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Taxonomy and molecular systematics of three oligotrich (s.l.) ciliates including descriptions of two new species, Strombidium guangdongense sp. nov. and Strombidinopsis sinicum sp. nov. (Protozoa, Ciliophora)

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Research Article

Taxonomy and molecular systematics of three oligotrich (s.l.) ciliates including descriptions of two new species, *Strombidium guangdongense* sp. nov. and *Strombidinopsis sinicum* sp. nov. (Protozoa, Ciliophora)

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In this study we investigated the morphology of three oligotrich (s.l.) ciliates, *Strombidium guangdongense* sp. nov., *Cyrtostrombidium paralongisomum* Tsai *et al.*, 2015 and *Strombidinopsis sinicum* sp. nov. *Strombidium guangdongense* sp. nov. is characterized by its elongate obconical to obovoidal body shape and widely spaced dikinetids in the girdle and ventral kineties. Another new species, *Strombidinopsis sinicum* sp. nov. is diagnosed by its small size and semi-globular body shape without mineral envelopes. Some additional morphological data of the recently described species *Cyrtostrombidium paralongisomum* Tsai *et al.*, 2015, such as the endoral membrane, are supplied based on our population. We also analysed the molecular phylogeny of each species based on small subunit rRNA (SSU rRNA) gene sequence data. The monophyly of *Cyrtostrombidium* is supported by our phylogenetic analyses, but the monophyly of *Strombidinopsis* and of the family Strombidinopsidae are both rejected by AU tests. In addition, *Strombidium* species have a tail branch separately from one another in phylogenetic trees, whereas strombidiids with a pigment spot group together, suggesting the latter character is a synapomorphy for this group of strombidiids.

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Key words: Cyrtostrombidiidae, phylogeny, Strombidiidae, Strombidinopsidae, SSU rRNA

Introduction

In recent years, molecular ecological methods such as clone library construction and high throughput sequencing have been increasingly used to evaluate ciliate diversity in environmental samples and this has led to the discovery of new ciliate assemblages (Orsi et al., 2011; Stoeck & Epstein, 2003). However, for many of these newly discovered 'molecular species' or 'operational taxonomic units (OTUs)' no morphological or behavioural data exist, preventing us from inferring their ecological roles and ecosystem function (Worden et al., 2015). Thus, detailed morphological investigations paired with gene sequences analyses are necessary, both for new and for insufficiently known species.

Oligotrich (s.l.) ciliates are often the dominant group in marine planktonic protozoan communities (Song, Wang, & Warren, 2000; Song, 2005; Suzuki & Song, 2001). Since most are effective grazers of bacteria, phytoplankton, and nanoflagellates, they are known to be an important component in the pelagic microbial food loop (Agatha & Struder-Kypke, 2014; Jeong, Shim, Lee, Kim, & Koh, 1999; Lee et al., 2015; Montagnes, Berger, & Taylor, 1996; Pierce &

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Turner, 1992; Stoecker & Capuzzo, 1990). Recently, many new oligotrich (s.l.) ciliate species have been reported, indicating that their biodiversity is greater than previously assumed (Gao, Gong, Lynn, Lin, & Song, 2009; Liu et al., 2013; 2015a; 2015b; Song et al., 2015a; 2015b). Some studies have concluded that more than 83−89% of the oligotrich species diversity is unknown (Agatha, 2011).

During faunistic studies on planktonic ciliates in coastal waters of China, two oligotrich (s.str.) ciliates, namely *Strombidium guangdongense* sp. nov., and *Cyrtostrombidium paralongisomum* Tsai et al., 2015, and one choreotrich ciliate, *Strombidinopsis sinicum* sp. nov., were found. Their morphological characters were investigated based on observations *in vivo* and following silver staining and their phylogenetic relationships were analysed based on SSU rRNA gene sequence data.

Materials and methods

Sample collection

All samples were collected using 20-μm mesh plankton nets from the upper 0.5 m of coastal waters off Zhanjiang, China. *Strombidium guangdongense* sp. nov. was found on 16 December 2009 (water temperature 19.0 °C, salinity 24.7‰, and pH 8.4). *Cyrtostrombidium paralongisomum* Tsai et al., 2015 was collected on 26 March 2010 (water temperature 19.7 °C, salinity 23.9‰, and pH 7.8). *Strombidinopsis sinicum* sp. nov. was collected on 21 March 2010 (water temperature 26.0 °C, salinity 25.9‰, and pH 8.9). Using micropipettes, specimens were directly isolated from the samples for further study. No cultures of them were established.

Morphological investigations

The behaviour of each of the three species was observed in Petri dishes (9 cm across; water depth 1 cm) under a dissecting microscope at about 20 °C. Cell morphology was investigated with a compound microscope equipped with bright-field and differential interference contrast optics. Protargol staining followed the protocol of Wilbert (1975). Counts and measurements on protargol-stained cells were performed at 1000× magnification; *in vivo* measurements were made at 40−1000× magnification. Drawings of live specimens were based on photomicrographs. Drawings of protargol-stained cells were made with help of a camera lucida at 1000× magnification. Terminology is mainly according to Agatha and Riedel-Lorjé (2006) and systematics follows Adl et al. (2012).

Extraction, amplification, and sequencing of DNA

DNA was isolated according to Gao et al. (2009); Gao, Gao, Wang, Katz, and Song (2014). Universal eukaryotic primers (Medlin, Elwood, Stickel, & Sogin, 1988) were used to amplify the SSU rRNA gene. PCR conditions followed Zhao, Gao, Fan, Strueder-Kypke, and Huang (2015). The PCR product was purified using the TIAN gel Midi Purification Kit (Tiangen Bio., Shanghai, China) and inserted into a pUCm-T vector (Sangon Bio., Shanghai, China). DNA from plasmids was sequenced at the Invitrogen sequencing facility in Shanghai, China.

Phylogenetic analyses

All available SSU rRNA gene sequences of oligotrich (s.l.) ciliates from GenBank databases were included in present analyses. Representative species of Prostomatea, Phacodiniidia, Euplotia, and Hypotrichia were used as the outgroup taxa.

Sequences were aligned using Clustal X 1.83 (Jeanmougin, Thompson, Gouy, Higgins, & Gibson, 1998). Ends of alignments were trimmed and ambiguous sites were removed manually using Bioedit 7.0 (Hall, 1999) yielding a matrix of 1621 characters. Maximum likelihood (ML) analyses were carried out using RAxML-HPC2 on XSEDE v 8.0.24 (Stamatakis, 2006; Stamatakis, Hoover, & Rougemont, 2008) with the GTRGAMMA model on the online server CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010) (http://www.phylo.org/portal2/home.action). The reliability of internal branches was assessed using a multi-parametric bootstrap method with 1000 replicates, and searches for the best tree were conducted starting from 100 random trees. Bayesian inference (BI) analysis was performed with MrBayes 3.2.2 on XSEDE v 3.2.2 (Ronquist & Huelsenbeck, 2003) provided on the CIPRES Science Gateway with the model GTR+I+G selected by Akaike Information Criterion (AIC) in MrModeltest v2 (Nylander, 2004). Markov chain Monte Carlo was run for 1,000,000 generations with two parallel runs, each with four simultaneous chains, sampling every 100 generations. The first 2500 trees were discarded as a burn-in. The remaining trees were used to calculate the posterior probabilities (PP) applying the majority rule consensus.

PAUP* 4.0b 10 was used to generate the constrained ML trees under the GTR+I+G model to test the hypotheses that the genus *Strombidinopsis* and the family Strombidinopsidae are both monophyletic. The best-constrained trees, that is, those with the lowest lnLikelihood values, were compared with the unconstrained ML trees using the Approximately Unbiased (AU) test (Shimodaira, 2002) as implemented in CONSEL v. 0.1i (Shimodaira & Hasegawa, 2001).

Results

Taxonomy

Order Strombidiida Petz & Foissner, 1992
Family Strombidiidae Fauré-Fremiet, 1970
Genus *Strombidium* Claparède & Lachmann, 1859

Strombidium guangdongense sp. nov.
(Figs 1–23; Table 1)

Diagnosis. Marine *Strombidium* with cell size usually 20–35 × 10–20 μm *in vivo* and 24–41 × 12–20 μm after protargol staining; body shape variable from elongate obconical to obovoidal with small apical protrusion. Brown to black pigment spot located within apical protrusion. Extrusomes rod-shaped, about 10 × 0.5 μm. Macronucleus oblong to ovoid. 12–15 collar and 3–5 buccal membranelles; girdle kinety equatorial and ostensibly closed with 13–23 dikinetids; ventral kinety extending onto right ventral side and occupying posterior 2/5 portion of cell, composed of 4–7 dikinetids; all somatic dikinetids widely spaced, i.e., the distance between two neighbouring dikinetids up to five times the length of a dikinetid.

Type locality. Coastal waters off Zhanjiang (21°12′N, 110°25′E), Guangdong Province, China.

Etymology. The specific epithet 'guangdongense' refers to the fact that this species was discovered in the water of Guangdong.

Deposition of slides. A protargol slide containing the holotype specimen (marked with a black circle) is deposited at the Natural History Museum, London, with registration number NHMUK 2015.7.7.1. One protargol slide with paratype specimens is deposited in the Laboratory of Protozoology, Ocean University of China, Qingdao, with registration number LWW09121601.

Deposition of SSU rRNA gene sequence data. The SSU rRNA gene sequence is deposited in GenBank with accession number KJ609049; its length and G+C content are 1749 bp and 47.3% respectively.

Description. Cell size *in vivo* 20–35 × 10–20 μm, after protargol staining 24–41 × 16–23 μm. Body shape variable, usually elongate obconical to obovoidal (Figs 1, 8, 10–12). Cell anterior transversely truncated, collar region slightly domed to form an apical protrusion about 2 μm high *in vivo* (Fig. 6) but undetectable after protargol staining. Posterior end of cell normally bluntly rounded (Figs 10–12). In about 30% of individuals, a thorn-like tail found in the posterior end, which is thin and hyaline, often directed to left *in vivo* (Figs 13, 17, arrowheads). Tails contractile, generated from the hemitheca stretching posterior (Figs 8, 13, 14, arrowheads), and the length could extend up to 30% of cell length, undetectable after staining.

Pellicle thin and hyaline. Hemitheca covers posterior region of cell below girdle kinety (Figs 1, 17, arrow). Polygonal platelets not observed. Distended cell surface not recognizable in protargol-stained cells. Cytoplasm colourless, filled with numerous red granules (~1 μm in diameter); granules densely clustered in apical protrusion forming a brown to black pigment spot that appears black following protargol staining (Figs 1, 6, 12, 19, 23, arrows). Extrusomes prominent and rod-shaped, about 10 × 0.5 μm (Fig. 18). Extrusomes obliquely oriented to cell surface, not clustered, forming an equatorial funnel in mid-region of cell (Figs 1, 7, 15, arrow). Extrusome attachment sites produce a ~1 μm-wide strip located ~3 μm above girdle kinety after protargol staining (Figs 2, 3, arrow). Macronucleus oblong to ovoid, about 16–23 × 5–9 μm after protargol staining, centrally located and containing numerous chromatin granules ~1 μm across (Figs 3, 22). Contractile vacuole, cytopyge and micronucleus not recognized. In Petri dish with *in situ* water at room temperature, cells swim forward while rotating about main cell axis, interrupted by sudden changes of direction (Fig. 5).

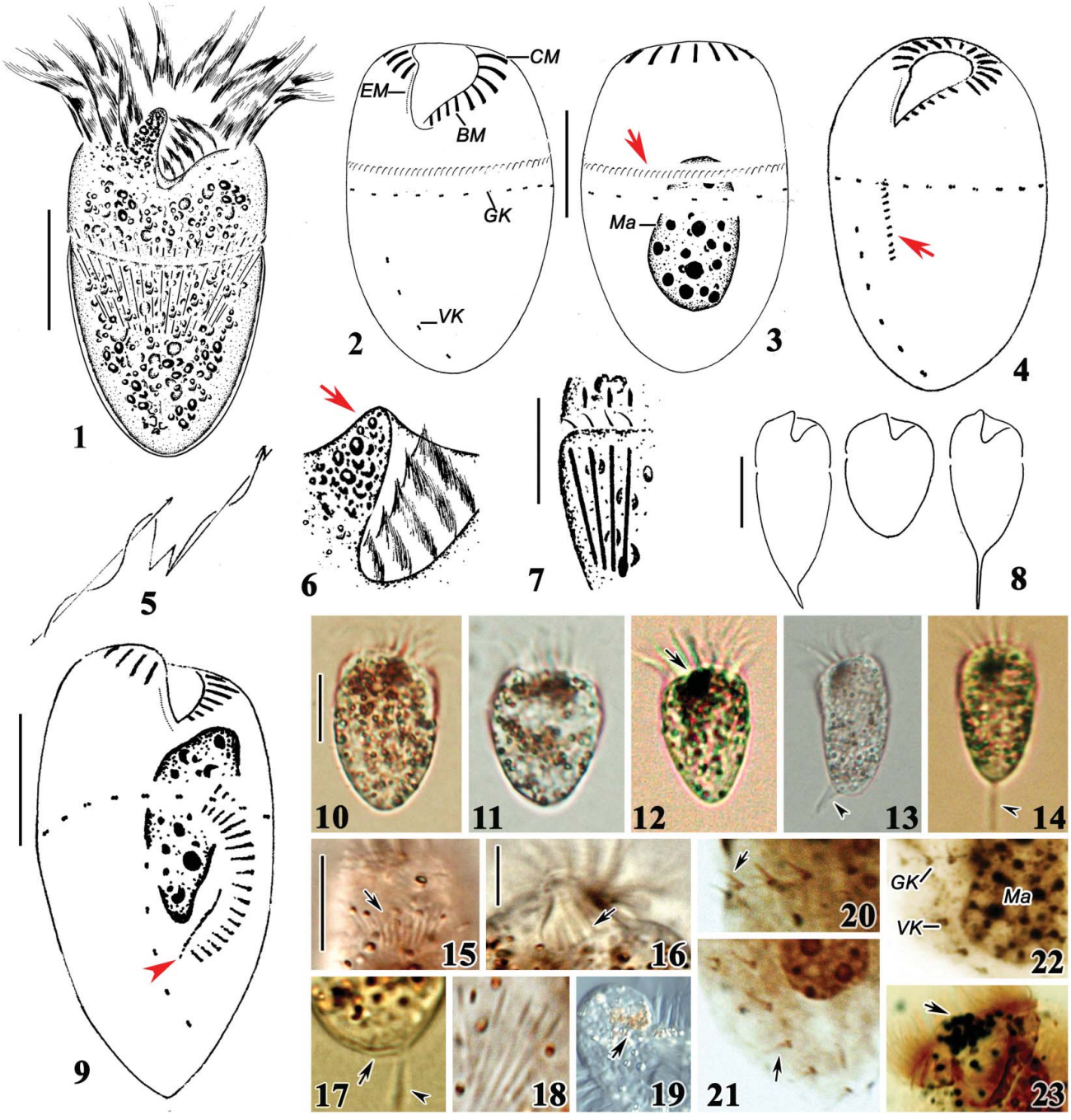
Buccal cavity narrow, extending about 20% down cell length (Figs 1, 2). Membranellar zone consists of 12 or 13 collar membranelles and 4 or 5 buccal membranelles, collar and buccal zones not separated. Collar membranelles with cilia up to 10 μm long *in vivo* which typically extend anteriorly (Fig. 1). Bases of collar membranelles about 4 μm long. Buccal membranelles located within oblique, shallow groove (Figs 2, 6, 16, arrow). Cilia of buccal membranelles about 2–4 μm long *in vivo*, bases about 2–3 μm long, decreasing in length progressively towards cytostome. Endoral membrane located on inner wall of right buccal lip, composed of a single row of kinetosomes (Fig. 2), rarely recognizable *in vivo*. Pharyngeal fibres not observed.

Somatic kineties composed of dikinetids, cilia ~1 μm long *in vivo*. Girdle kinety equatorial, horizontally oriented, ostensibly continuous (Figs 2, 3). Girdle kinety composed of 13–19 widely spaced dikinetids; within each dikinetid, only left basal body is ciliated (Figs 20, arrow; 22). Ventral kinety located in posterior 2/5 portion of cell, commencing about 5 μm below girdle kinety and right of buccal vertex, extending posteriad onto right ventral side and terminating at posterior end of cell; composed of 4–7 widely spaced dikinetids, only anterior basal body of each dikinetid is ciliated (Figs 2, 21, arrow; 22).

Morphogenesis. Some early dividers were observed. The oral primordium develops in a transient subsurface tube posterior to the girdle kinety and left of the ventral kinety (Figs 4, arrow, 9). The adoral zone of the opisthe is inverse C-shaped and longitudinally oriented. The new endoral membrane is positioned to the right of the proximal end of the opisthe's adoral zone (Fig. 9, arrowheads).

Family Cyrtostrombidiidae Agatha, 2004
Genus *Cyrtostrombidium* Lynn & Gilron, 1993

Cyrtostrombidium paralongisomum Tsai et al., 2015
(Figs. 24–44; Table 1)



Figs. 1–23. *Strombidium guangdongense* sp. nov. from life (1, 5–8, 10–19) and after protargol staining (2–4, 9, 20–23). (1, 10) Ventral views of typical specimen. (2, 3) Ventral (2) and dorsal (3) views of the holotype specimens showing the ciliary pattern and the macronucleus, arrow marks the stripe of argyrophilic fibres. (4, 9) Ventral views of an early and an early middle divider. The oral primordium (arrow) is located below the girdle kinety and left of the ventral kinety with the new endoral membrane (arrowhead) to the right of the adoral zone. (5) Swimming trace. (6, 19) Detail of apical protrusion showing the pigment spot (arrows). (7) Detail of extrusomes attached above the girdle kinety. (8, 11–14) Different body shapes, arrowheads mark the tail and arrow marks the pigment spot. (15) Anterior portion of cell showing the extrusomes (arrow). (16) Detail of anterior portion of cell, arrow marks the buccal membranelles. (17) Posterior portion of cell showing the tail (arrowhead) generated from the the hemitheca (arrow) stretching posterior. (18) Resting extrusomes. (20–22) Detail of girdle and ventral kineties and macronucleus, arrows mark the somatic cilia. (23) Anterior portion of cell showing the pigment granules (arrow). BM, buccal membranelles; CM, collar membranelles; EM, endoral membrane; GK, girdle kinety; Ma, macronucleus; VK, ventral kinety. Scale bars: Figs 1–4, 8–15, 17: 10 μm; Figs 6, 7, 16, 18–23: 5 μm.

Table 1. Morphometric characterizations of *Strombidium guangdongense* sp. nov., *Cyrtostrombidium paralongisomum* Tsai et al., 2015 (Zhanjiang population), and *Strombidinopsis sinicum* sp. nov.

Characters	Species name	Min	Max	Mean	SD	N
Cell length	*S. guangdongense*	24	41	33.5	4.3	22
	C. paralongisomum	76	95	84.3	7.3	7
	S. sinicum	33	46	40.2	4.2	23
Cell width	*S. guangdongense*	16	23	19.9	1.8	22
	C. paralongisomum	26	39	32.6	4.6	7
	S. sinicum	37	46	42.3	2.6	23
Macronucleus, length	*S. guangdongense*	16	23	18.5	2.2	17
	C. paralongisomum	21	36	28.8	5.5	5
	S. sinicum	16	22	19.0	1.5	20
Macronucleus, width	*S. guangdongense*	5	9	6.7	1.1	17
	C. paralongisomum	16	22	18.6	2.3	5
	S. sinicum	8	15	12.0	1.7	20
Collar membranelles, number	*S. guangdongense*	12	13	12.4	0.5	14
	C. paralongisomum	13	15	14	0.8	7
	S. sinicum	15	18	16.2	0.8	22
Buccal membranelles, number	*S. guangdongense*	4	5	4.3	0.5	12
	C. paralongisomum	-	-	-	-	-
	S. sinicum	1	1	1.0	0.0	15
Anterior cell end to cytostome, distance	*S. guangdongense*	6	9	7.4	0.8	21
	C. paralongisomum	13	16	15.6	2.0	12
Anterior cell end to girdle kinety, distance	*S. guangdongense*	11	17	14.2	1.7	20
	C. paralongisomum	18	22	19.8	1.5	5
Posterior cell end to anterior end of ventral kinety, distance	*S. guangdongense*	9	17	13.2	2.5	20
	C. paralongisomum	45	61	54.8	6.7	5
Girdle kinety, number of dikinetids	*S. guangdongense*	13	19	16.7	1.6	17
	C. paralongisomum	49	60	54.6	4.3	5
Ventral kinety, number of dikinetids	*S. guangdongense*	4	7	4.8	0.9	20
	C. paralongisomum	39	50	46	4.4	5
Cytopharyngeal rods, number	*C. paralongisomum*	14	16	14.8	0.8	5
Cytopharyngeal basket length	*C. paralongisomum*	25	32	28.0	2.7	5
Cytopharyngeal basket width	*C. paralongisomum*	11	12	11.6	0.6	5
Somatic kineties, number	*S. sinicum*	20	26	23.3	1.7	21
Dikinetids in somatic kinety 1, number	*S. sinicum*	9	14	11.5	1.4	22

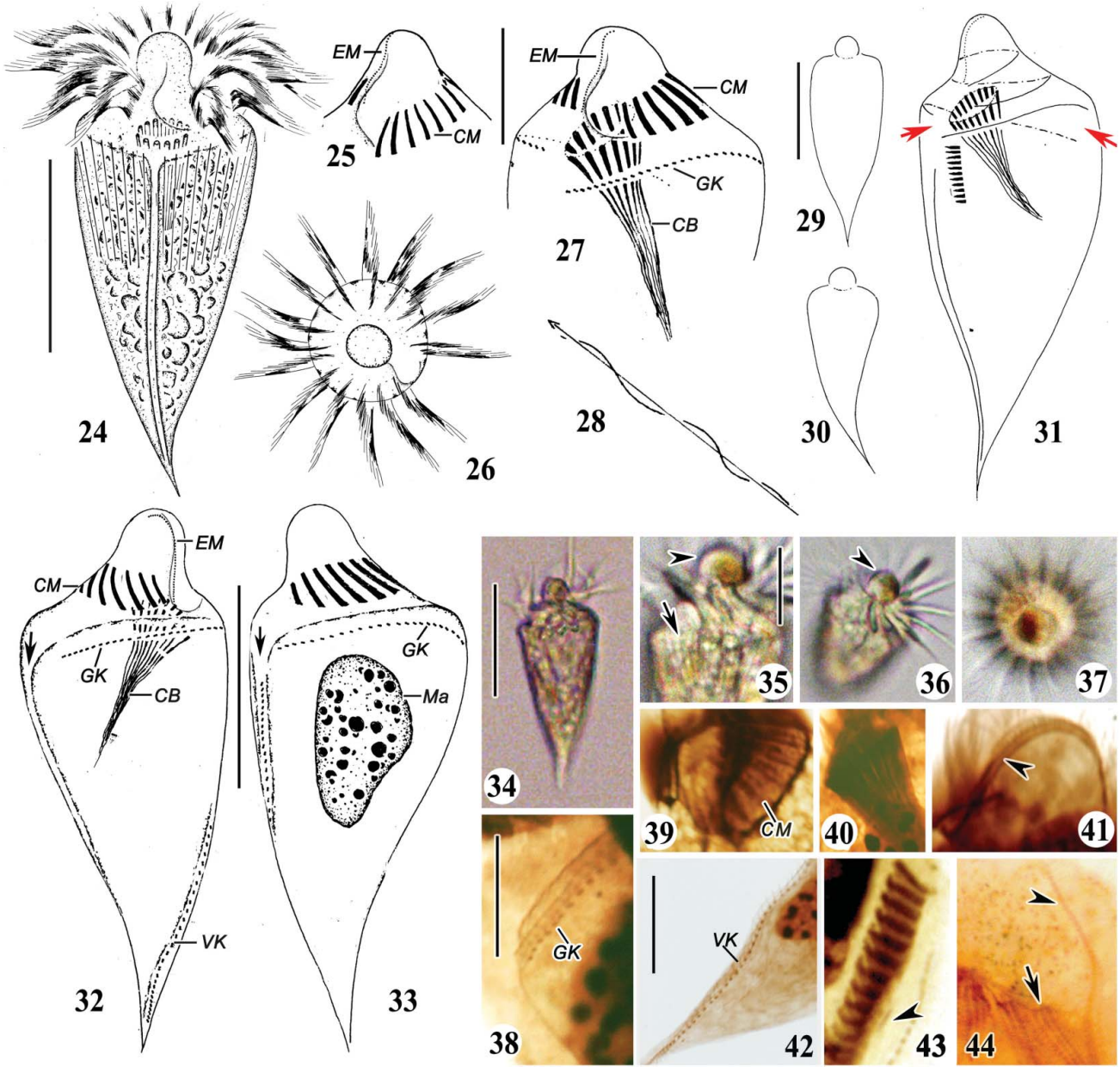
Data based on protargol-stained and randomly selected specimens. Measurements in μm.
Max, maximum; Mean, arithmetic mean; Min, minimum; N, number of specimens measured; SD, standard deviation.

Although the morphology of *Cyrtostrombidium paralongisomum* was studied by Tsai et al. (2015), some new or unique features have been found in Zhanjiang population. Thus a brief description of the new population is here supplied along with a phylogenetic analysis based on its SSU rRNA gene sequence.

Deposition of voucher specimen. One protargol slide containing the voucher specimen is deposited in the Laboratory of Protozoology, Ocean University of China, Qingdao, with registration number LWW2010032601.

Description of the Zhanjiang population. Cell size 60−80 × 20−30 μm *in vivo* and 76−95 × 26−39 μm after protargol staining. Anterior end domed centrally to form a conspicuous global apical protrusion about 6 μm high *in vivo* (Figs 24, 35, 36, arrowheads). Posterior portion slightly flattened bilaterally with posterior end pointed, forming a tail that is not contractile but can wiggle freely (Figs 24, 29, 30).

On the cell surface two longitudinal furrows beginning at ventral and dorsal gaps in girdle kinety respectively and extending to posterior end of cell (Figs 24, 32, 33, arrows). No polygonal platelets observed. Extrusomes about 20 × 1 μm each, oriented slightly obliquely to cell surface and evenly spaced (not in bundles) (Figs 24, 35, arrow). Macronucleus ellipsoidal to ovoidal, about 29 × 19 μm after protargol staining (Fig. 33). In Petri dish with



Figs. 24–44. *Cyrtostrombidium paralongisomum* Tsai et al., 2015 (Zhanjiang population) from life (24, 26, 28–30, 34–37) and after protargol staining (25, 27, 31–33, 38–44). (24, 34) Ventral views of typical specimen. (25) Lateral view of anterior portion to show the buccal zone and collar membranelles. (26, 37) Adoral view. (27) Anterior portion of cell to show the detail of oral apparatus with cytopharyngeal basket and girdle kinety. (28) Swimming trace. (29, 30) Different body shapes. (31) Ciliary pattern of an early divider, note the ventral and dorsal gaps (arrows) of girdle kinety. (32, 33) Right (32) and left (33) lateral views showing the ciliary pattern and the macronucleus, arrows mark the longitudinal furrows on the ventral and dorsal side. (35, 36) Anterior portion of cell, arrowheads indicate the apical protrusion and arrow marks the extrusomes. (38) Detail of the girdle kinety. (39) Collar membranelles. (40) Detail of cytopharyngeal basket. (41) Detail of apical protrusion, arrowhead marks the endoral membrane. (42) Posterior half part of cell. (43) Oral primordium with the new endoral membrane (arrowhead) of an early divider. (44) Detail of anterior portion of cell, arrowhead marks the endoral membrane and arrow marks the collar membranelles. CB, cytopharyngeal basket; CM, collar membranelles; EM, endoral membrane; GK, girdle kinety; Ma, macronucleus; VK, ventral kinety. Scale bars: Figs 24, 26, 29–34, 36, 37: 40 μm; Figs 25, 27, 35, 38–41, 43, 44: 10 μm; Fig. 42: 20 μm.

in situ water at room temperature, cells swim forward in spirals while rotating about main cell axis (Fig. 28).

Buccal cavity narrowed, lying underneath apical protrusion and above cytopharynx (Figs 24, 25, 27). Adoral zone of membranelles surrounding apical protrusion, almost closed but with a ventral gap at location of buccal cavity, composed of 13−15 collar membranelles (Figs 25−27). Adoral zone slightly dextrally spiralled; proximal end of adoral zone positioned slightly lower than distal end. Bases of membranelles about 5−7 μm long (Fig. 44, arrow); length of last three proximal membranelles decreases slightly from left to right (Figs 25, 27, 39). Cilia of membranelles ~25 μm long *in vivo*, stretching laterally giving a whorl-like appearance in apical view when swimming (Figs 26, 37). Endoral membrane on right inner wall of buccal cavity, beginning near distalmost membranelle, extending upward, and terminating on the top of apical protrusion (Figs 25, 27, 32, 41, 44, arrowheads). Endoral membrane composed of a single row of kinetosomes, each bearing a cilium about 5 μm long after protargol staining. Cytopharynx 12 μm in diameter and surrounded by 15 cytopharyngeal rods (nematodesmata) each about 28 μm long, extending obliquely backwards and terminating in mid-region of cell (Figs 27, 31, 32, 40).

Girdle kinety split into two parts by two gaps which are located at sites of ventral and dorsal furrows of hemitheca, each about 5 μm wide (Figs 27, 31, arrows). Girdle kinety consisting of 49−60 dikinetids (left part 24−31, right part 25−29), asymmetrically arranged with region to right of ventral gap about 3 μm higher than region to left (Fig. 27). Ventral kinety positioned along ventral furrows, composed of 39−50 dikinetids. Only anterior basal body of each dikinetid is ciliated, bearing a ~3 μm-long rod-shape cilium.

Morphogenesis. Some early dividers were observed. The oral primordium develops as a cuneate, longitudinally oriented field of basal bodies with a short endoral membrane (Figs 31, 43).

Order Choreotrichida Small & Lynn, 1985
Family Strombidinopsidae Small & Lynn, 1985
Genus *Strombidinopsis* Kent, 1881

Strombidinopsis sinicum sp. nov.
(Figs. 45−62; Table 1)

Diagnosis. Marine species, cell size 25−40 × 30−45 μm *in vivo*, 33−46 × 37−46 μm after protargol staining. Body semi-globular without mineral envelope on the body surface. One buccal membranelle and 15−18 collar membranelles without elongated collar membranelles extending into the oral cavity. 20−26 somatic kineties with 9−14 dikinetids each. Two ellipsoidal macronuclear nodules connected by funiculus. Two globular micronuclei, one each in an indentation of macronuclear nodules.

Type locality. Coastal waters off Zhanjiang (21°12′N, 110°25′E), Guangdong, China.

Etymology. The specific epithet ‘sinicum’ refers to the fact that this species was discovered in Chinese waters.

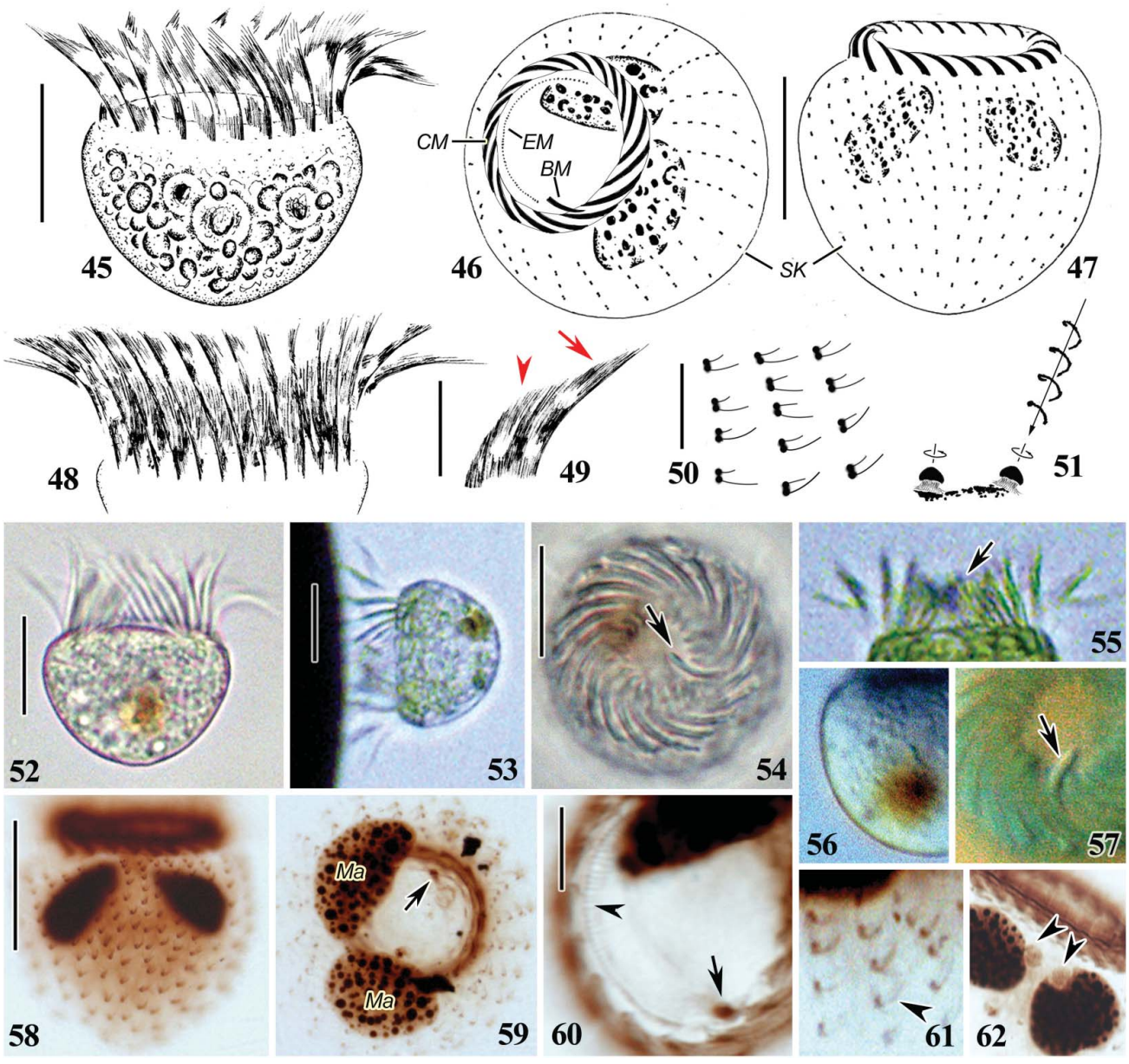
Deposition of slides. One protargol slide containing the holotype specimen (marked with a black circle) is deposited in the Laboratory of Protozoology, Ocean University of China, Qingdao, with registration number LWW2010032102. All other specimens on this slide are paratypes.

Deposition of SSU rRNA gene sequence data. The SSU rRNA gene sequence has been deposited in GenBank with accession number KR263893; its length and G+C content are 1748 bp and 46.0% respectively

Description. Size 25−40 × 30−45 μm *in vivo* and 33−46 × 37−46 μm after protargol staining. Body semi-globular or bowl-shaped with adoral region narrower than body portion; length:width ratio about 1:1−1.5 *in vivo* (Figs 45, 52, 53). Anterior end of body transversely truncated, centrally domed to form a 2 μm-high apical protrusion that is arched and slightly contractile, moving up and down during feeding. Posterior end broadly rounded (Figs 45, 52).

Cytoplasm colourless, with abundant lipid droplets 2−4 μm across and food vacuoles 5−9 μm across containing remnants of ingested bacteria and flagellates (Fig. 45). Cell surface smooth, without mineral envelope (Figs 52, 56). Extrusomes and contractile vacuole not observed. Two ellipsoidal macronuclear nodules connected by funiculus, lying in a V-shaped configuration below adoral zone, each nodule containing numerous nucleoli, 0.5−2 μm across (Figs 46, 59). Two globular micronuclei about 2−3 μm across, each lying in an indentation of macronuclear nodules (Fig. 62). In Petri dish with *in situ* water at room temperature, cells swim forward in spirals while rotating about main cell axis, or glide over the surface of substrate to which cell attaches via its membranelles while constantly rotating (Fig. 51).

Oral apparatus occupying anterior end of cell. Oral cavity extending posteriad to about 10% of cell length. Zone of collar membranelles closed, comprising 15−18 membranelles. Each membranelle divided into a narrow outer portion with ~15 μm long cilia bending distinctively outwards (Figs 48 and 49, arrow), and a wide inner portion with ~10 μm long cilia which often project above the oral cavity giving a flame-like appearance (Fig. 55, arrow); all polykinetids of same structure and length, i.e., composed of three rows of basal bodies, none extending into oral cavity (Fig. 46). Single buccal membranelle with outer end located between two buccal membranelles and inner end curved into oral cavity (Figs 46, 54, 57, 59, 60,



Figs. 45–62. *Strombidinopsis sinicum* sp. nov. from life (45, 48, 49, 51–57) and after protargol staining (46, 47, 50, 58–62). (45, 52) Lateral views of typical specimen. (46, 59) Adoral views showing the ciliary pattern and the macronucleus, arrow marks the buccal membranelle. (47, 58) Lateral views of holotype specimen. (48, 55) Cilia of collar membranelles, arrow marks the inner portions of oral cilia. (49) Detail of collar membranelles, arrow marks the long outer portion and arrowhead marks the short inner portion. (50, 61) Detail of somatic cilia (arrowhead). (51) Swimming trace. (53) Specimen attaching to substrate. (54, 57) Adoral views of collar membranelles, arrows mark the buccal membranelle. (56) Detail of cortex to show the smooth cell surface. (60) Detail of oral zone to show the buccal membranelle (arrow) and endoral membrane (arrowhead). (62) Macronuclear nodules and micronuclei (arrowheads). BM, buccal membranelle; CM, collar membranelles; EM, endoral membrane; Ma, macronuclear nodule; SK, somatic kinety. Scale bars: Figs 45–47, 52–58, 62: 20 μm, Figs 48, 49: 10 μm, Figs 50, 60: 5 μm.

arrows). Paroral membrane inconspicuous, composed of a single row of basal bodies lying within furrow at bottom of peristome, around which it performs nearly 1/2 turn before descending into oral cavity (Figs 46, 60, arrowhead).

Somatic cilia arranged in 20–26 kineties which generally commence below membranellar zone and extend to posterior end of cell, spiralling slightly dextrally (Figs 47, 57). Each kinety consisting of 9–14 dikinetids which are more densely arranged in anterior portion and sparsely arranged in posterior portion; each dikinetid lies parallel to kinety axis; in each dikinetid, anterior basal body bears a ~2 μm-long cilium, posterior basal body bears a ~3 μm-long cilium (Figs 50, 61, arrowhead).

SSU rRNA gene sequence analyses (Fig. 63)

The pairwise distances of SSU rRNA gene sequences between *Strombidium guangdongense* and other sequenced *Strombidium* species ranged from 90.4% to 96.0%, in which *S. purpureum* had the highest similarity with *S. guangdongense* (Table S1, see online supplemental material, which is available from the article's Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2016.1162872). The SSU rRNA gene sequence of the Zhanjiang population of *Cyrtostrombidium paralongisomum* had a 99.8% similarity (i.e., differed by four nucleotides) with that of the Taiwan population, and a 99.6% similarity with *C. longisomum*, from which it differed by six nucleotides (Table S2, see supplemental material online). Among the choreotrichids, *Strombidinopsis sinicum* had a highest similarity with *Parastrombidinopsis minima* (95.2%) and lowest with *Pelagostrobilidium minutum* (92.0%); similarities between *S. sinicum* and its congeners *S. acuminata* and *S. jeokjo* were both 92.4% (Table S3, see supplemental material online).

In the SSU rRNA gene trees, the subclass Oligotrichia is sister to Lynnellidae. Within Oligotrichia, the families Cyrtostrombidiidae and Tontonnidae are both monophyletic whereas Stombidiidae is polyphyletic. *Strombidium guangdongense* is nested within the clade containing *Williophrya meadai* and nine *Strombidium* species, although other species of *Strombidium* are distributed among several different clades suggesting that this genus is not monophyletic. The most closely related species to *S. guangdongense* in our phylogenetic tree is *S. purpureum* (Fig. 63), although their kinship is only weakly supported (ML 58% and BI 0.55). The representatives of *Cyrtostrombidium* form a clade within which the two populations of *C. paralongisomum* group together, followed by C. *longisomum.* Within Choreotrichia, *Strombidinopsis sinicum* does not cluster with its congeners but instead is more closely related to the strobilidiid clade, albeit with only low support (ML 55% and BI 0.68), followed by the clade comprising *Parastrombidinopsis shimi* and *P. minima* (ML 59% and BI 0.89). *Strombidinopsis acuminata* and *S. jeokjo* cluster together, forming the basal branch of the choreotrichs (Fig. 63). The monophylies of the genus *Strombidinopsis* and of the family Strombidinopsidae are both rejected by the AU test ($P = 0.005$ and 0.003, respectively).

Discussion

Comparison of *Strombidium guangdongense* sp. nov. with similar species

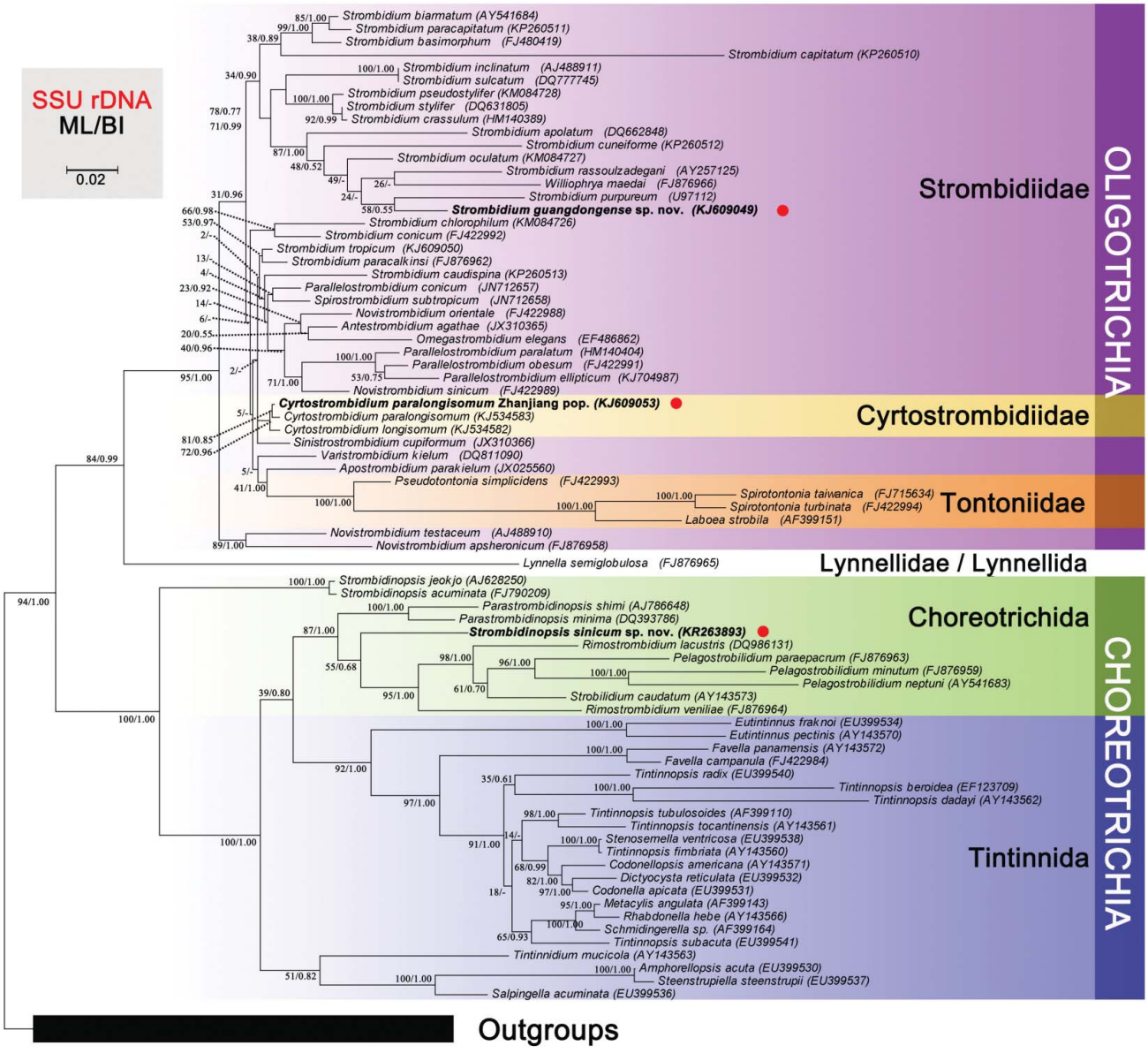
In terms of the pigment spot in the apical protrusion, *S. guangdongense* sp. nov. should be compared with four congeners, namely *S. cuneiforme*, *S. apolatum*, *S. oculatum* and *S. rassoulzadegani* (McManus, Xu, Costas, & Katz, 2010; Montagnes, Lowe, Poulton, & Jonsson, 2002; Song et al., 2015a; 2015b). However, *S. guangdongense* can be distinguished from each of them by its widely spaced dikinetids, both in the girdle and in the ventral kinety, i.e., the distance between two neighbouring dikinetids in *S. guangdongense* is up to five times the length of a dikinetid (vs. up to two to three times dikinetid length). Hitherto, the possession of widely spaced dikinetids in the girdle and ventral kineties has only been reported in *Strombidium globosaneum* (Song & Packroff, 1997). However, *S. guangdongense* sp. nov. differs from *S. globosaneum* by: (1) the elongate obconical body shape (vs. globular shape); (2) presence (vs. absence) of extrusomes; (3) the position of the ventral kinety on the right (vs. in the middle) of the body when viewed ventrally; (4) the distinct gap between the girdle kinety and the anterior end of the ventral kinety (vs. girdle kinety close to the anterior end of the ventral kinety) (Song & Packroff, 1997).

Comparison of tails in oligotrichs

In some individuals of *Strombidium guangdongense* sp. nov., a thorn-like tail was found in the posterior cell end. Detailed observation reveals that this tail comes from the elongation of the hemitheca and is contractile with the length variable from 0−30% of cell length. Consequently it is variable in length among different individuals and is undetectable after protargol staining. Up to now, eight congeners have been reported to possess tails, namely *S. pseudostylifer*, *S. caudispina*, *S. rapulum*, *S. rassoulzadegani*, *S. stylifer*, *S. parastylifer*, *S. foissneri*, and *S. minor* (Kahl, 1935; McManus et al., 2010; Song & Packroff, 1997; Song, Warren, & Hu, 2009; Song et al., 2015a, 2015b; Xu, Song, Sun, & Chen, 2006; Xu, Sun, Song, & Warren, 2008). However, their tails are generated from the elongation of cell plasmogen (thus cannot disappear), and non-contractile. Thus, the tail of *Strombidium guangdongense* can be easily distinguished from them.

The contractile tail is also possessed by tontoniids. However, their tails are parts of cell plasmogen without the ability of disappearance and extremely flexible (the length completely extended is up to 10−15 times of cell length) (Agatha, 2004). Consequently their tails are different from that of *Strombidium guangdongense*.

In some individuals of *Omegastrombidium elegans*, the hemitheca stretches posterior forming a spine which is short and easily disappeared (Song, Warren, & Hu, 2009). These characters are so similar with tails of *Strombidium guangdongense* that the tails of *Omegastrombidium elegans* and *Strombidium guangdongense* belong to the same tail type. The function of this kind of tail is not mentioned in previous studies. Nevertheless, the tail of *Strombidium guangdongense* extends and retracts more frequently



Figs. 63. Maximum likelihood tree inferred from small subunit rRNA gene sequences indicating the phylogenetic positions of *Strombidium guangdongense* sp. nov., *Cyrtostrombidium paralongisomum*, and *Strombidinopsis sinicum* sp. nov. Numbers at the nodes represent support values in the following order: Maximum likelihood (ML) bootstrap values, and Bayesian inference (BI) posterior probabilities. Nodes absent from one of the two phylogenies are indicated by a hyphen. The scale bar indicates 2 substitutions per 100 nucleotides.

under compression by cover-glass than when free swimming, and thus we supposed that the tail probably has something to do with pressure sensing of the cells.

Comparison of *Cyrtostrombidium paralongisomum* Tsai et al., 2015 with similar species

Cyrtostrombidium species are difficult to distinguish due to their similar body shapes and somatic kinety patterns, so morphometric data are usually needed in order to identify them. The Zhanjiang population of *C. paralongisomum* is similar both to the original (Taiwan) population and to *C. longisomum* in terms of the numbers of collar membranelles (13−15 vs. 12−15 and 11−14) and cytopharyngeal rods (14−16 vs. 14−20 and 15−17), but differs in cell size after protargol staining (76−95× 26−39 μm vs. 82−126 × 17−37 μm and 36−62 × 13−32 μm), the length of the ventral kinety (45−61 μm vs. 58−104 μm and 30−45 μm), and the number of dikinetids in the ventral kinety (39−50 vs. 45−54 and 27−39) (Tsai, Chen, & Chiang, 2015). However, there is

a considerably higher overlap in morphometry between the present population and *C. paralongisomum* than with *C. longisomum*. Indeed, we conclude the present population and *C. paralongisomum* to be so similar as to be conspecific.

Dissimilarities in the SSU rRNA gene sequences of the three populations of *Cyrtostrombidium* were below 1%, which was suggested as the threshold for OTU discrimination of ciliates in environmental molecular analyses (Doherty, Costas, McManus, & Katz, 2007; Tamura, Katz, & McManus, 2011). Differences in morphometry, however, suggest that *C. paralongisomum* is clearly separated from the two *C. longisomum* populations at species level. A similar situation was found in two tintinnid ciliates, both nominally identified as *Helicostomella subulata* due to their high similarity both in lorica morphology and SSU rRNA gene sequences (~99.5%). However, the high dissimilarity of their internal transcribed spacer 2 gene sequences indicates they represent two different (cryptic) species (Xu, Sun, Shin, & Kim, 2012). A subsequent study supported this conclusion and further discriminated three clusters within the genus *Helicostomella* based on multiple gene markers (Santoferrara, Tian, Alder, & McManus, 2015). Greater species sampling and data for additional gene markers are therefore required in order to gain a better understanding of the diversity and systematics of *Cyrtostrombidium*.

The endoral membrane in *Cyrtostrombidium* is documented here for the first time. Because of its unusual location, i.e., almost overlapping the right buccal lip, it is likely that the endoral membrane was overlooked in the Taiwan population of *C. paralongisomum* and perhaps in other species (Kim, Suzuki, & Taniguchi, 2002; Tsai et al., 2015). In the original report of *C. paralongisomum*, an argentophilic line is present on the apical protrusion in the illustration of protargol-stained specimens (Tsai et al., 2015), which is probably the endoral membrane. However, whether the endoral membrane has been overlooked, or is absent, in other cyrtostrombidiids requires further investigation.

Comparison of *Strombidinopsis sinicum* sp. nov. with similar species

Members of the genus *Strombidinopsis* are usually difficult to distinguish from one another because of their similar morphologies and the scarcity of characters for species separation. Six congeners, i.e., *S. azerbaijanica*, *S. elegans*, *S. minima*, *S. batos*, *S. sphaira*, and *S. chilorhax*, have a small cell size and thus should be compared with *S. sinicum* sp. nov. (Table 2) (Agatha, 2003; Alekperov & Asadullayeva, 1997; Lynn, Montagnes, Dale, Gilron, & Strom, 1991; Song & Bradbury, 1998).

Strombidinopsis azerbaijanica can be separated from *S. sinicum* sp. nov. by having three elongated collar membranelles that extend into the oral cavity (vs. no elongated collar membranelles extending into the oral cavity) and the absence (vs. one in *S. sinicum*) of buccal membranelles (Alekperov & Asadullayeva, 1997).

Strombidinopsis elegans differs from *S. sinicum* sp. nov. by having one elongated collar membranelle that extends into the oral cavity (vs. no elongated collar membranelles extending into the oral cavity), much higher numbers of collar membranelles (26 or 27 vs. 15−18), and only one micronucleus located between the two macronuclei (vs. two micronuclei, one each in an indentation of macronuclear nodules) (Song & Bradbury, 1998).

The body surface of *S. minima* is covered by a unique mineral envelope which is absent in specimens of *S. sinicum* sp. nov., both *in vivo* and following protargol staining. Moreover, their body shapes are remarkably different (broadly obconical or cylindical in *S. minima* vs. semiglobular in *S. sinicum* sp. nov.), thus these two species can easily be separated (Agatha, 2003).

Strombidinopsis sinicum sp. nov. can be distinguished from *S. batos*, *S. sphaira*, and *S. chilorhax* by its large body size (33−46 × 37−46 μm in protargol-stained specimens vs. 12−20 × 10−17 μm for *S. batos*, 18−25 × 16−28 μm for *S. sphaira*, 24−35 × 17−39 μm for *S. chilorhax*), more somatic kineties (20−26 vs. 10−16 in *S.*

Table 2. Morphological comparison among seven small *Strombidinopsis* species.

Species	Length	Width	ME	nCM	nBM	nECM	nSK	nDk	Reference
S. sinicum	33−46	37−46	absent	15−18	1	0	20−26	*c.* 12	Present study
S. azerbaijanica	15−20	15−20	absent	15−16	0	3	18−20	–	Alekperov and Asadullayeva (1997)
S. elegans	27−31	27−35	absent	26−27	1	1	19−24	*c.* 8	Song and Bradbury (1998)
S. minima	40−64	43−62	present	26−32	1	0	20−29	*c.* 15	Song and Bradbury (1998)
S. batos	12−20	10−17	present	14−17	1	0	10−16	*c.* 6	Lynn et al. (1991)
S. sphaira	18−25	16−28	present	13−15	1	0	13−15	*c.* 6	Lynn et al. (1991)
S. chilorhax	24−35	17−39	present	15−18	1	0	15−18	*c.* 10	Lynn et al. (1991)

Data based on protargol-stained specimens. Measurements in μm. ME, mineral envelope; nCM, number of collar membranelles; nBM, number of buccal membranelles; nECM, number of elongated collar membranelles; nSK, number of somatic kineties; nDk, number of dikinetids per somatic kinety; –, data unavailable.

batos, 13−15 in *S. sphaira*, 15−18 in *S. chilorhax*) and more dikinetids in a single somatic kinety (~12 vs. 6 in *S. batos* and *S. sphaira*, 10 in *S. chilorhax*). Although the mineral envelope was not mentioned in descriptions of *S. batos*, *S. sphaira*, and *S. chilorhax*, some tiny granules can be observed on their body surfaces in the original illustrations of protargol-stained specimens (Lynn et al, 1991). Considering that their living morphology has not been documented, the presence of a mineral envelope may have been overlooked in each of these three species. Therefore, the presence of a mineral envelope could be another character to separate *S. sinicum* sp. nov. from *S. batos*, *S. sphaira*, and *S. chilorhax*.

Phylogenetic analyses

In our phylogenetic trees, the tailed *Strombidium* species, i.e., *S. pseudostylifer*, *S. caudispina*, *S. rassoulzadegani*, *S. stylifer*, and *S. guangdongense*, do not cluster with tontoniids but instead nest within the oligotrich clade (Fig. 63). It is likely, therefore, that tails have evolved independently in these groups as an adaptation to life in pelagic biotopes. Furthermore, the pigment spot in the apical protrusion is shared in many strombidiids. In our phylogenetic trees, all species with a pigment spot, i.e., *S. guangdongense*, *S. cuneiforme*, *S. apolatum*, *S. oculatum*, *S. rassoulzadegani*, and *Williophrya maedai*, cluster together (Fig. 63), although *S. purpureum*, which is not known to have a pigment spot, is also nested within this clade. This finding suggests that the pigment spot might be a synapomorphy for this clade of strombidiids.

The family Cyrtostrombidiidae was established because species of this family possess unique cyrtos-like pharyngeal fibres and lack ventral membranelles compared with the family Strombidiidae (Agatha, 2004). In our phylogenetic trees, the three populations of *Cyrtostrombidium* for which SSU rRNA gene sequence data are available form a highly supported clade (Fig. 63). However, both in the present and in previous phylogenetic analyses (Tsai et al., 2015), *Cyrtostrombidium* nests within the family Strombidiidae. This suggests that the development of cyrtos-like pharyngeal fibres, and the disappearance of ventral membranelles probably happened late in oligotrichid evolution.

Morphologically, strombidinopsids differ from strobilidiids by having numerous longitudinal somatic kineties (composed of dikinetids) that extend the entire length of the cell (vs. some somatic kineties that spiral around the cell and are composed of monokinetids) (Lynn et al., 1991). However, the genus *Parastrombidinopsis*, which is currently assigned to the family Strombidinopsidae based on its morphology, clusters with Strobilidiidae in the SSU rRNA gene tree. Additionally, *Strombidinopsis sinicum* sp. nov. does not cluster with its congeners *S. acuminata* and *S. jeokjo*, but instead is more closely related to the strobilidiid clade (Fig. 63), and the monophylies of the genera *Strombidinopsis* and the family Strombidinopsidae were both rejected by our AU test. Thus the systematics of strombidinopsids remains unresolved pending the availability of more data, i.e., morphological, morphogenetic, and molecular, including sequence data from more taxa and from additional genes.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental data

Supplemental data for this article can be accessed http://dx.doi.org/10.1080/14772000.2016.1162872.

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