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Taxonomy and phylogeny of three heterotrich ciliates (Protozoa, Ciliophora), with description of a new *Blepharisma* species

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The morphology and phylogeny of three heterotrich ciliates, *Anigsteinia clarissima* (Anigstein, 1912) Isquith, 1968, ***Blepharisma penardi* sp. nov.**, and *Blepharisma undulans* Stein, 1867, were investigated based on living morphology, infraciliature, and small subunit (SSU) rDNA sequence data. The new species ***B. penardi* sp. nov.** is recognized by the following combination of characters: size about 150–180 × 45–55 μm *in vivo*, cell colour variable from colourless to pale pink to dark brownish; peristome extending to middle of body; 36–63 adoral membranelles; 24–34 somatic kineties; single macronucleus; cortical granules tiny and colourless; freshwater habitat. *Anigsteinia clarissima* and *B. undulans* are both reported from China for the first time and are redescribed based on a combination of previous descriptions and new data from the Chinese populations. Phylogenetic analyses based on SSU rDNA sequence data show that ***B. penardi* sp. nov.** and *B. undulans* are both located within a clade comprising only congeners, thus supporting the monophyly of the genus *Blepharisma*. *Anigsteinia clarissima* clusters with its only congener forming a clade that is sister to the *Spirostomum* assemblage. Both the morphological and the molecular data support the placement of *Anigsteinia* in the family Spirostomidae.

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ADDITIONAL KEYWORDS: *Anigsteinia clarissima* – ***Blepharisma penardi* sp. nov.** – *Blepharisma undulans* – morphology.

INTRODUCTION

The ciliate class Heterotrichea Stein, 1859, is characterized by the possession of somatic dikinetids associated with postciliodesmata, and an oral apparatus consisting of a paroral membrane and a prominent adoral zone of membranelles (Lynn, 2008). These conspicuous organisms are mostly large, free-living, and seemingly cosmopolitan. Nonetheless, relatively few species have been investigated using modern methods such as silver staining and molecular techniques (Silva-Neto *et al*., 2012; Fernandes *et al*., 2013, 2015; Boscaro *et al*., 2014; Fernandes, da Silva Neto & Schrago, 2014; Shazib *et al*., 2014; Yan *et al*., 2015). *Blepharisma* Perty, 1849, is characterized by its large body size and its complex buccal ciliature with an extensively developed membranellar band on the left side of the oral area and an undulating membrane on the right side. Certain species of this genus have long been used as model organisms for studying cytology and cell

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biology (Giese, 1973; Small & Lynn, 1985; Lynn, 2008). Its close relative, *Anigsteinia* Isquith, 1968, with five known species, was originally separated from *Blepharisma* and established as a separate genus by Isquith (1968). It is characterized by its elongate body shape and lacunar contractile vacuole system. These two widespread genera have attracted much attention for over a century, with several recent studies focusing on their molecular phylogeny (Stein, 1867; Anigstein, 1912; Giese, 1973; Isquith & Repak, 1974; Foissner, 1989; Aescht & Foissner, 1998; Dragesco, 2002; Schmidt *et al*., 2007; Miao *et al*., 2009; Zhou *et al*., 2010). By contrast, studies of their taxonomy and biodiversity have become less common in recent years that most, if not all, species have been described (Lee & Shin, 2009; Fernandes *et al*., 2013; Shazib *et al*., 2014).

In the present study, three heterotrichs, namely *Anigsteinia clarissima* (Anigstein, 1912) Isquith, 1968, *Blepharisma penardi* sp. nov., and *Blepharisma undulans* Stein, 1867, were isolated from a marine intertidal area and from freshwater lakes in China, giving the opportunity to investigate their morphology, taxonomy, and phylogeny based on small subunit (SSU) rDNA sequence data.

MATERIAL AND METHODS

Anigsteinia clarissima was collected from the intertidal zone of Silver Beach, Qingdao (35°55′N, 120°12′E), China, on 7 May 2012; the water temperature was 18 °C and salinity 31 psu.

Blepharisma penardi sp. nov. was isolated from a small freshwater pond at Baihuayuan Garden, Qingdao (36°04′N, 120°20′E), China, on 29 October 2013; the water temperature was 16 °C.

Blepharisma undulans was collected from Huguang Lake, Zhanjiang (21°09′N, 110°18′E), China, on 13 March 2013; the water temperature was 27 °C.

Observations on living cells were carried out using bright field and differential interference contrast microscopy (100–1000× magnification). The protargol staining method of Wilbert (1975) was used to reveal the infraciliature, the protargol reagent having been synthesized following the protocol of Pan, Bourland & Song (2013). Counts and measurements of the morphological characteristics of stained specimens were performed at a magnification of 1000× . Drawings were made with the help of a camera lucida. Terminology mainly follows Lynn (2008).

Genomic DNA was extracted from cells using a DNeasy Tissue kit (Qiagen, CA) according to the manufacturer's instructions. PCR amplification of the SSU rDNA was performed using the primers 82F (5′-GAA ACT GCG AAT GGC TC-3′) and Euk B for *B. penardi* sp. nov., and Euk A (5′-AAC CTG GTT GAT CCT GCC AGT-3′) and Euk B (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) for *B. undulans* and *A. clarissima* (Elwood, Olsen & Sogin, 1985; Medlin *et al*., 1988). Cloning and sequencing were carried out according to Gao & Katz (2014).

A total of 50 taxa was used for phylogenetic analysis, including the three newly sequenced species. The other nucleotide sequences used in the analyses were obtained from the GenBank database (for accession numbers see Fig. 7). Three karyorelictean species were selected as the outgroup taxa. Sequences were first aligned on the web server Phylogeny.fr (http://www.phylogeny.fr/index.cgi) with the alignment algorithm MUSCLE (Edgar, 2004; Dereeper *et al*., 2008, 2010) using the default parameters and further modified manually using BioEdit 7.0.5.2 (Hall, 1999). The final alignment of 1646 characters with both ends trimmed and 48 taxa was used to construct phylogenetic trees (the alignment file is available upon request). Bayesian inference (BI) and maximum likelihood (ML) analyses were carried out online on the CIPRES Science Gateway (Miller, Pfeiffer & Schwartz, 2010). The model GTR + Invariant + Gamma was selected as the best-fit model for nucleotide substitution based on the Akaike information criterion using MrModeltest 2 (Nylander, 2004). The BI analysis was performed using MrBayes 3.2.2 (Ronquist *et al*., 2012) with a chain length of 5 000 000 generations and a sampling frequency of 100 generations. The first 25% of sampled trees were discarded as burn-in. The ML analysis was carried out using RAxML-HPC2 (8.0.24) (Stamatakis, 2014). Searches for the best tree were conducted from 1000 random trees. Support for the best ML tree came from 1000 bootstrap replicates.

RESULTS TAXONOMY

Order Heterotrichida Stein, 1859

Family Spirostomidae Stein, 1867

Genus *Anigsteinia* Isquith, 1968

Anigsteinia clarissima (Anigstein, 1912) Isquith, 1968 (Figs 1A–D, 2–3, 5A–F, H–N, Q; Tables 1, 2)

Anigsteinia clarissima was originally described by Anigstein (1912) as *Blepharisma clarissimum*. Numerous subsequent papers dealt with this species but none provided a clear definition (Kahl, 1928, 1932; Yagiu, 1943; Fenchel, 1969; Isquith & Repak, 1974; Dragesco & Dragesco-Kernéis, 1986). Thus, an improved diagnosis based on previous reports and on new data is supplied here.

Improved diagnosis Body length about 160–560 μm *in vivo*, length to width ratio 7–10:1; buccal cavity about half body length; 63–101 adoral membranelles; 18–32 somatic kineties; macronucleus moniliform with 14–50 nodules; conspicuous lacunar contractile vacuole system; marine habitat.

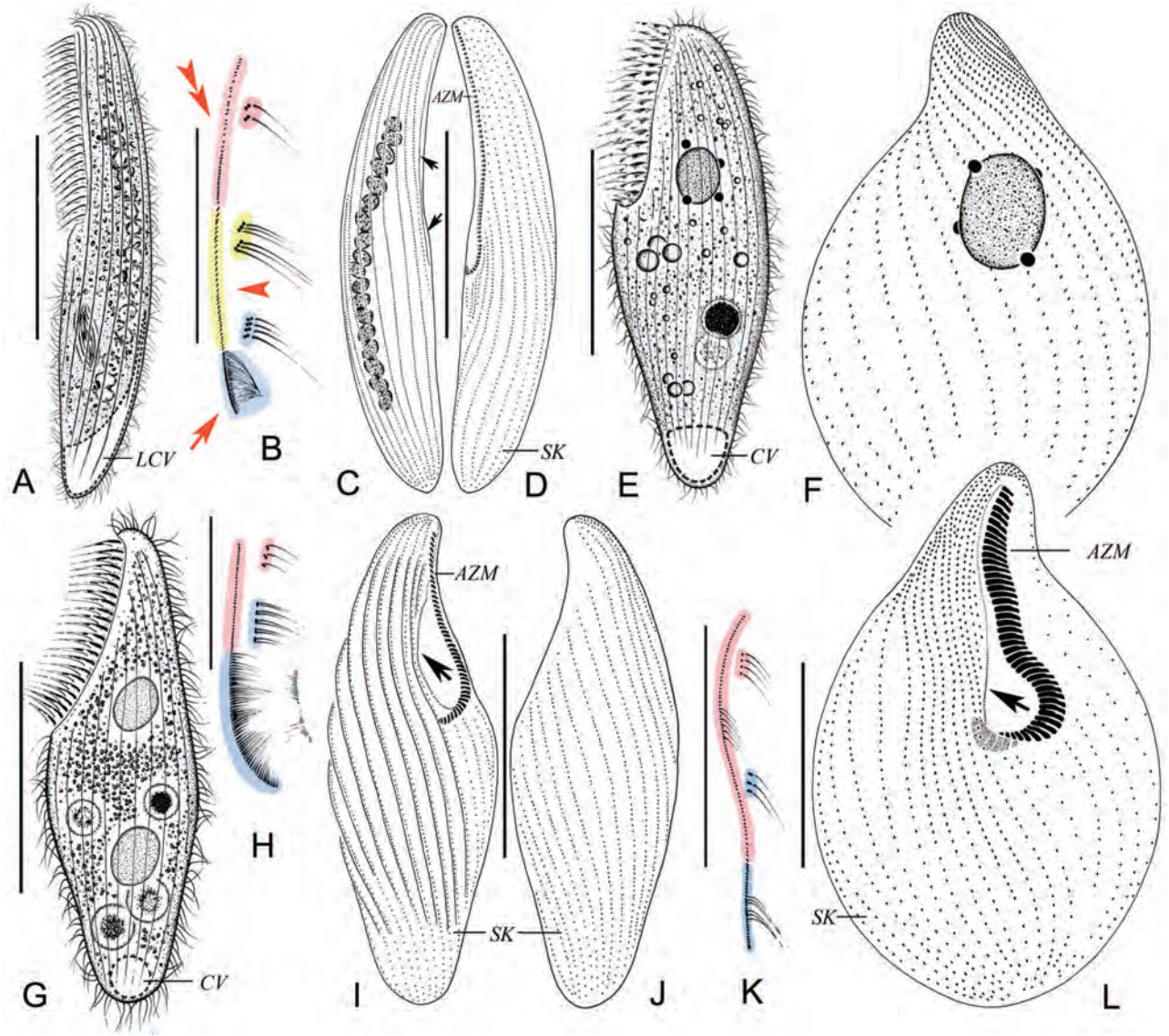

Figure 1. Morphology and infraciliature of *Anigsteinia clarissima* (A–D), ***Blepharisma penardi* sp. nov.** (E, F, K, L), and *Blepharisma undulans* (G–J). A, left view of a typical individual of *A. clarissima*. B, paroral membrane of *A. clarissima*, anterior (double arrowheads) and posterior (arrow) parts are formed of dikinetids with only left basal body ciliated, middle part (arrowhead) formed by trikinetid fragments with all basal bodies ciliated. C, D, general infraciliature of *A. clarissima*, arrows show paroral membrane. E, left view of a typical individual of ***B. penardi* sp. nov.** F, L, dorsal (F) and ventral (L) views of infraciliature of ***B. penardi* sp. nov.** from the same specimen, arrow marks paroral membrane. G, left view of a typical individual of *B. undulans*. H, paroral membrane of *B. undulans*, anterior part with only right basal bodies ciliated and posterior part with both basal bodies ciliated. I, J, right (I) and left (J) views of *B. undulans* showing the infraciliature, arrow points to paroral membrane. K, paroral membrane of ***B. penardi* sp. nov.**, anterior part consists of ciliated monokinetids, posterior part consists of dikinetids with only left basal body ciliated. Abbreviations: AZM, adoral zone of membranelles; CV, contractile vacuole; LCV, lacunar contractile vacuole system; SK, somatic kineties. Scale bars = 100 μm (A, C, D), 70 μm (E, G, I, J, L), 50 μm (B, H, K).

*Description based on Qingdao population*Body 230–300 × 35–50 μm *in vivo*: slender and slightly contractile with tapered posterior end (Figs 1A, 2A–D); bilaterally flattened, width to thickness ratio about 3:1. Pellicle flexible, with numerous colourless, spherical cortical granules (0.8–1 μm in diameter) arranged in three or four longitudinal rows between the kineties (Fig. 2H). Peristome long and narrow, commencing slightly below anterior end of body and extending to mid-body region (Fig. 2A–E). About 60–100 adoral

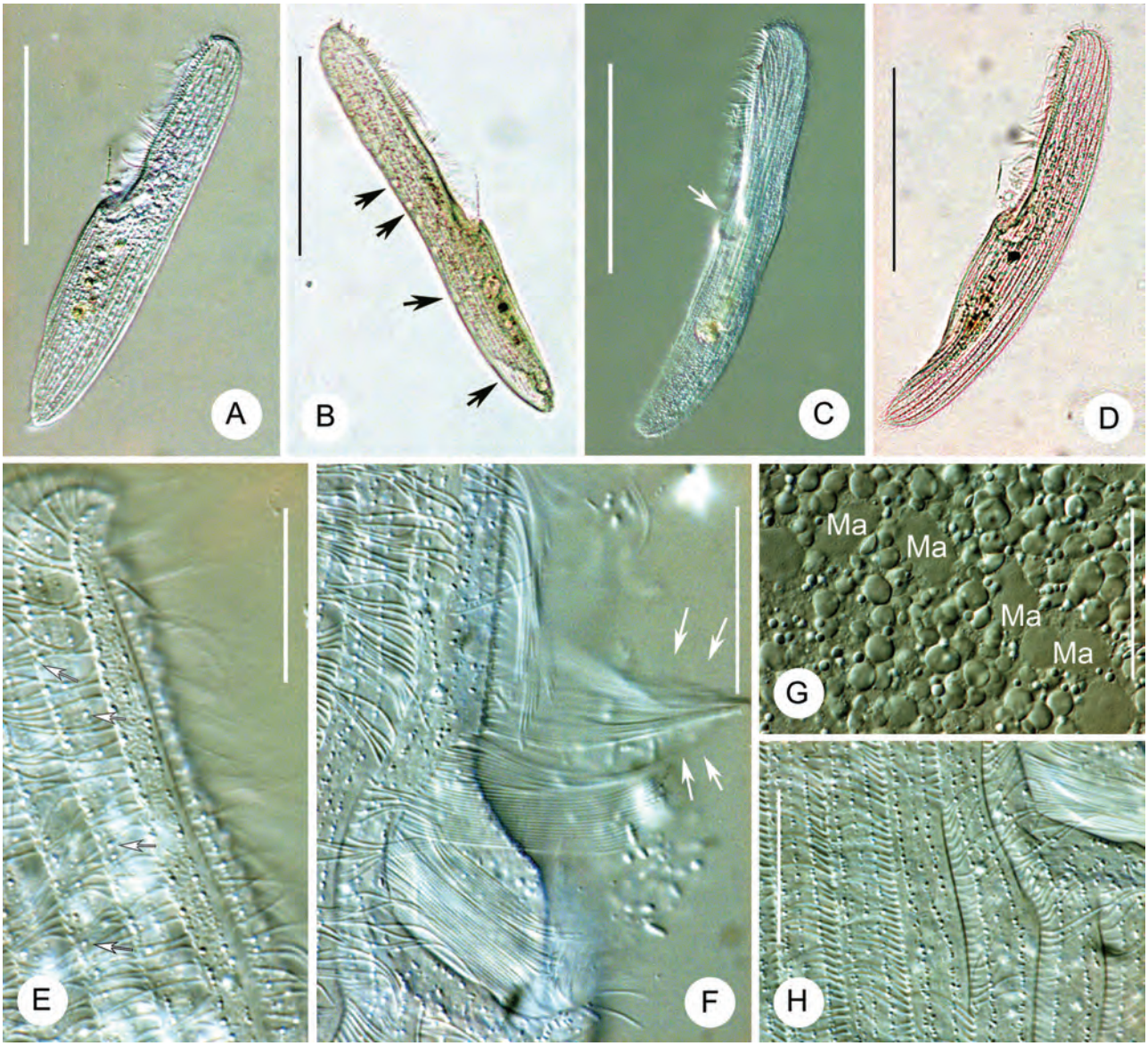

Figure 2. Photomicrographs of *Anigsteinia clarissima* from life. A–D, general views of typical individuals, arrows in B show lacunar contractile vacuole system, arrow in C indicates the paroral membrane. E, anterior region of cell, arrows mark the colourless cortical granules. F, posterior portion of buccal field, arrows show paroral membrane. G, macronuclear nodules. H, portion of cortex (right view), showing cortical granules between somatic kineties. Abbreviation: Ma, macronucleus. Scale bars = 120 μm (A–D), 25 μm (E–H).

membranelles. Paroral membrane conspicuous (Figs 1B, 2F, 3B, C, H). Cytoplasm greyish and opaque, packed with globular granules and food vacuoles that are frequently abundant (Fig. 2D, G). Macronucleus moniliform with 14 to 26 interconnected nodules, each nodule ellipsoidal and about 7–10 μm in diameter; micronuclei not detected (Figs 1C, 2G, 3A, H). Conspicuous lacunar contractile vacuole (CV) system, CV terminally located (Figs 1A, 2B). Locomotion by gliding over substratum, i.e. sand grains, organic debris, bottom of Petri dish.

Eighteen to 26 somatic kineties, including six to 11 (right) shortened postoral rows (Figs 1C, D, 3A, E–G). Cilia about 12 μm long *in vivo*. Each adoral membranelle consists of one short and two long rows of basal bodies. Paroral membrane divided into three parts (Fig. 1B): anterior and posterior parts consist of dikinetids with only left basal body of each pair ciliated; middle part consists of several trikinetid fragments with all three basal bodies in each trikinetid ciliated.

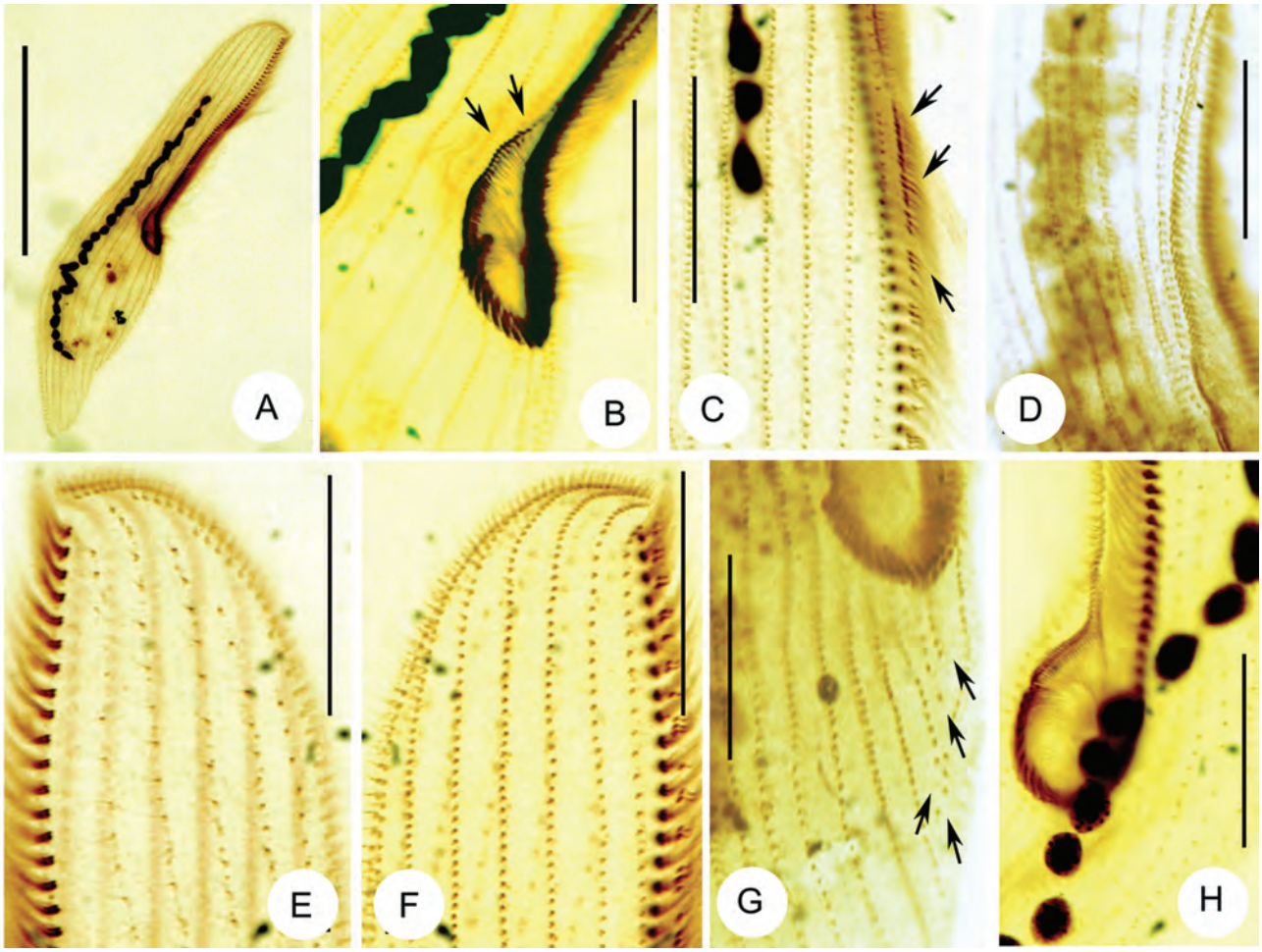

Figure 3. Photomicrographs of *Anigsteinia clarissima* after protargol staining. A, right view of a typical individual. B–D, anterior of cell (right view), arrows show paroral membrane. E, F, left (E) and right (F) views of anterior portion. G, portion of infraciliature, arrows mark postoral kineties. H, posterior end of oral field and the moniliform macronucleus. Scale bars = 140 μm (A), 30 μm (B–H).

ORDER HETEROTRICHIDA STEIN, 1859

FAMILY BLEPHARISMIDAE JANKOWSKI IN SMALL & LYNN, 1985

GENUS *BLEPHARISMA* PERTY, 1849

***BLEPHARISMA PENARDI* SP. NOV.** (FIGS 1E, F, K, L, 4A–I, 6A, C, D; TABLES 1, 3)

Synonyms: Blepharisma lateritium sensu Penard, 1922

BLEPHARISMA STEINI FORMA *PENARDI* KAHL, 1932

Diagnosis Body about 80–180 × 45–55 μm *in vivo*; cells colourless to dark brownish; peristome extending to mid-body; 36–63 adoral membranelles; 24–34 somatic kineties; single macronucleus; three to five micronuclei nodules; granules pale pink to colourless; habitat freshwater or moss.

Type locality A freshwater pond in Baihuayuan Garden (36°4′N, 120°20′E), Qingdao, China.

Type material A protargol slide containing the holotype specimen marked with an ink circle is deposited in the Laboratory of Protozoology, Ocean University of China (OUC), China (slide number YY2013102901). A paratype slide is deposited in the Natural History Museum, London, UK (registration number NHMUK 2015.4.23.1).

Etymology The species name *penardi* is named in honour of Dr Eugene Penard who first described this organism.

Gene sequence The SSU rDNA sequence, derived from a single cell isolated from the same sample as the holotype, is deposited in GenBank (accession number KR815913).

Description Body about 150–180 × 45–55 μm *in vivo*, slender and irregularly sigmoid, flexible and slightly

Table 1. Morphometric characteristics of *Anigsteinia clarissima* (upper line in each row), ***Blepharisma penardi* sp. nov.** (middle line), and *Blepharisma undulans* (lower line) from protargol-stained specimens

Characters	Min.	Max.	Mean	M	CV	*N*
Body length	181	411	268.6	234.0	28.3	15
	101	158	128.2	126.0	11.5	26
	163	257	214.0	214.0	13.1	20
Body width	41	80	59.5	61.0	22.7	15
	54	89	72.6	74.5	14.4	26
	54	126	82.8	80.0	21.8	20
Buccal field, length	114	192	147.9	143.0	16.6	15
	48	79	66.1	70.5	15.0	26
	52	99	84.2	85.5	14.4	20
Adoral membranelles, number	63	101	86.7	90.0	12.5	15
	36	63	52.1	54.0	11. 4	20
	42	68	51.7	51.0	12.0	20
Distance from anterior body margin to Ma	49	111	64.2	54.5	28.6	16
	21	51	33.9	33.0	23.8	26
	31	132	65.9	64.0	30.5	19
Somatic kineties, number	18	26	21.3	21.5	10.4	16
	24	34	29.5	29.0	8.9	17
	21	27	23.7	24.0	6.8	18
Shortened somatic kineties, number	6	11	8.4	8.0	17.3	15
	2	5	3.7	4.0	19.6	14
	9	12	10.2	10.0	10.5	13
Macronuclear nodules, number	14	26	18.9	17.5	19.8	12
	1	1	1.0	1.0	0	26
	2	2	2.0	2.0	0	14
Macronuclear nodule, length	6	13	9.5	10.0	21.3	11
	20	37	26.7	25.5	21.9	14
	18	47	31.4	31.0	21.9	14
Macronuclear nodule, width	5	13	7.3	7.0	32.0	11
	17	30	21.0	20.0	16.1	14
	16	34	23.0	21.0	24.3	14
Micronuclei, number	–	–	–	–	–	–
	3	5	4.1	4.5	24.0	8
	–	–	–	–	–	–

All measurements in μm. Abbreviations: CV, coefficient of variation in %; M, median; Ma, macronucleus; Max., maximum; Mean, arithmetic mean; Min., minimum; *N*, number of specimens; –, data not available.

bilaterally flattened (Figs 1E, 4A, C, D). Buccal area about 50% body length. Cell coloration in both freshly isolated and cultivated (for about 1 to 2 weeks) samples rather variable from almost colourless to dark-brownish at low magnification using bright-field microscopy (Fig. 4A, C, D) although most individuals are slightly brownish or pale pink. It remains unclear what causes the cell coloration as neither pigments nor coloured food vacuoles were detected. Cortical granules colourless, round, *c.* 0.2–0.3 μm in diameter, densely arranged in rows located between kineties (Fig. 4E). Paroral membrane inconspicuous, difficult to detect *in vivo* (Fig. 4A, C, D). Macronucleus located slightly above mid-body region, spherical to ovoid, about 20 μm in diameter, with three to five closely associated globular micronuclei (Fig. 1F). Contractile vacuole conspicuous, *c.* 20 μm in diameter, terminally located (Figs 1E, 4C, D). Locomotion mainly by gliding slowly on bottom of Petri dish.

Infraciliature consists entirely of dikinetids except for anterior two-thirds of paroral membrane, which is composed of monokinetids (Fig. 1F, K, L). In posterior third of paroral, only left basal body of each dikinetid is ciliated (Fig. 1K). Adoral zone composed of 36–63 membranelles, each of which consists one short and two long rows of basal bodies (Figs 1L, 4H). Twenty-four to 34 longitudinal somatic kineties including two to five shortened postoral (right) rows (Figs 1F, L, 4F, G), with cilia about 12–13 μm long *in vivo*.

Table 2. Morphometric comparison of different populations of *Anigsteinia clarissima*

Species	BL (μm)	No. of SK	No. of Ma	Data source
A. clarissima	230–300	18–26	14–26	Present work
A. clarissima	160–380	22–25	32–47	Anigstein (1912)
A. clarissima	500–600	–	30*	Kahl (1928)
A. clarissima forma *arenicola*	200–400	–	30–50	Kahl (1932)
A. clarissima	200–400	–	30–50	Yagiu (1943)
A. clarissima var. *arenicola*	200–400	–	–	Fauré-Fremiet (1950)
A. clarissima var. *arenicola*	270	–	>50	Dragesco (1960)
A. clarissima forma *arenicola*	176–560	–	35–51	Hartwig (1973)
A. clarissima aff. *arenicola*	176	–	15–16	Hartwig & Parker (1977)
A. clarissima	300	32	9	Ricci *et al*. (1982)
A. clarissima (?)	350–600	32	35*	Dragesco & Dragesco-Kernéis (1986)

Abbreviations: BL, body length; Ma, macronucleus; SK, somatic kineties; –, data not available.
*data from the drawings.

Blepharisma undulans Stein, 1867 (Figs 1G–J, 4J–M, 6E–K; Tables 1, 4)

The morphology and infraciliature of a South Korean population of this species were recently described in detail (Lee & Shin, 2009). Therefore, only a brief note of the Qingdao population is documented here.

Description based on Qingdao population Cells about 120–250 × 35–60 μm *in vivo*, body shape relatively stable: anterior conspicuously pointed, posterior end widely rounded (Figs 1G, 4J); width to thickness ratio about 2:3. Cells invariably bright pink at low magnification as a result of the presence of cortical granules. Cortical granules about 0.5 μm across, mostly dark rose-red in colour although some are white (arrows in Fig. 4M), distributed in numerous longitudinal rows between kineties (Fig. 4J, M). Pellicle soft. Peristome narrow, extending to mid-body region (Figs 1G, I, 4J, K). Paroral membrane difficult to detect *in vivo* (Fig. 4J). Cytoplasm usually packed with several food vacuoles (Fig. 4J). Two ellipsoidal macronuclear nodules, about 30 × 20 μm in stained individuals, one located in anterior and the other in posterior half of body, nuclear connective strand not detected (Figs 1G, 4K). Micronucleus not detected. Contractile vacuole *c.* 15 μm in diameter terminally located (Figs 1G, 4J). Locomotion mainly by gliding slowly on bottom of Petri dish.

Infraciliature consists entirely of dikinetids. Adoral zone composed of 42–68 membranelles, each of which consist of one short and two long rows of basal bodies. Paroral membrane composed of dikinetids: in anterior half only right basal body of each pair is ciliated, in posterior half both pairs are ciliated (Figs 1H, 4L). Twenty-one to 27 ciliary rows arranged longitudinally (Fig. 1I, J), cilia about 10 μm long *in vivo*. Amongst these about nine to 12 postoral (right side) rows are shortened, i.e. only 11–16 kineties extend complete length of cell (Fig. 1I, J).

Molecular data and phylogenetic analyses

The three new SSU rDNA sequences in this study have been deposited in the GenBank database. Their lengths, G + C contents, and accession numbers are as follows: *A. clarissima*, 1673 bp, 46.44%, KR815914; *B. penardi* sp. nov., 1642 bp, 47.81%, KR815913; *B. undulans*, 1661 bp, 47.26%, KR815912. Pairwise comparison of the SSU rDNA sequences revealed that *B. penardi* differs from *Blepharisma steini* by 28 nucleotides (1.74%).

The BI and ML analyses of the SSU rDNA data set generated phylogenetic trees with nearly identical topologies; therefore, only the BI tree is shown here (Fig. 7). *Anigsteinia clarissima* clusters with *Anigsteinia* sp. (HM140405) in a clade that is sister to the *Spirostomum* assemblage. *Anigsteinia* and *Spirostomum* thus form a well-supported (99% ML, 1.00 BI) monophyletic group that represents the family Spirostomidae.

All species of *Blepharisma* group together with full statistical support forming a sister branch to Stentoridae, which is consistent with previous studies (Schmidt *et al*., 2007; Zhou *et al*., 2010; Shazib *et al*., 2014; Yan *et al*., 2015). The relationships within Blepharismidae, however, are still not consistently resolved. *Blepharisma steini* branches off first in both analyses with full support and the next most deeply branching taxon is *B. penardi* sp. nov. (94% ML, 0.95 BI). The Qingdao population (KR815913) and German population (AM713183) of *B. undulans*, *Blepharisma musculus* (KJ651813), *Blepharisma japonicum* (AM713185), and *Blepharisma*

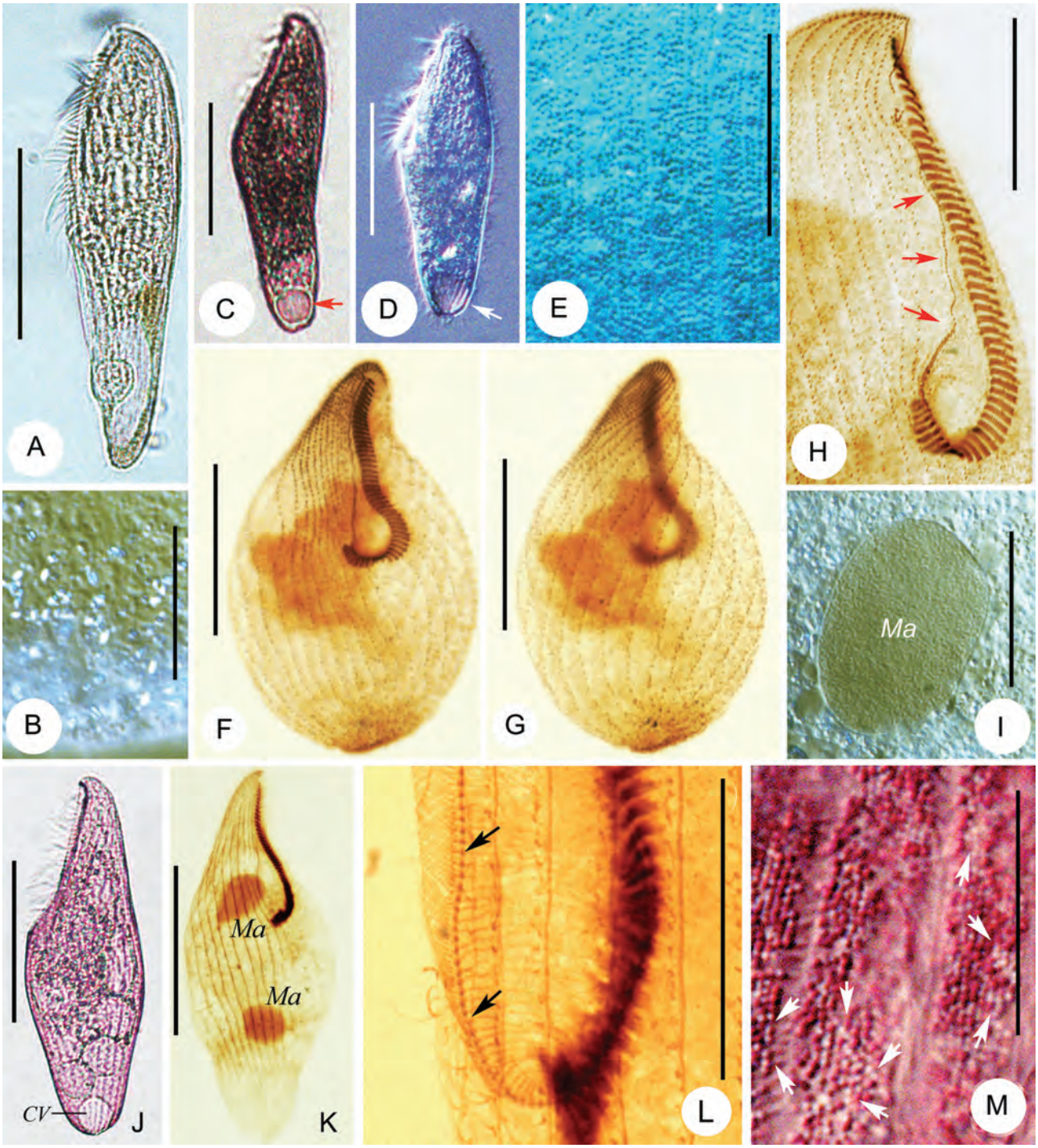

Figure 4. Photomicrographs of ***Blepharisma penardi* sp. nov.** from life (A–E, I) and after protargol staining (F–H), and *Blepharisma undulans* from life (J, M) and after protargol staining (K, L). A, C, D, left view, to show the shape variants, arrows in C, D mark the contractile vacuole. Note the cell colour: colourless (A) and dark-brownish (C). B, cytoplasmic granules. E, portion of cortex, showing the tiny, densely arranged cortical granules. F, G, general infraciliature. H, portion of buccal field, arrows point to paroral membrane. I, macronucleus. J, left view of a typical individual. K, right view, to show the two macronuclei. L, posterior portion of oral field, arrows indicate paroral membrane. M, to show cortical granules, note there are some white granules (arrows) amongst the coloured ones. Abbreviations: CV, contractile vacuole; Ma, macronucleus. Scale bars = 70 μm (A, C, D, F, G, J, K), 30 μm (H), 15 μm (B, E, I, L, M).

Table 3. Morphometric comparison of ***Blepharisma penardi* sp. nov.** and different populations of *Blepharisma steini*

Species	BL (μm)	No. of SK	No. of membranelles	Data source
***B. penardi* sp. nov.**	150–180	24–34	36–63	Present work
*B. penardi**	80–120	–	–	Penard (1922)
B. steini var. *penardi*	80–120	–	–	Kahl (1932)
B. steini	150–200	–	–	Kahl (1932)
B. steini	100–184	*c.* 25	*c.* 50	Larsen & Nilsson (1983)
B. steini	170	18–23	35–47	Foissner (1989)
B. steini	67–91	20–26	40–50	Al-Rasheid (2001)
B. steini	68–120	18–22	33–45	Lee & Shin (2009)

*Reported as *Blepharisma lateritium*.
Abbreviations: BL, body length; SK, somatic kineties; –, data not available.

Table 4. Morphometric comparison of different populations of *Blepharisma undulans*

Species	BL (μm)	No. of SK	No. of Ma	No. of membranelles	Data source
B. undulans	120–200	21–27	2	42–68	Present work
B. undulans	150–300	–	2	–	Kahl (1932)
B. undulans undulans	70–250	24–30	2	–	Suzuki (1954)
B. undulans	90–140	–	2	–	Bhandary (1962)
B. undulans	150	18–25	2–5	–	Kennedy (1965)
B. undulans	234–379	–	–	–	Cela (1972)
B. undulans	100–300	25–28	2	53–63	Foissner (1989)
B. undulans	–	16–36	2	34–51	Dragesco & Dragesco-Kernéis (1991)
B. undulans	150–250	22–27	2	47–56	Lee & Shin (2009)

Abbreviations: BL, body length; Ma, macronucleus; SK, somatic kineties; –, data not available.

sinuosum (JN627438) cluster with *Blepharisma americanum* (AM713182) in a subclade that is sister to the subclade comprising *Blepharisma hyalinum* (AM713184) and *Blepharisma elongatum* (AM713186).

DISCUSSION

COMMENTS ON *BLEPHARISMA PENARDI* SP. NOV.

Blepharisma penardi sp. nov. was first recorded as a variety of *B. lateritium* by Penard (1922) (Fig. 6A, C, D; Table 3). According to the original description, the organism is '80–120 μm, shape variable, reddish, sometimes almost colourless, the shape of macronucleus like date-palm seed, mostly 4–6 small micronuclei' (Penard, 1922). Kahl (1932) described it as a 'form' of the newly defined species, *B. steini* Kahl, 1932, i.e. *B. steini* forma *penardi*. The Qingdao isolate matches the original description in body shape and general morphology, coloration, and habitat. The only significant difference is the cell size (150–180 vs. 80–120 μm *in vivo*). As cell size is thought to be largely food- or population-dependent, we believe that these forms are conspecific and that our identification is correct.

Blepharisma penardi sp. nov. can be clearly distinguished from its closely related congener, *B. steini*, by its higher number of somatic kineties (24–34 vs. 18–22 in the Korean population, 18–23 in the Austrian population, and *c.* 25 in the Danish population of *B. steini*), the size of the contractile vacuole (large and conspicuous vs. small and inconspicuous in *B. steini*) and the body shape (slender and sigmoidal vs. widely oval in *B. steini*) (Kahl, 1932; Larsen & Nilsson, 1983; Foissner, 1989; Al-Rasheid, 2001; Lee & Shin, 2009; Fig. 6B; Table 3). Another possible difference is cell colour, that is, *B. penardi* is variable from colourless to dark-brownish whereas *B. steini* is invariably red in colour (Kahl, 1932; Larsen & Nilsson, 1983; Foissner, 1989; Al-Rasheid, 2001; Lee & Shin, 2009). In addition, the separation of these two species is strongly supported by molecular data, their SSU rDNA sequences differing by 28 nucleotides (98.26% similarity) (Schmidt *et al.*, 2007).

COMMENTS ON *ANIGSTEINIA CLARISSIMA* (ANIGSTEIN, 1912) ISQUITH, 1968

Anigsteinia clarissima, which is commonly found in intertidal regions of sandy beaches, was originally reported as *Blepharisma clarissimum* (Anigstein, 1912), but was reassigned by Isquith (1968) who established the genus *Anigsteinia* with *A. clarissima* as the type species. During the past century, numerous 'populations' of this taxon have been reported worldwide with rather conspicuous variations in morphology, from highly slender to elongated oval body shape, from widely rounded to pointed caudal end, and from 15 to over 30 macronuclear nodules (Anigstein, 1912, see our Fig. 5B; Kahl, 1928, Fig. 5H; Kahl, 1932, Fig. 5D–F; Yagiu, 1943, Fig. 5A; Bock, 1952, Fig. 5I; Dragesco, 1960, Fig. 5C; Raikov, 1960, Fig. 5J; Agamaliev, 1968, Fig. 5K, L; Czapik & Jordan, 1976, Fig. 5M, N; Dragesco & Dragesco-Kernéis, 1986, Fig. 5Q). As most descriptions lack details of the ciliature or morphology *in vivo*, and because molecular data are lacking for almost all, evaluating these isolates remains a huge challenge. The Qingdao population corresponds extremely closely with the original description, the only difference being that the former has fewer macronuclear nodules (14–26 vs. 32–47). As this feature is population-dependent in most ciliates with multiple macronuclear nodules, we believe that the Qingdao isolate is a population of *A. clarissima*.

According to Isquith & Repak (1974), who provided a taxonomic revision of *Anigsteinia* as well as a key to the species, and Dragesco (2002), who supplied the most recent review of *A. clarissima*, the original form described by Anigstein (1912) and the populations reported later by Kahl (1932) are clearly conspecific (Fig. 5B, E, F, H). Another isolate described by Kahl (1932), *B. clarissimum* forma *arenicola* Kahl, 1932 (Fig. 5D), is defined only by its body shape and ratio of body length to width. We believe that such differences are very likely to be population-dependent and hence this form to be conspecific with *A. clarissima*.

The form reported by Dragesco (2002) that was collected from Aber Bay, Roscoff (Fig. 5O) might belong to another species, *Anigsteinia candida* (Yagiu & Shigenaka, 1956) Isquith & Repak, 1974, because it has a very high number of macronuclear nodules (62 on average in Roscoff population; 181–287 in a Japanese population of *A. candida* described by Yagiu & Shigenaka, 1956; 200–260 in a Saudi Arabian population described by Al-Rasheid, 2000). Furthermore, Dragesco (2002) noted that the nodules 'are not interconnected but freely disposed throughout the cell, especially in the central region', which is also characteristic of *A. candida* (Yagiu & Shigenaka, 1956; Al-Rasheid, 2000).

Since the review by Kahl (1932), over ten 'populations' have been reported under the names of *B. clarissimum*, *B. clarissima*, *A. clarissimum*, or *A. clarissima* (Yagiu, 1943; Fauré-Fremiet, 1950; Bock, 1952; Dragesco, 1960, 1966; Raikov, 1960; Agamaliev, 1968; Fenchel, 1969; Hartwig, 1973; Czapik & Jordan, 1976; Hartwig & Parker, 1977; Dragesco & Dragesco-Kernéis, 1986; Fig. 5A, C, G, I, J–M, O–Q). Amongst these, the identity of one of the isolates reported by Czapik & Jordan (1976) is questionable because it has an evidently wider body shape than is usual for *A. clarissima* (Fig. 5M). By contrast, another individual described by the same authors (Czapik & Jordan, 1976) is probably correctly assigned because, as is typical for *A. clarissima*, it has a slender body with a pointed caudal region (Fig. 5N). Based on the diagrams supplied by Czapik & Jordan (1976), both of these isolates have significantly fewer adoral membranelles than is normal for *A. clarissima* (Fig. 5M, N). These drawings, however, were based on observations *in vivo* and so probably do not accurately represent the true number of membranelles present. Data based on silver-stained specimens are not available for either isolate.

Dragesco (1966) reported an organism from Thonon-les-Bains, France, that he called *Blepharisma clarissimum*, which has an obvious neck area and only about ten macronuclear nodules (Fig. 5P). Thus, it clearly differs from *A. clarissima*, which has no neck and about 15–45 macronuclear nodules. Isquith & Repak (1974) established a new species for this isolate, *Anigsteinia oligonucleata* (Dragesco, 1966) Isquith & Repak, 1974. The number of macronuclear nodules is not, however, a reliable character for species separation amongst ciliates with multiple macronuclear nodules (Berger, 2008). Consequently the validity of *A. oligonucleata* awaits further support. The organism reported as *A. clarissima* by Ricci, Santangelo & Luporini (1982) has only nine macronuclear nodules and therefore is probably a population of *A. oligonucleata* if this is a valid species (Fig. 5G; Table 2).

COMMENTS ON *BLEPHARISMA UNDULANS* STEIN, 1867

This well-known organism has been reported on numerous occasions from sites all over the world and thus its identity is relatively easy to establish (Stein, 1867; Kahl, 1932; Suzuki, 1954; Bhandary, 1962; Cela, 1972; Dragesco & Dragesco-Kernéis, 1991; Lee & Shin, 2009). The Qingdao population corresponds closely with both the original report (Stein, 1867) and the redescription by Kahl (1932) in terms of its bright pink coloration and the pinkish cortical granules, general morphology, pattern of ciliature, possession of two macronuclear nodules, and habitat (Fig. 6; Table 4).

Compared with the South Korean and Argentinian populations (Cela, 1972; Lee & Shin, 2009), the Qingdao population has only one dissimilar feature, i.e. the body shape: the Qingdao population is stably lanceolate or elongate-pyriform with the anterior end pointed and

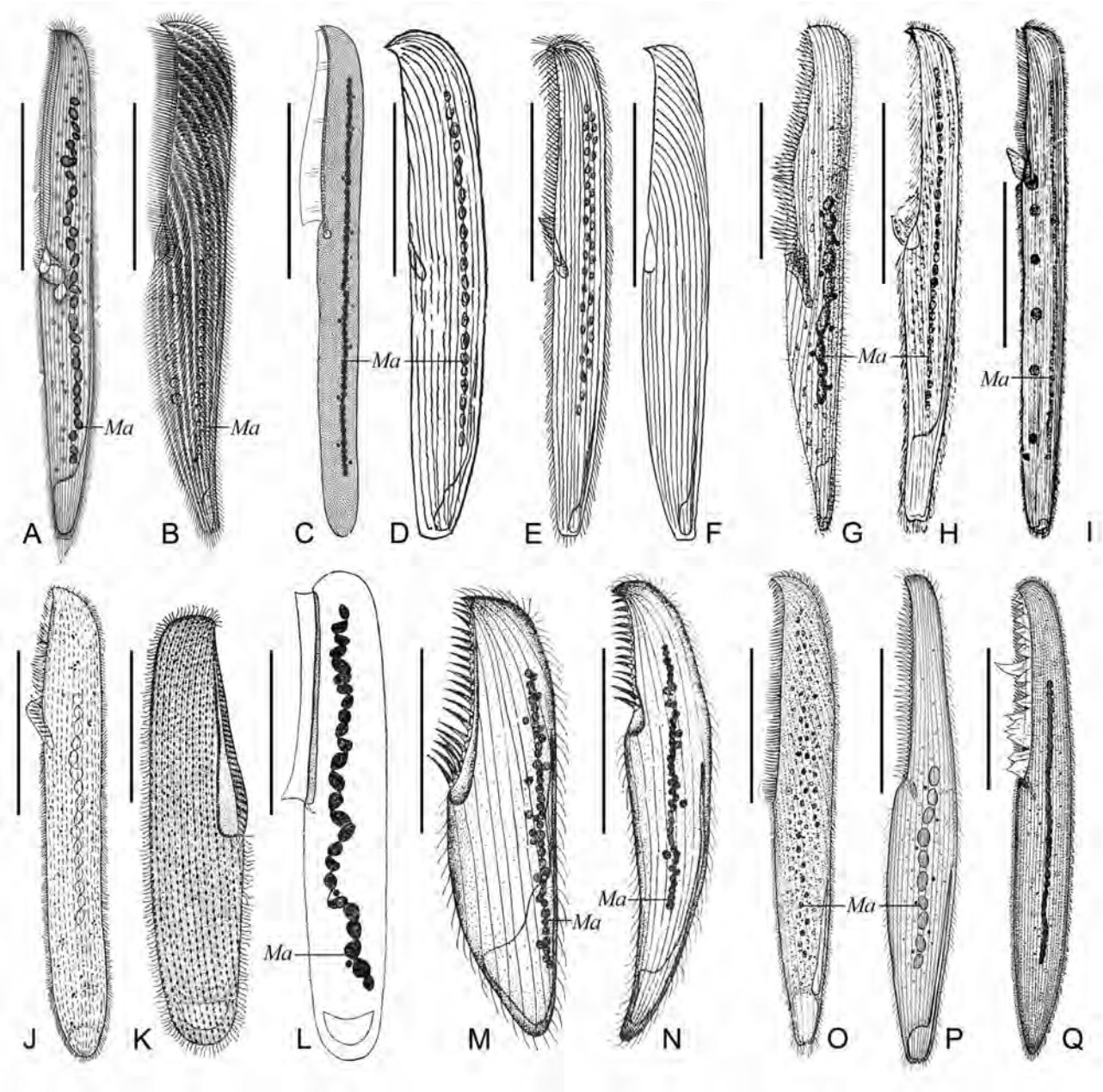

Figure 5. Different isolates under the names of *Anigsteinia clarissima* (A–N, Q), *Anigsteinia candida* (O), and *Anigsteinia oligonucleata* (P). A, from Yagiu (1943). B, from Anigstein (1912). C, from Dragesco (1960). D, reported as *Blepharisma clarissimum* forma *arenicola* by Kahl (1932). E, from Kahl (1932). F, from Kahl (1932), after Anigstein (1912). G, from Ricci *et al*. (1982). H, from Kahl (1928). I, from Bock (1952). J, from Raikov (1960). K, L, from Agamaliev (1968). M, N, from Czapik & Jordan (1976). O, *A. candida*, reported as *A. clarissima* (misidentification) by Dragesco (2002). P, *A. oligonucleata*, reported as *A. clarissima* (misidentification) by Dragesco (1966). Q, from Dragesco & Dragesco-Kernéis (1986). Abbreviation: Ma, macronuclear nodules. Scale bars = 375 μm (O), 200 μm (E, I, M, N), 180 μm (H), 150 μm (D, G, J), 110 μm (F, Q), 80 μm (B), 60 μm (C, K, L, P), 40 μm (A).

posterior end widely rounded (vs. spindle- or bat-shaped with the posterior third slightly narrowed in the South Korean and Argentinian populations; Fig. 6F, H). It has long been known that body shape in *Blepharisma undulans* is variable and largely dependent upon nutrition, with well-nourished cells usually assuming a pyriform shape, expanded posteriorly, and underfed organisms having an elongate spindle shape

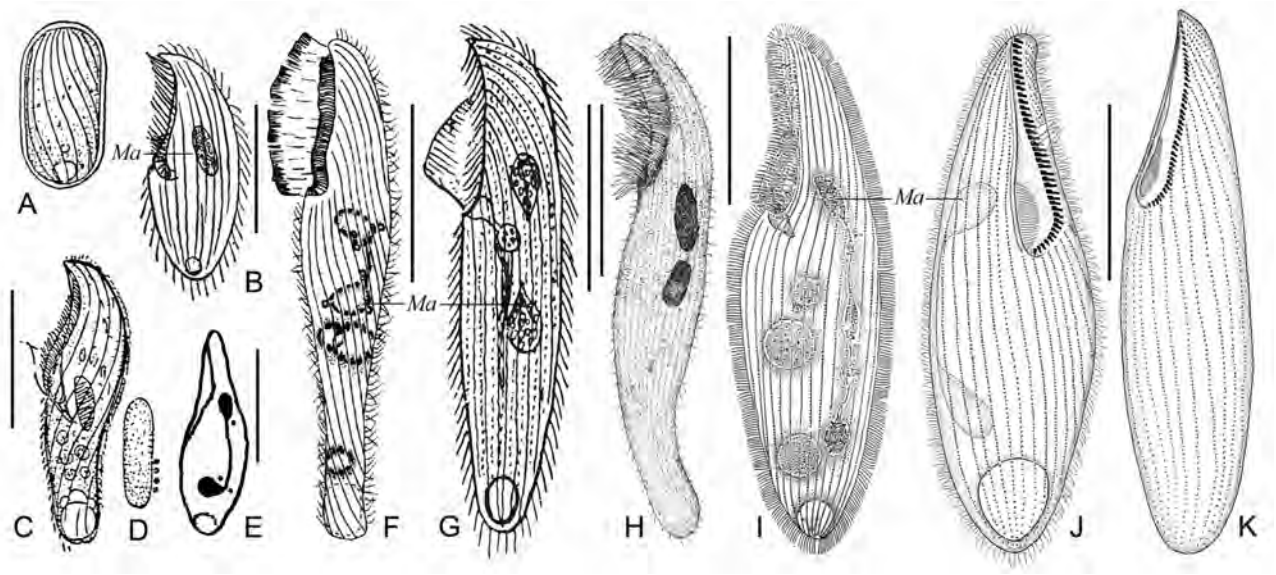

Figure 6. Nominal isolates of ***Blepharisma penardi* sp. nov.** (A, C, D), *Blepharisma steini* (B), and *Blepharisma undulans* (E–K). A, C, D, ***B. penardi* sp. nov.**, originally described as an unnamed variety of *Blepharisma lateritium*, from Penard (1922). B, *B. steini*, from Kahl (1932). E, *B. undulans*, from Bhandary (1962). F, *B. undulans*, from Lee & Shin (2009). G, *B. undulans*, from Kahl (1932). H, *B. undulans*, from Cela (1972). I, *B. undulans*, from Suzuki (1954). J, K, *B. undulans*, from Dragesco & Dragesco-Kernéis (1991). Abbreviation: Ma, macronuclear nodules. Scale bars = 90 μm (B, I), 80 μm (G, F) 50 μm (C, E, H), 40 μm (J, K).

(Giese, 1973). We therefore believe that the difference in body shape between the Qingdao population of *B. undulans* and the South Korean and Argentinian populations to be population- or environment-dependent and not significant for taxonomy. The conservative nature of *B. undulans* is also supported by the molecular data, the SSU rDNA sequence obtained in this study being identical to that provided by Schmidt *et al*. (2007).

Phylogenetic analyses

The overall topology of our phylogenetic tree is congruent with that of Shazib *et al*. (2014). The newly sequenced species *A. clarissima* clusters with its congener, *Anigsteinia* sp. (HM140405), on a branch that is deeply divergent from *Blepharisma*, thus supporting the morphological evidence for separating *Anigsteinia* from *Blepharisma* (Isquith, 1968). The two *Anigsteinia* spp. form a clade that is sister to the *Spirostomum* assemblage and so the family Spirostomidae is monophyletic with *Anigsteinia* included. Therefore, the molecular data also support the morphological evidence, i.e. the uniquely posteriorly thickened paroral membrane and the terminal contractile vacuole system with a long canal, for the assignment of the former to the family Spirostomidae, as suggested previously (Isquith, 1968; Isquith & Repak, 1974; Shazib *et al*., 2014).

The addition of two new sequences of *Blepharisma* verifies the monophyly of this genus. As shown in Figure 7, *B. penardi* sp. nov. occupies a separate branch within the *Blepharisma* clade and is clearly separated from its congeners (including the morphologically similar species, *B. steini*), which supports our morphological evidence for its establishment as a new species. Furthermore, apart from the difference in sequence length (the Chinese population is 47 bp shorter at the 5′ end and 33 bp longer at the 3′ end), the SSU rDNA sequence of the Chinese population of *B. undulans* is identical to that of the German population (AM713183) investigated by Schmidt *et al*. (2007). It is noteworthy, however, that phylogenetic relationships within the genus *Blepharisma* are largely unresolved because the SSU rDNA sequences of several species are either identical or differ only negligibly (Fernandes *et al*., 2013). Consequently, sequence data of genes that evolve more rapidly than SSU rDNA, e.g. the mitochondrial cytochrome oxidase I gene or the ribosomal internal transcribed spacer regions (ITS1 and IT2), are needed in order to resolve this problem.

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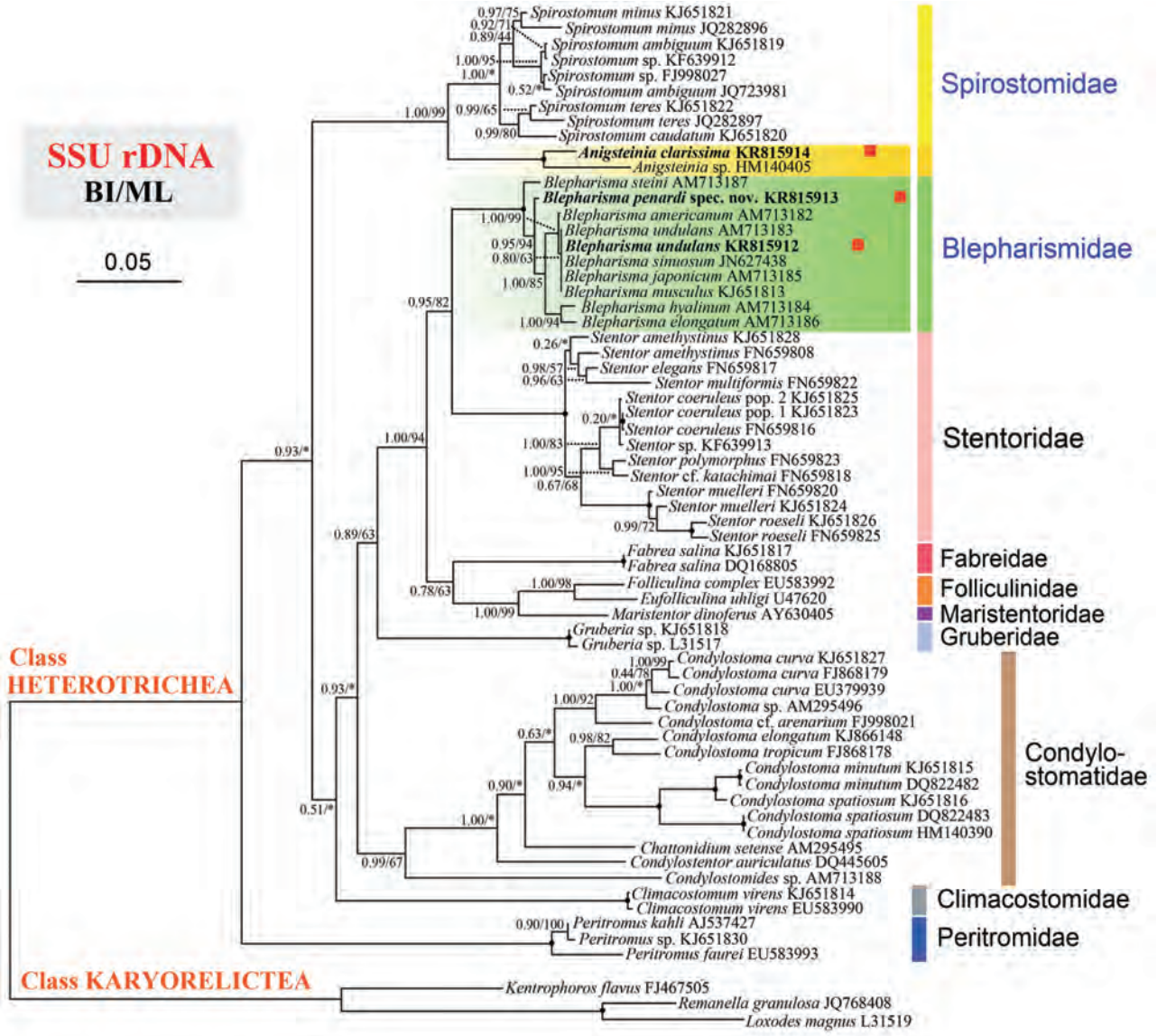

Figure 7. Bayesian inference (BI) tree inferred from small subunit (SSU) rDNA sequences showing the positions of *Anigsteinia clarissima*, ***Blepharisma penardi* sp. nov.**, and *Blepharisma undulans* (all three taxa in bold). Members of the class Karyorelictea are the outgroup taxa. Numbers near branches show the posterior probabilities of BI and bootstrap values from maximum likelihood (ML). Black circles indicate full support (1.00 BI, 100% ML) in both analyses. Disagreements between BI and ML are shown by asterisks. All branches are drawn to scale. The scale bar corresponds to five substitutions per 100 nucleotide positions.

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