Parauronema longum is another relative of *Uronema marinum* in the family Uronematidae. Both species resemble each other in terms of general morphology and infraciliature, but the former differs from the latter in body size (30–50 μm long vs. 25–35 μm long), in the numbers of kineties in membranelle 1 (2 vs. 1) and in the somatic ciliary rows (18–21 vs. 12–14) (Song 1995).

Kahl (1931) first detailedly redescribed the living morphology of *U. marinum* and stated that the pellicle was not notched. But he very likely studied a species-complex, of three or more species, because his three drawings of *U. marinum* are quite different. From his illustrations (Figs 2A–C), the cell shape of Fig. 2C is pear-like while Fig. 2B has a larger ratio of length to width; moreover, both specimens apparently have distinct extrusomes. Only Fig. 2A is *U. marinum* and very similar to Dujardin's and our populations in its ovoid shape and the inconspicuous extrusomes.

Parducz (1939) described a population of *U. marinum* and observed many details of the silverline system (Fig. 2D). Unfortunately, he did not supply any essential traits of the buccal apparatus. According to the limited data, his isolate is possibly *U. marinum*.

Borror (1963) first gave a simple illustration of infraciliature of *U. marinum*. But his samples were collected from 17 stations and his living observations lack detail. His population might be a mix of different species, which caused the fairly high variation in the number of somatic kineties (12–15). We postulate that the smaller forms might be the same species as our isolates, and that the larger forms with 15 ciliary rows are likely to be a different species.

Thompson (1964) studied a small marine ciliate and identified it as *U. marinum* (Fig. 2F), based on his silver impregnated specimens. He supplied details of its infraciliature, but living observation data is still lacking, and the range in the number of somatic kineties is too wide (13–16). Most likely, his form is a mix of at least two species, including *U. marinum*.

Jankowski (1964) found a small scuticociliate in fresh water, which he called *Uronema marinum* (Fig. 2G). He reported its life cycle and silverline system, but gave no details of live morphology and the buccal apparatus. Therefore, this species identification still remains uncertain.

Czapik (1964) reported a form named *Uronema marium*, whose size, shape and number of ciliary rows are similar to our populations (30–40 μm long vs. 25–35 μm, 12–15 vs. 12–14 somatic kineties). It may be a population of *U. marinum*, although its buccal apparatus has not been described.

Dragesco and Dragesco-Kernéis (1986) redescribed *U. marinum* in their book (Figs 2H, I), although still with an inadequate description of living features. Their isolate is possibly a mix of different species.

Coppellotti collected a ciliate in 1990, which he identified as *Uronema marinum* (Fig. 2E). He supplied not only infraciliature but also scanning electron micrographs. However, he ignored important living features

Table 1. Morphometric characterization of *Pleuronema setigerum* (upper row), *Uronema heteromarinum* nov. spec. (middle row) and *U. marinum* (lower row). Data according to protargol impregnated specimens. All measurements in µm. CV – coefficient of variation in %, Ma – macronucleus, Max – maximum, Min – minimum, M1–3 – membranelle 1–3, n – number of specimens measured, PK – preoral kineties, Ps – *Pleuronema setigerum*, Sc – scutica, SD – standard deviation, SK – somatic kineties, Uh – *Uronema heteromarinum*, Um – *Uronema marinum*.

Character	Min	Max	Mean	SD	CV	n	Species
Body length	40	55	45.5	17.4	0.4	21	Ps
	30	50	40.8	5.9	0.1	25	Uh
	28	39	34.1	3.2	0.1	25	Um
Body width	16	30	20.9	9.4	0.4	20	Ps
	20	30	23.7	3.1	0.1	25	Uh
	14	20	17.4	1.8	0.1	25	Um
Length of buccal field	31	40	35.9	7.0	0.2	21	Ps
	12	22	16.7	2.2	0.1	25	Uh
	13	16	14.6	0.7	0	25	Um
Width of buccal field	8	18	13.5	6.9	0.5	21	Ps
	–	–	–	–	–	–	Uh
	–	–	–	–	–	–	Um
No. of SK	14	16	14.9	0.6	0	21	Ps
	15	16	15.4	0.5	0	25	Uh
	12	14	13.0	0.3	0	25	Um
No. of PK	3	5	4.1	0.2	0	21	Ps
	–	–	–	–	–	–	Uh
	–	–	–	–	–	–	Um
No. of basal bodies in SK1*	–	–	–	–	–	–	Ps
	18	24	20	1.4	0.1	25	Uh
	17	21	19.4	1.1	0.1	25	Um
No. of basal bodies in SKn*	–	–	–	–	–	–	Ps
	20	25	22.8	1.4	0.1	25	Uh
	15	21	18.8	1.5	0.1	25	Um
No. of basal bodies in M1	–	–	–	–	–	–	Ps
	4	7	5.4	0.8	0.2	25	Uh
	3	6	5.0	0.9	0.2	25	Um
No. of kinety rows in M3	3	3	3	0	0	21	Ps
	–	–	–	–	–	–	Uh
	–	–	–	–	–	–	Um
No. of Ma	1	12	2.0	3.0	1.5	21	Ps
	1	1	1	0	0	25	Uh
	1	1	1	0	0	25	Um
No. of basal bodies in Sc	–	–	–	–	–	–	Ps
	3	3	3	0	0	25	Uh
	3	3	3	0	0	25	Um

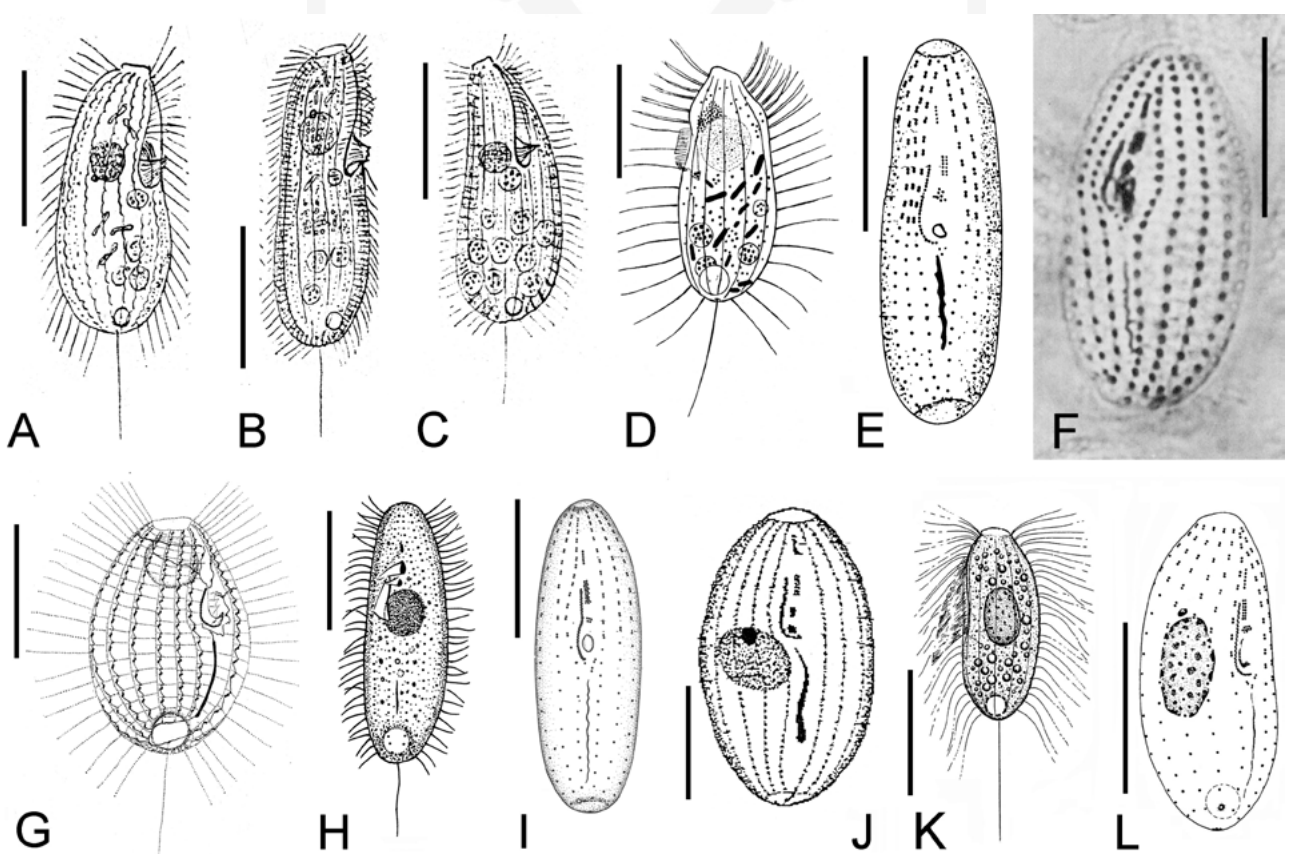
* Basal body pairs counted as single ones.

Table 2. List of species under the name "*Uronema marinum*". Only the "true" *Uronema marinum* marked in **bold**.

Species name	Remark
U. marinum sensu Kahl, 1931	Very likely a species–complex, including *U. marinum*
***U. marinum* sensu Parducz, 1931**	Possibly *U. marinum*
U. marinum sensu Borror, 1963	Species-complex, including *U. marinum*
U. marinum sensu Thompson, 1964	Species-complex, including *U. marinum*
U. marinum sensu Jankowski, 1964	An insufficiently described form, possibly an unknown one
***U. marinum* sensu Czapik, 1964**	Possibly *U. marinum*
U. marinum sensu Dragesco and Dragesco-Kernéis, 1986	Species-complex, including *U. marinum*
U. marinum sensu Coppellotti, 1990	Species-complex, including *U. marinum*
U. marinum sensu Song and Packroff, 1997	Likely two different species, the smaller form is *U. marinum*
U. marinum sensu Alekperov and Asadullayeva, 1997	An insufficiently described form, likely an undefined *U. marinum* sensu Thompson, 1964

Table 3. Morphological and molecular comparison of *Uronema heteromarinum* nov. spec. with *U. marinum*.

Characters	*Uronema heteromarinum*	*Uronema marinum*
Pellicle	Strongly notched with reticulate ridges	Generally smooth
Extrusomes	Conspicuous, recognizable *in vivo*	Inconspicuous, invisible *in vivo*
Position of cytostome	Subequatorial located	Equatorial located
Position of membranelles	M1 conspicuously apart from M2	M1 near M2 and M3
Somatic kineties, number	15–16	12–14



(e.g. extrusomes). According to the high degree of variation in size and numbers of somatic kineties, his form might include other species as well.

Very likely, *Uronema marinum* sensu Alekperov and Asadullayeva, 1997 is the same species as *U. marinum* sensu Thompson, 1964 (Fig. 2J).

Song and Packroff (1997) reported two populations of *Uronema marinum* (Figs 2K, L). They noticed that the smaller form has fewer (12–13) somatic kineties than the larger form (15–16). Unfortunately, they did not state live observation of the pellicle and extrusomes. Compared with our population, the larger form may be a different species.

***Uronema heteromarinum* nov. spec. (Figs 3, 4, 8; Tables 1, 3)**

Diagnosis: Medium-sized, cylindrical or kidney-shaped marine *Uronema*, *in vivo* about 25–50 × 10–25 μm with a truncated apical plate; buccal field about 1/2 of body length, with cytostome constantly subequatorial located, pellicle notched with conspicuous reticulate ridges; 15–16 somatic kineties; membranelle 1 apart from other membranelles; contractile vacuole subcaudally positioned near ventral margin with its opening pore at posterior end of somatic kinety 2.

Type location: Coastal waters of Qingdao (36°18′N, 120°43′E), China.

Type specimens: One holotype (registration no. FXP-2008082901-01) and several paratype (registration no. FXP-2008082901-02) slides with protargol-impregnated specimens is deposited in the Laboratory of Protozoology, Ocean University of China, China.

Etymology: The name *heteromarinum* recalls the fact that this species is similar to *Uronema marinum*.

Description: Cell size *in vivo* 28–50 × 10–22 μm; body shape usually elliptical to cylindrical, or kidney-shaped when laterally viewed; ventral side slightly concave in mid-body, while dorsal side convex; anterior end flat, with a conspicuous apical plate, dorsal area broadly rounded (Figs 3A–C, 4A–D). Buccal field about half of body length, with cytostome slightly posterior to mid-body level (Fig. 3A). Pellicle thick and strongly notched in outline with conspicuous reticulate ridges (Figs 3G, 4I, J). Extrusomes bar-shaped, about 2 μm long, and closely arranged beneath pellicle (Figs 3G, 4F).

Cytoplasm colorless to grayish, containing several food vacuole and bar- or dumbbell-like crystals, which are usually 2 μm long (Figs 3B, C, 4F). Macronucleus large and rounded, mostly located at anterior region (Figs 3A, D, E). Contractile vacuole about 5 μm in diameter, caudal positioned; contracting interval very short, less than five seconds.

Somatic cilia about 8 μm long, densely arranged; single caudal cilium ca. 10–15 μm long. Oral cilia within buccal cavity about 6 μm long, usually difficult to be recognized (Fig. 3A). Swimming moderately fast while rotating about main body axis, sometimes crawling on debris, or even quiet on the bottom.

Fifteen to sixteen somatic kineties arranged longitudinally, which usually have dikinetids in anterior 1/3–1/2 of each row and monokinetids posteriorly. Buccal apparatus as shown in Figs 3D, I: membranelle 1 (M1) distinct sub-apically positioned and remote from other membranelles, consisting of ca. 4–7 basal bodies, which are usually arranged in one row (Figs 3D, 4G, H); membranelles 2 and 3 (M2, M3) short, and close to each other; M2 composed of two longitudinal rows of basal bodies; M3 composed of four transverse rows. Paroral membrane of stichodyad type, on right of shallow buccal cavity, extending anteriorly to about middle of M2. Scutica consisting of 3 pairs of basal bodies (Figs 3D, I, arrow).

Silverline system typical for genus, cytopyge (CyP) subterminally as thin argentophilic patch between SK1 and SKn. Contractile vacuole pore (CVP) positioned at end of 2nd somatic kinety (Figs 3I, 4M).

Comparison: Considering the morphology, infraciliature and habitat, four species should be compared with our form: *Uronema marinum* Dujardin, 1841, *U. gallicum* Pérez-Uz and Song, 1995, *U. elegans* Maupas, 1883, and *Uronemella filificum* (Kahl, 1931).

Unlike *U. marinum*, *Uronema heteromarinum* has reticulate ridges on notched pellicle, subequatorially positioned cytostome, more somatic kineties (15–16 vs. 12–14 in *U. marinum*), and its M1 is remote from M2 (Table 2).

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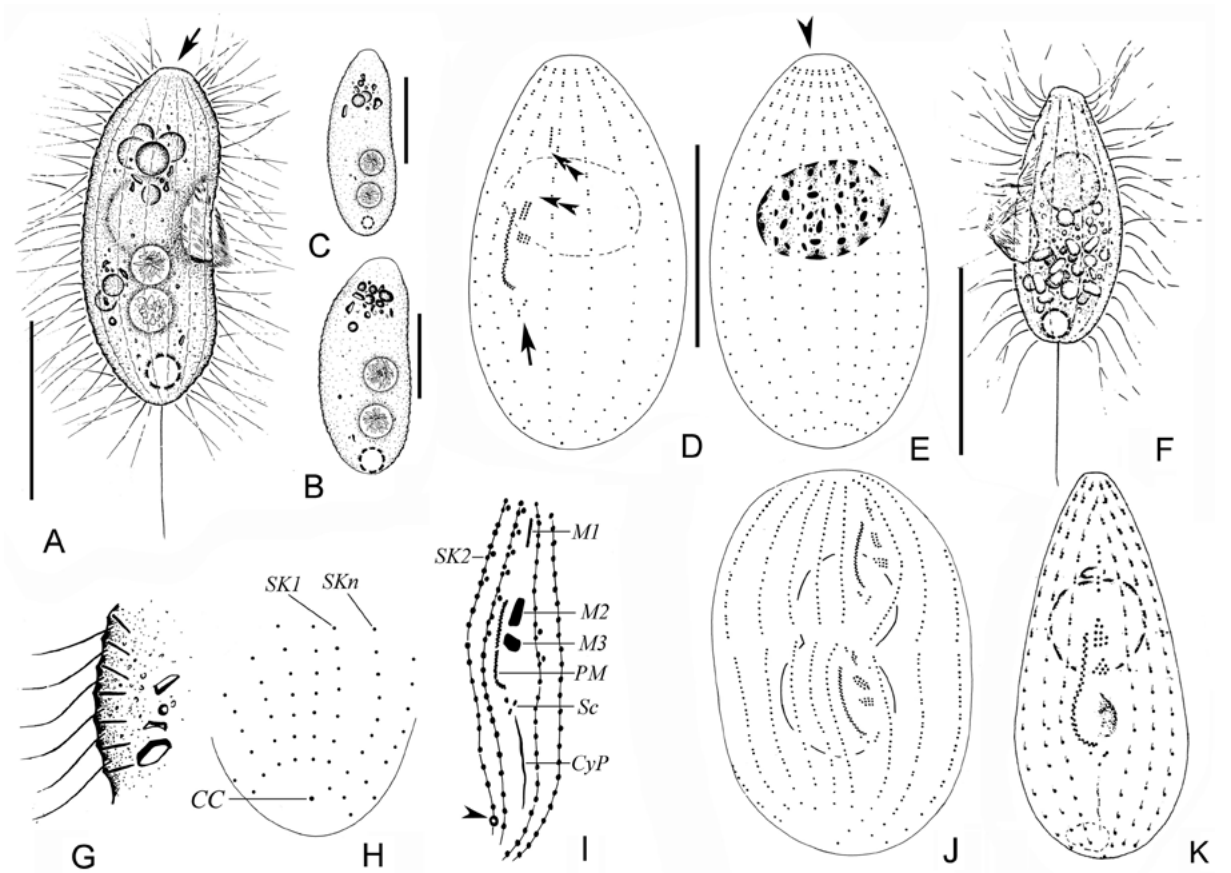
Figs 2A–L. Some Scuticociliates under the name *Uronema marinum*. **A**, **B**, **C** – from Kahl 1931; **D** – from Parducz 1939; **E** – ventral view, infraciliature, from Coppellotti 1990; **F** – photograph after Chatton-Lwoff impregnation, from Thompson 1964; **G** – silverline system, from Jankowski 1964; **H**, **I** – from Dragesco and Dragesco-Kernéis 1986; **J** – infraciliature from Alekperov 1997; **K**, **L** – from Song and Packroff 1997. Scale bars: 20 μm.



Figs 3A-K. *Uronema heteromarinum* nov. spec. (A–E, G–J) and *U. gallicum* (F, K) from life (A–C, F, G), after protargol (D, E, H, J–K) and silver nitrate impregnation (I). **A** – ventral view of a typical cell, arrow points to glabrous apical plate; **B** – large individual; **C** – small individual; **D**, **E** – ventral and dorsal views of the same specimen, arrow in (D) marks scutica, arrowhead in (E) refers to glabrous apical plate; **F** – left view, typical individual, from Pérez-Uz and Song 1995; **G** – part of cortex, to show extrusomes; **H** – caudal view; **I** – structure of oral apparatus, arrowhead shows pore of contractile vacuole; **J** – dividing cell; **K** – ventral view, infraciliature, from Pérez-Uz and Song 1995. M1–3 – membranelles 1–3, PM – paroral membrane, SK1, 2, n – somatic kinety 1, 2, n, Sc – scutica, CC – caudal cilium, CyP – cytopyge. Scale bars: 20 μm.

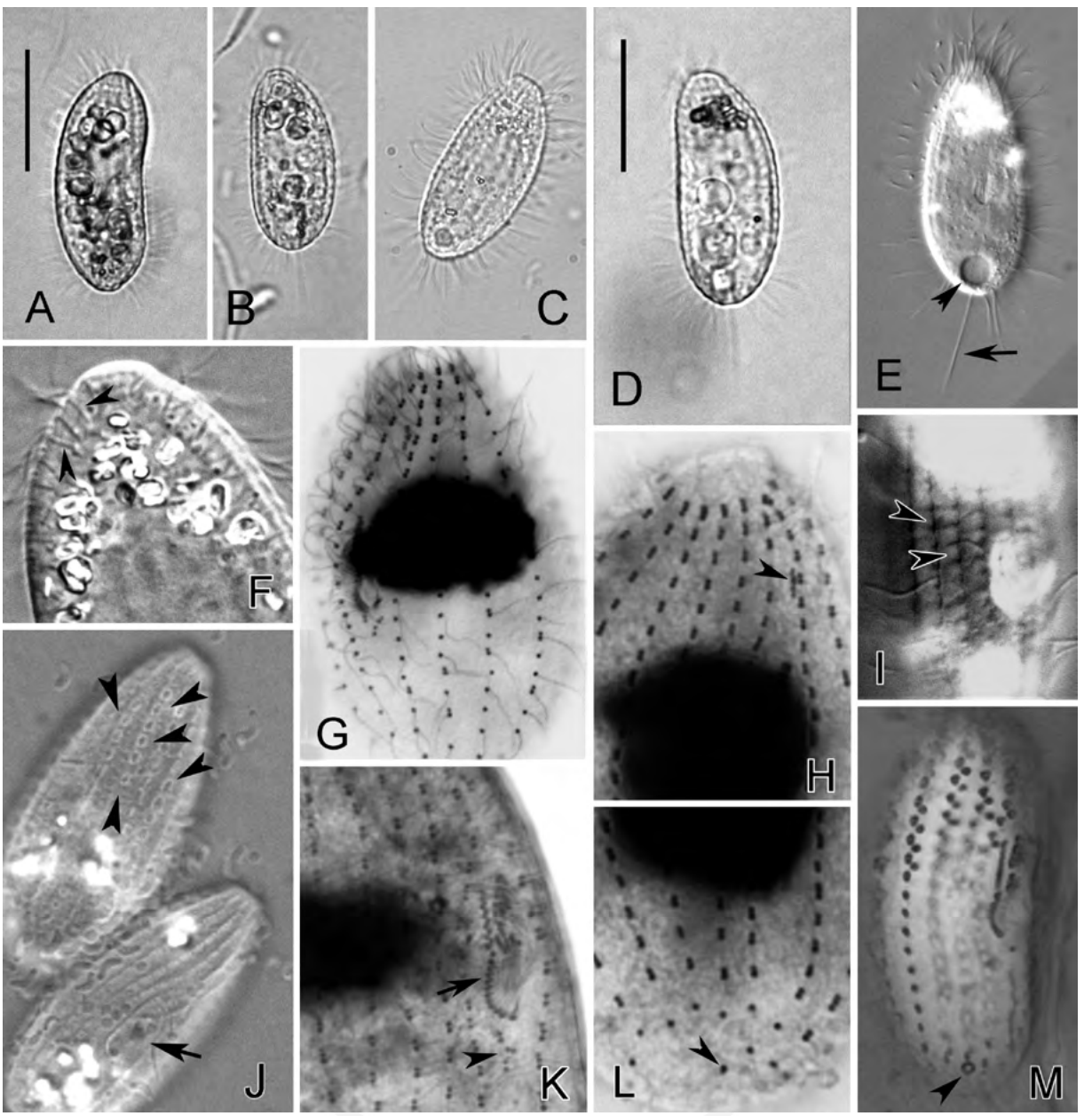
Uronema gallicum differs from the new species *U. heteromarinum* in having widely spaced basal bodies in M1 (vs. close-arranged in the latter), three rows of basal bodies in M2 (vs. 2 rows), and a pointed anterior end (vs. truncate) (Pérez-Uz and Song 1995).

Though *Uronema elegans* is similar to *U. heteromarinum* in its buccal apparatus, the conspicuous extrusomes, and in having distinct reticulate pellicular ridges, it can be distinguished by its fatter body shape (ratio of body length to width 1.5:1 vs. 2.5:1 in the latter) and a higher more somatic kineties (23–26 in the former vs. 15–16 in the latter) (Song *et al*. 2002).

Uronemella filificum can be separated from the new species in cell shape (inverted pear- shaped vs. cylindrical or kidney-shaped), more somatic kineties (16–23 vs. 15–16), larger apical plate and the behavior (thigmotactic vs. non-thigmotactic) (Song *et al*. 2002).

Sequence comparison and phylogeny of the genus *Uronema*

Phylogenetic trees show that the genus *Uronema* is not monophyletic, in which the new isolate *U. heteromarinum* clusters with *U. elegans* in all the parsimony, maximum likelihood, and Bayesian methods with high posterior probability and high bootstrap support (BI/ML/MP, 1.00/100/99). The pair-wise sequence similarities between *U. heteromarinum* and other two forms (*U. elegans* and our population of *U. marinum*) range



Figs 4A–M. *Uronema heteromarinum* nov. spec. *in vivo* (A–F, I, J), after protargol (G, H, K, L) and after Chatton-Lwoff impregnation (M). **A** – lateral view of a typical body; **B–D**, **J** – to show different cell shapes, arrow in (J) refers to cytostome, arrowheads in (J) show the notched pellicle; **E** – ventral view, arrow points to caudal cilia, arrowhead marks contractile vacuole; **F** – anterior part of cell, arrowheads refer to extrusomes; **G** – ventral view, to show infraciliature; **H** – infraciliature of anterior part of cell, arrowhead shows membranelle 1; **I** – detailed view of cortex, arrowheads mark notched pellicle; **K** – detailed view of oral apparatus, arrow points to paroral membranelle, arrowhead refers to scutica; **L** – caudal view of cell, arrowhead shows basal body of caudal cilia; **M** – right-ventral view, arrowhead points to pore of contractile vacuole. Scale bars: 30 μm.

from 89.1% to 93.9%, i.e. the differences range from 6.1% to 10.9% which are considered sufficient for species separation.

Five available SSU rDNA sequences of *Uronema marinum* in GenBank (DQ867072, DQ867073, DQ867074, AY551905, and Z22881), submitted by three different institutes, form a strong supported clade in all the trees and differ in 34, 35, 36, 37, 39 nucleotides from our Qingdao isolate, respectively. Considering that none of them has any relevant morphological

descriptions published while our isolate is from pure culture and identified lying on living observation and modern staining method, we highly suspect all of them are non-*U. marinum* speices.

The Qingdao isolate of *U. marinum* groups with the *Parauronema virginianum* and then followed by *Entodiscus borealis*, although support values for the former relationship are not significant (BI/ML/MP, 0.85/60/79). Morphologically, the genus *Uronema* and the genus *Parauronema* are nearly the same, except in the former M1 one-rowed and in the latter M1 two-rowed. Additionally, *U. marinum* is similar with *P. virginianum* morphologically; the numbers of their somatic kineties are the same and they both have inconspicuous extrusomes (Song *et al*. 2009). Considering these, it is acceptable that *U. marinum* clusters with *P. virginianum*.

Pleuronema setigerum Calkins, 1902 (Figs 5, 6, 7, 8; Table 1)

Since first reported, this species has been redescribed on three occasions (Kahl 1931, Noland 1937, Borror 1963). However, it is still inadequately studied in terms of living observations, as well as infraciliature and silverline system features. Hence, based on the current study, an improved diagnosis and a detailed description are provided.

Improved diagnosis: *In vivo* ca. 30–50 × 15–30 μm in size, slender oval in outline; buccal field occupying four-fifth of body length; about 9–13 prolonged caudal cilia; three to five preoral kineties and 14–22 somatic kineties; membranelle 1 about 20% of the anterior part of membranelles 2 (M2a) in length, consisting of 3 longitudinal rows of basal bodies; posterior end of M2a ring-like; membranelle 3 three-rowed; paroral membrane about four-fifth of cell length, forming a sail-like structure; contractile vacuole subcaudally positioned; one macronucleus; marine habitat.

Description of the Qingdao population: Body shape and size quite constant, *in vivo* 40–50 × 15–20 μm, slender oval to elliptical in outline, widest at midbody; both ends broadly rounded (Figs 5A, 6A–C, F, G, N, O); in lateral view, the ventral side almost flat, while the dorsal side convex (Figs 6F, G, O). Buccal field occupying about 80% of body length and almost half of body width (Fig. 5I). Cilia of paroral membrane prominent, about 35 μm long and forming a typical sail-like structure (Figs 5A, 6A, O). Extrusomes 5 μm long, lying beneath notched pellicle and closely arranged between ciliary rows (Figs 5D, 6E). Cytoplasm colorless to slightly grayish, containing greasily shining globules (mostly 3–4 μm across) and irregularly-shaped crystals (about 5 μm long) (Figs 6D, N). Food vacuole usually in middle of cell. Single spherical macronucleus with many globular nucleoli anterior to cell equator, but ten to twelve spherical macronuclear nodules packed together existing in two of twenty-one specimens (Figs 5B, C, 6J, L). Contractile vacuole about 13 μm in diameter, located subcaudally near dorsal cell margin (Figs 5A, F, 6C, D). Somatic cilia about 13 μm long; 13 prolonged caudal cilia on average, each about 35 μm in length, stretched always in radial manner (Fig. 6H).

Mostly lying motionless on debris with sail-like structure open, somewhat drifting or wobbling, at intervals its oral cilia not stretching for a few seconds (Figs 5F, 6C); when swimming moderately fast, rotating about main body axis and keeping cilia of buccal field closed.

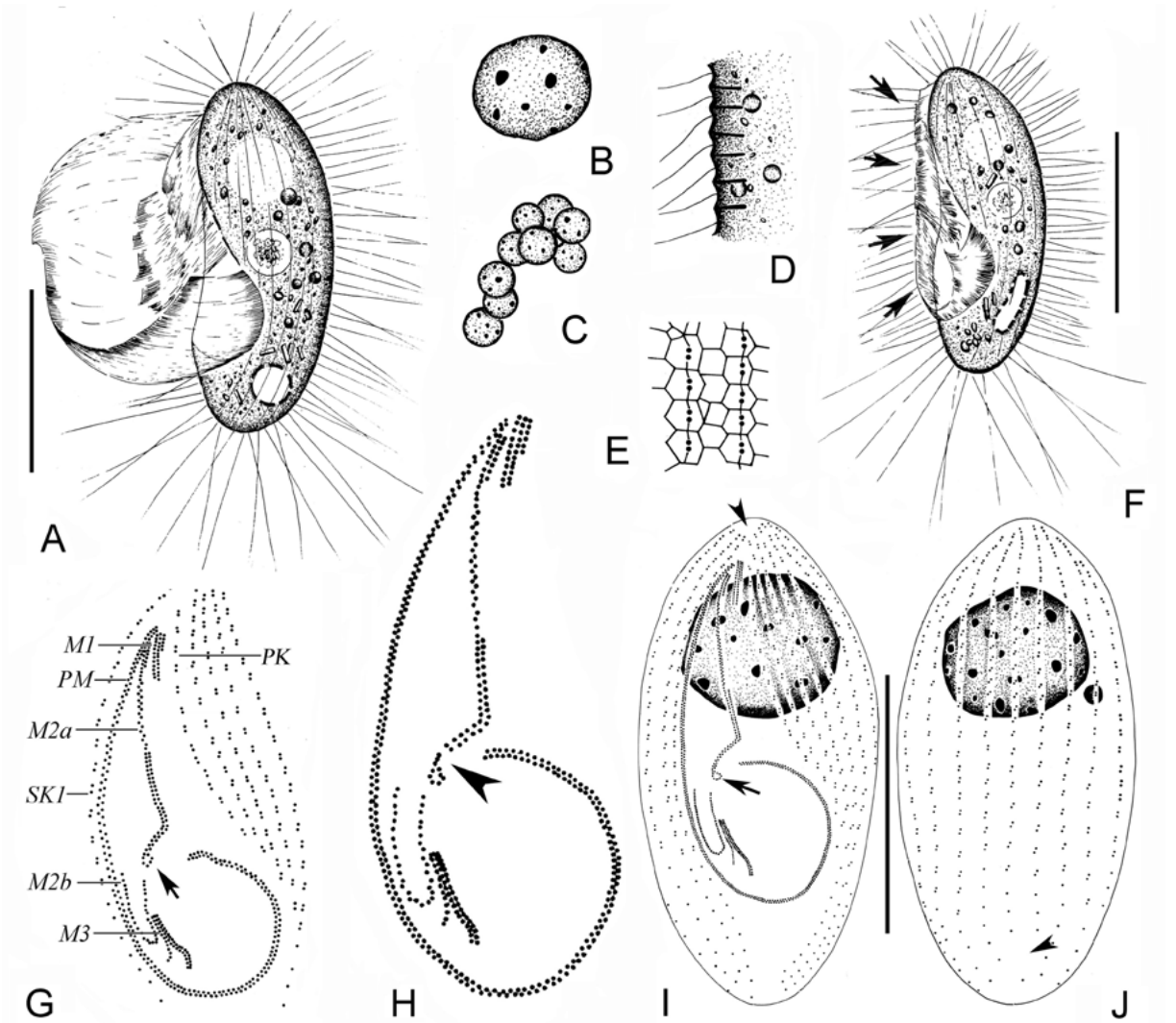
Fourteen to sixteen somatic kineties extending over entire length of the cell, terminating at small glabrous apical plate; each kinety composed of dikinetids in anterior 3/4 of body, while loosely-arranged monokinetids in posterior quarter. Three to five preoral kineties to left of the buccal field (Figs 5I, J, 6K, L).

Oral apparatus typical for genus: membranelle 1 (M1) with one short and two longer rows of basal bodies; M2a mostly two-rowed but with the middle section that is single-rowed in 'zigzag' pattern, and its posterior end characteristically ring-like; in some abnormal cells, a gap existing in posterior region of M2a (Figs 5H, 6P); M2b V-shaped, separated from M2a; M3 three rowed. Paroral membrane prominent, about eighty percent of body length (Figs 5G, I, 6M).

Silverline system with a near-hexagonal honeycomb pattern (Figs 5E, 6I).

Comparison and remarks: *Pleuronema* is a species-rich scuticociliates genus. Up to date, over 20 species have been studied using silver staining techniques (Dragesco 1968; Grolière and Detcheva 1974; Small and Lynn 1985; Song 2000; Wang *et al*. 2008a, b, 2009a). *P. setigerum* was first reported by Calkins in 1902 and then redescribed by Kahl (1931) who cited Calkins's drawing (Fig. 7A). Our population is very similar to the previous ones in its body length (40–50 μm vs. 45–50 μm), and the number of caudal cilia (13 vs. 9–10 in Kahl 1931), thus they are affirmably conspecific.

Noland (1937) reported a form, which Small and Lynn named *Pleuronema nolandi* (Small and Lynn 1985). Borror (1963) described its infraciliatrue but gave only a diagram of the buccal morphology. Small

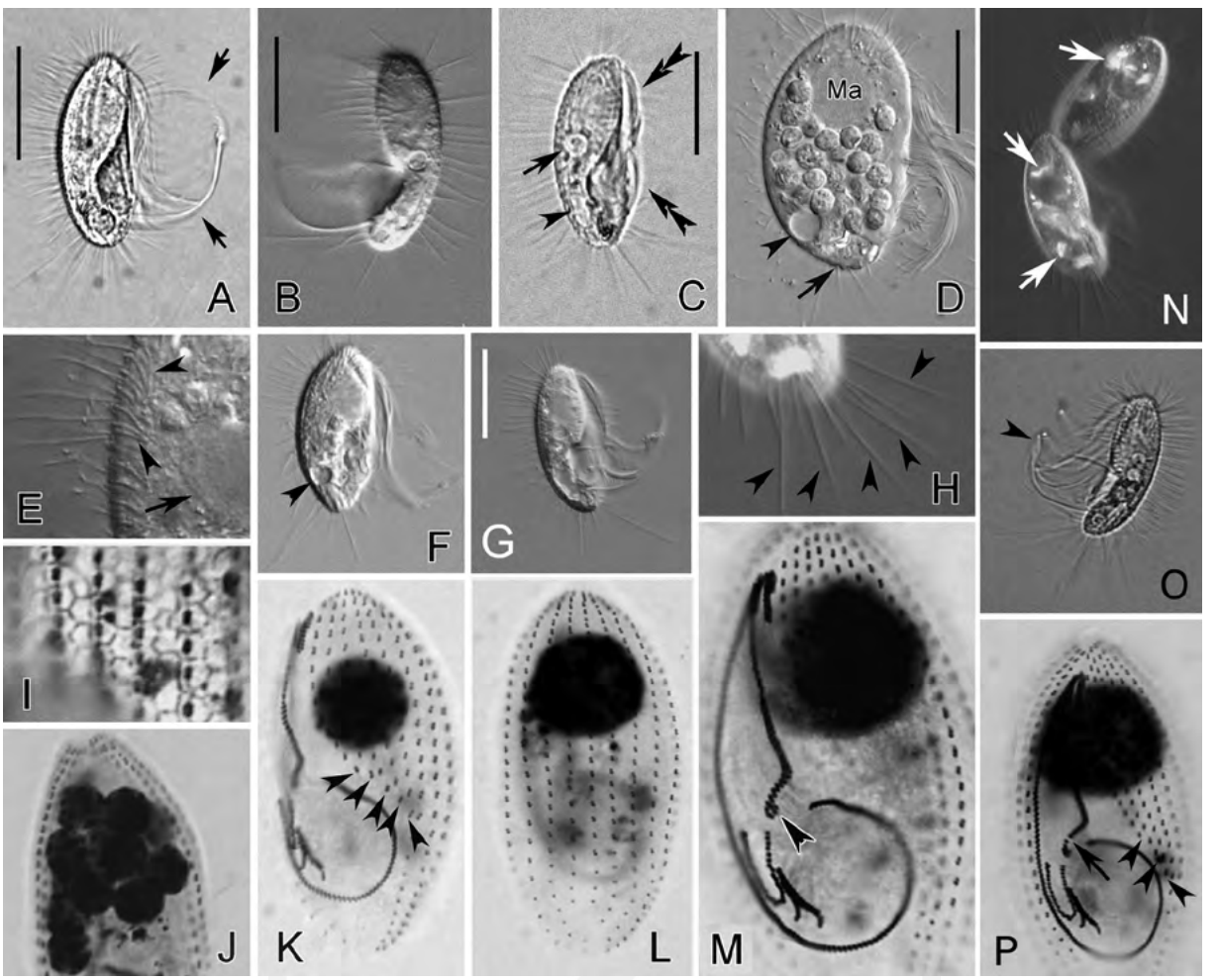


Figs 5A–J. *Pleuronema setigerum* from life (A, D, F), after protargol (B, C, G–J) and silver nitrate impregnation (E). **A** – ventral view of a typical individual; **B**, **C** – different appearance of macronuclear apparatus; **D** – detailed view of cortex to demonstrate the arrangement of extrusomes; **E** – part of silverline system; **F** – ventral view of an oral cilia folded cell, arrows marks folded oral cilia; **G**, **H** – detailed view of oral apparatus, arrow in (G) shows ring-like posterior end of M2a, arrowhead in (H) marks the gap in posterior of M2a; **I**, **J** – ventral and dorsal views of the specimen, showing infraciliature and nuclear apparatus, arrow in (I) points to ring-like posterior end of M2a, arrowhead in (H) refers to glabrous apical plate, arrowhead in (J) marks loose arrangement of basal bodies in cell posterior. M1–3 – membranelles 1–3, M2a – the upper part of membranelle 2, M2b – the lower part of membranelle 2, PM – paroral membrane, SK1 – somatic kinety 1, PK – preoral kinety. Scale bars: 30 μm.

(1964) restudied this species and considered it as a new one, *Pleuronema nolandi*. He also provided a detailed line drawing based on Borror's protargol stained specimen. They are nearly the same as ours in both living and infraciliature data, except minor difference in the number of ciliary rows (25 vs. 20), and thus these three forms are conspecific.

In terms of body shape, general infraciliature and marine habitat, at least four species should be compared with *Pleuronema setigerum*: namely *P. smalli* Dragesco, 1968; *P. coronatum* Kent 1881; *P. puytoraci* Grolière and Detcheva, 1974; *P. czapikae* Wang *et al.* 2008.

Among these species, both *Pleuronema smalli* (Fig. 7E) and *Pleuronema coronatum* (Figs 7F, G) have a larg-

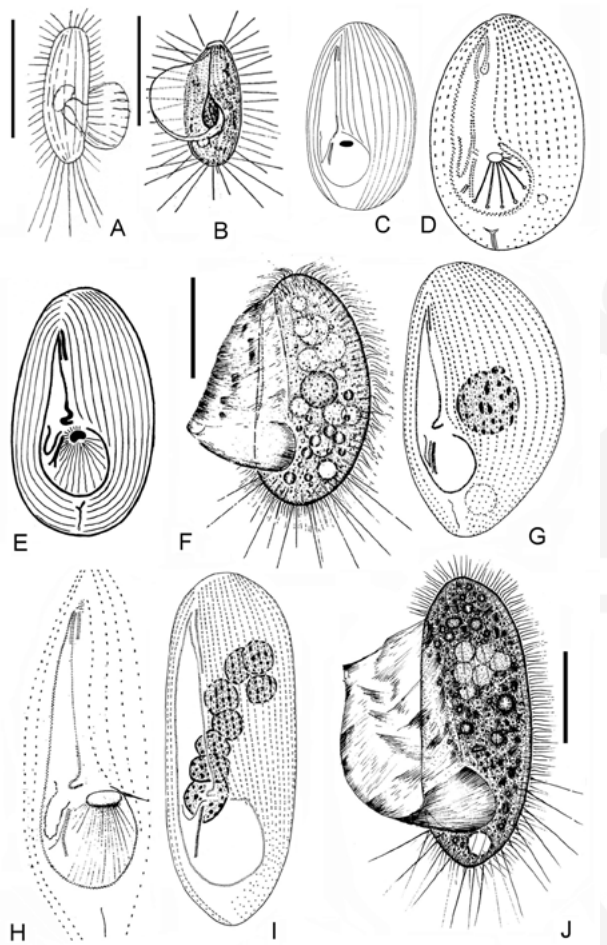


Figs 6A–P. *Pleuronema setigerum in vivo* (A–H, N, O), after Chatton-Lwoff impregnation (I) and after progtargol impregnation (J–M, P). **A** – right-ventral view of a typical individual, arrows points to cilia of paroral membranelle; **B**, **C**, **F**, **G**, **N**, **O** – to show different body shapes, arrow in (C) marks food vacuole, arrowhead in (C) refers to contractile vacuole, double arrowheads point to folded cilia of oral apparatus, arrowhead in (F) denotes contractile vacuole, arrows in (N) mark crystals, arrowhead in (O) refers to paroral membrane; **D** – lateral view, arrow shows the notch pellicle, arrowhead points to contractile vacuole; **E** – detailed view of cortex, arrow refers to macronucleus, arrowheads marks extrusomes; **H** – posterior part, arrowheads show caudal cilia; **I** – part of silverline system; **J** – small spherical macronuclear segments; **K** – right-ventral view, arrowheads point to preoral kineties; **L –** dorsal view; **M** – oral apparatus, arrowheads refers to ring-like posterior end of M2a; **P** – ventral view, arrow shows the gap in posterior of M2a, arrowheads point to preoral kineties. Scale bars: 35 μm.

er cell size (49–70 × 28–41 μm, 60–90 × 30–50 μm vs. 34–50 × 15–30 μm), more somatic kineties (28–36 and ca. 40, respectively vs. 14–22), and a different structure of the posterior end of M2a (hook-like vs. ring-like), therefore, they cannot be confused with *P. setigerum* (Dragesco 1968, Song 2000).

Pleuronema puytoraci (Fig. 7H) differs from *P. setigerum* in its body size (70–120 × 45–70 μm vs. 34–50 × 15–30 μm), the number of somatic kineties (28 vs. 14–22) and shape of posterior end of M2a (hook-like vs. ring-like) (Grolière and Detcheva 1974).

Pleuronema czapikae (Figs 7I, J) is distinguished from *P. setigerum* by a having larger cell size (85–115 × 40–50 μm vs. 34–50 × 15–30 μm), more somatic kineties (32 vs. 14–22) and more macronuclei (6–16 vs. usually 1), the shape of M2a's posterior end (hook-like vs. ring-like), and the longer M1 relative to M2a (33% vs. 20%) (Wang *et al*. 2008b).



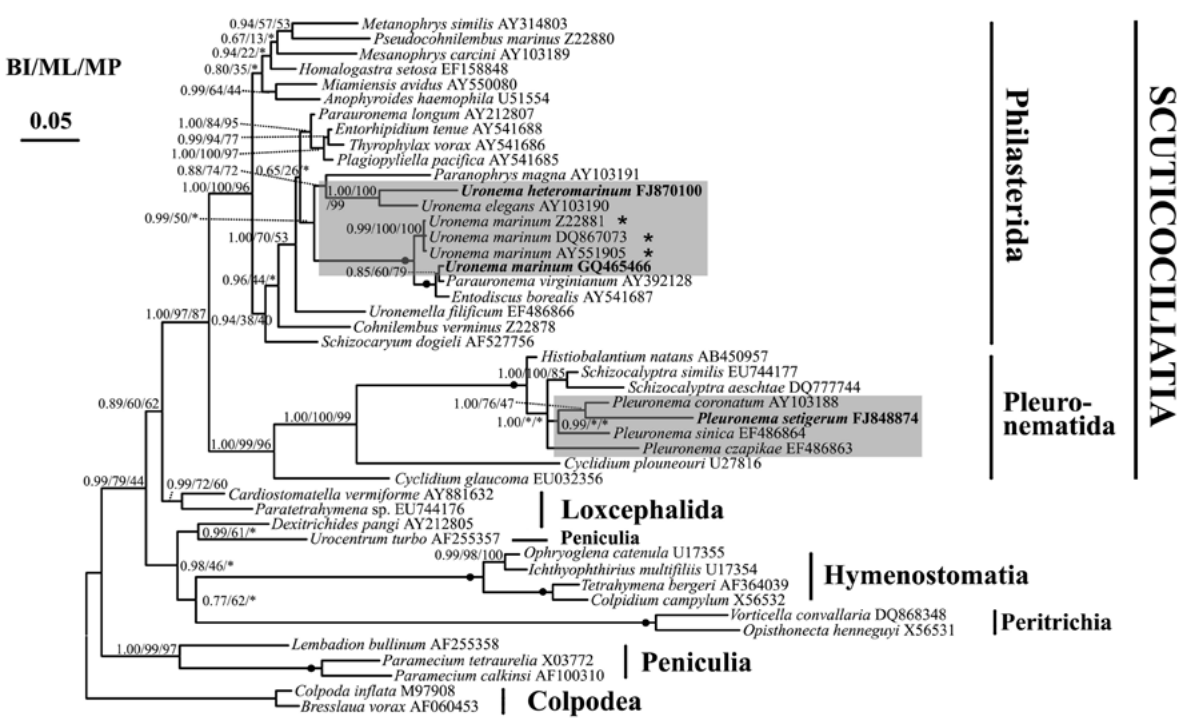
Figs 7A–H. *Pleuronema setigerum* and some species which are similar to *P. setigerum*. **A**, **B**, **C** – *P. setigerum* (A from Kahl 1931, B from Noland 1937, C from Borror 1963); **D** – after Small 1964, dissertation, called *P. nolandi;* **E** – infraciliature of *P. smalli* (from Dragesco 1968); **F**, **G** – *P. coronatum* (from Song 2000); **H** – oral apparatus of *P. puytoraci* (from Grolière and Detcheva 1974); **I**, **J** – *P. czapikae* (from Wang *et al*. 2008b). Scale bars: 40 μm.

Sequence comparison and phylogeny of the genus *Pleuronema*

The SSU rRNA gene sequence of *Pleuronema setigerum* differs from those of *P. coronatum*, *P. sinica* and *P. czapikae* in 119bp, 144bp and 169bp, respectively.

The organization of the *Pleuronema* species varies according to the analysis (Fig. 8). This is due to low

Fig. 8. Bayesian tree inferred from SSU rRNA gene sequences showing the position of *Uronema heteromarinum* nov. spec., *U. marinum* and *Pleuronema setigerum*. Nodal support for branches in the Bayesian Inference (BI), Maximum Likelihood (ML) and Maximum Parsimony (MP) trees are marked in order. Numbers near branches represent the posterior probabilities and the bootstrap values in the following order: BI/ML/MP. Clades with different topologies in the ML and MP trees relative to the BI tree are indicated with asterisks. Species for newly sequenced in this study are highlighted in bold text. Genus *Uronema* and *Pleuronema* are highlighted in gray. All branches are drawn to scale. The scale bar corresponds to 5 substitutions per 100 nucleotide positions.



bootstrap values within this clade, which results in polytomies in the MP tree. Independent from the algorithm used for the phylogenetic analysis, *P. setigerum* has a closer relationship to *P. coronatum* (BI/ML/MP, 1.00/76/47).

The polytomy (Fig. 8) formed with *Pleuronema* and *Schizocalyptra* in the SSU topology makes it difficult to access whether *Pleuronema* is monophyletic or paraphyletic. Therefore, a small tree only containing *Pleuronema* and *Schizocalyptra* along with *Histobalantium* as an outgroup was constructed by removing the masking of characters caused by the other ciliates in the full alignment, showing that the genus *Pleuronema* is monophyletic.

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