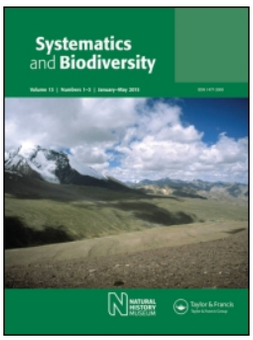

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Research Article

Morphology and molecular phylogeny of two new species of *Spirostrombidium* (Ciliophora, Oligotrichia), with a key to the species of *Spirostrombidium*

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Microscopic methods were used to investigate the morphological characterization of two novel oligotrich ciliates, *Spirostrombidium paraurceolare* sp. nov. and *Spirostrombidium faurefremieti* sp. nov., isolated from a mangrove wetland in Zhanjiang and an intertidal sandy beach in Qingdao, respectively. *Spirostrombidium paraurceolare* sp. nov. is characterized by three thigmotactic and 8–10 buccal membranelles, the girdle kinety spiralling around cell with one and a half whorls, and located at right anterior third of dorsal side anteriorly. *Spirostrombidium faurefremieti* sp. nov. can be recognized by a prominently deep and broad buccal cavity, two thigmotactic and 15–19 buccal membranelles, and the girdle kinety spiralling around cell with two whorls. The small subunit ribosomal RNA genes of these two species were sequenced and compared with those of their congeners to reveal nucleotide differences. Phylogenetic analyses revealed that the genus *Spirostrombidium* is non-monophyletic. *Spirostrombidium faurefremieti* sp. nov. falls into a clade comprising most congeners, but *Spirostrombidium paraurceolare* sp. nov. branches off and groups with *Varistrombidium kielum* with moderate support. A key to the identification of *Spirostrombidium* species is also provided.

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Key words: ciliature, planktonic ciliates, *Spirostrombidium paraurceolare* sp. nov., *Spirostrombidium faurefremieti* sp. nov., SSU rRNA gene, systematics

Introduction

Oligotrich ciliates are often an important component of the microbial loop among the marine plankton and contribute to the energy flux of the phytoplankton-based food web (Caron, Countway, Jones, Kim, & Schnetzer, 2012; Fenchel, 2008; Lynn, 2008). Although the somatic ciliature of the oligotrichs typically comprises only a girdle kinety and a ventral kinety, the diversity of ciliary patterns created by these two rows is considerable (Agatha, 2004a, b). More than 200 morphospecies have been reported so far (e.g., Agatha, 2011a, b; Gao et al., 2016a, 2016b; Liu et al., 2009, 2011, 2013, 2015a, 2015b, 2016, 2017, Song, 2005; Song et al., 2013, 2015a, 2015b, Tsai, Chen, & Chiang, 2015), but only about half of them have been well documented. Among these, the genus *Spirostrombidium* was established by Jankowski (1978), and redefined by Agatha (2004a). Its members are characterized by a long and spiralling girdle kinety, viz. 'girdle kinety dextrally spiralled, posterior portion inversely orientated and parallel to longitudinal ventral kinety' (Agatha, 2004a; Jankowski, 1978; Petz, Song, Wilbert, 1995). To date, although 15 species have been described as *Spirostrombidium*, for various reasons (e.g., researchers' expertise and equipment limitations at the time), several misidentifications or misdocumentations have been found among them (Agatha, 2004b; Corliss, 1986; Petz et al., 1995), and therefore most species of the genus need further taxonomic reinvestigations applying a combination of morphological and molecular data. In addition, molecular phylogenetic studies

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clarifying the relationships within the genus are also necessary (Gao et al., 2016a, 2017; Li, Liu, Gao, Warren, & Song, 2013; Xu, Song, Lin, & Warren, 2006a; Xu, Song, & Warren, 2006b).

In this work, two unknown *Spirostrombidium* taxa were investigated based on observations made on live cells and silver stained specimens. The SSU rRNA gene sequences were characterized and analysed in order to determine the phylogenetic position of the genus within Oligotrichia.

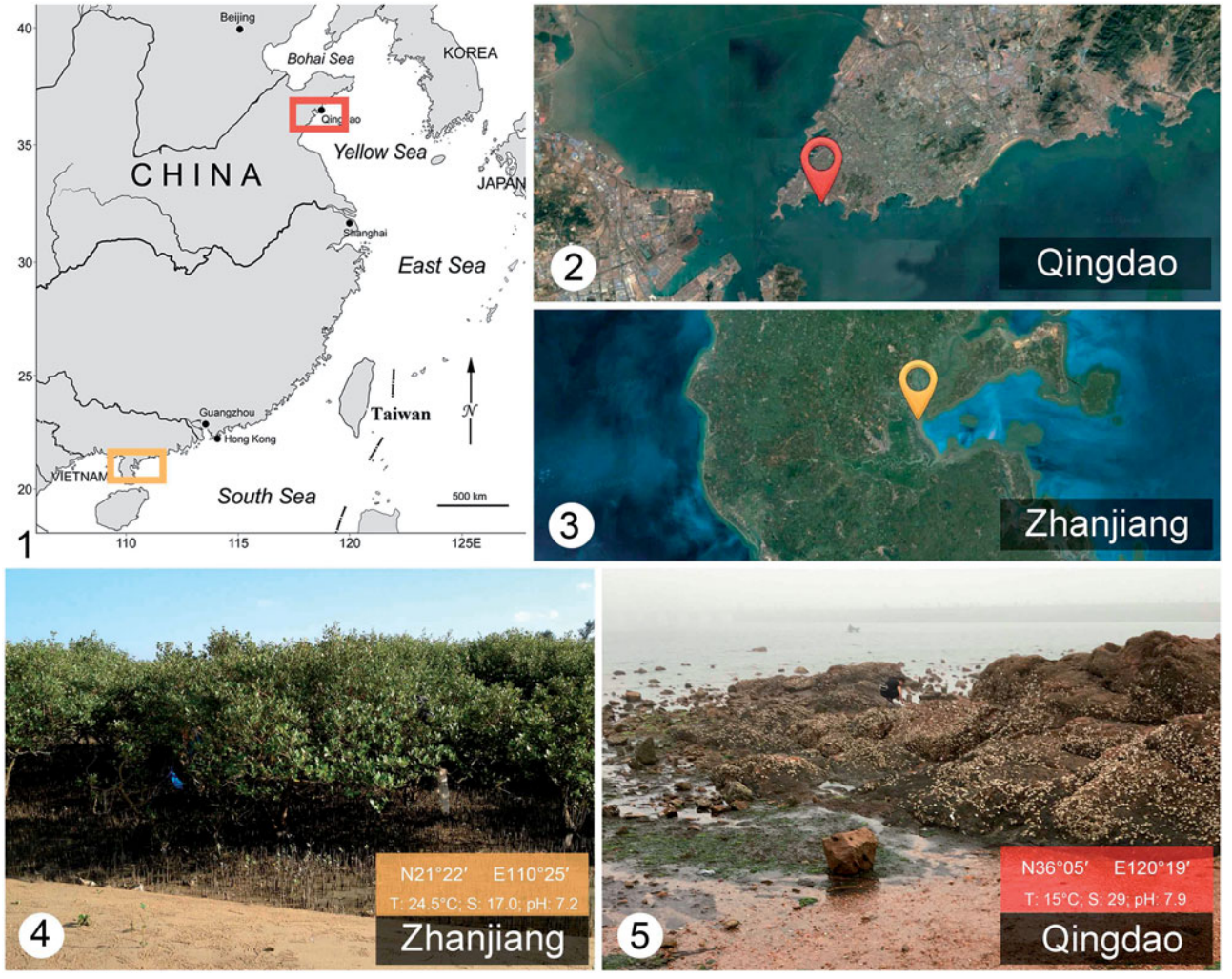
Materials and methods

Sample collection

Water samples were collected from coastal regions (Figs 1–3). *Spirostrombidium paraurceolare* sp. nov. was collected from a puddle in a mangrove wetland at low tide near Zhanjiang (21°22′N, 110°25′E), China (Fig. 4), in October 2013. Water samples were transferred with some rotten leaves to Petri dishes in the laboratory as raw cultures and then specimens were isolated for further study. *Spirostrombidium faurefremieti* sp. nov. was collected from an intertidal zone in Qingdao (36°05′N, 120°19′E), China (Fig. 5), in October 2016. Seawater was collected together with sandy sediments according to the method described by Xu, Fan, Al-Farraj, and Hu (2017).

Morphology

Living cells were isolated and observed using bright-field and differential interference contrast microscopy (Olympus BH-2, Japan) at 100–1000× magnifications.



Figs. 1–5. Locations of the sample sites. (1) Showing the locations of a mangrove wetland near Zhanjiang (21°22′N, 110°25′E), southern China and an intertidal zone in Qingdao (36°05′N, 120°19′E), northern China; (2) Landform of Qingdao in Google map; (3) Landform of Zhanjiang in Google map; (4, 5) The sample collecting sites.

The protargol staining method according to Wilbert (1975) was used to reveal the infraciliature and nuclear apparatus, with the protargol made according to Pan, Bourland, and Song (2013). Drawings of living cells were produced using free-hand sketches and photomicrographs, and drawings of silver-stained specimens were performed with the help of a camera lucida (Liu et al., 2016). Terminology is according to Agatha and Riedel-Lorjé (2006) and the classification follows Gao et al. (2016b).

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from cleaned cells using a DNeasy Tissue Kit (Qiagen, CA, USA). Primers 18s-F (5′-AAC CTG GTT GAT CCT GCC AGT-3′) and 18s-R (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) were used for SSU rRNA gene amplification according to Medlin et al. (1988). The PCR amplifications were performed according to the following protocol: 98 °C for 30 s, followed by 18 cycles of 98 °C for 10 s, 69 °C for 40 s with the remaining cycles stepping down by 1 °C for each cycle; then 72 °C for 90 s and 18 cycles of 98 °C for 10 s, 51 °C for 40 s, 72 °C for 90 s; and a final extension at 72 °C for 4 min. PCR products were purified by EasyPure Quick Gel Extraction Kit (Transgen Biotech, China), and then cloned using a pClone007 Blunt Simple Vector Kit (Tsingke Biological Technology, China). One clone was picked randomly and cultured in LB Broth medium for 12 h before sequenced in two directions by Tsingke Biological Technology Company (Beijing, China).

Phylogenetic analyses

All SSU rRNA gene sequences, including the two new sequences and 50 others obtained from the NCBI GenBank database, were used for the phylogenetic analyses. Five choreotrichid species, namely, *Strombidinopsis acuminate*, *Strobilidium caudatum*, *Rimostrombidium veniliae*, *Favella ehrenbergii* and *Eutintinnus frakno* were treated as the outgroup taxa. All 52 sequences were aligned using Bioedit (Hall, 1999) with default parameters, and the ends of alignments were trimmed yielding a matrix of 1,689 characters. Maximum likelihood (ML) analysis with 1,000 bootstrap replicates was performed in order to estimate the reliability of internal branches using RaxML-HPC2 on XSEDE 8.2.8 (Stamatakis, 2014), with the GTRGAMMA model provided on the online server CIPRES Science Gateway (Miller et al., 2010). Bayesian inference (BI) analysis was performed using MrBayes 3.2.6 on XSEDE v 3.2.6 (Ronquist et al., 2003) provided on the CIPRES Science Gateway with the model GTR + I+G selected by the Akaike Information Criterion (AIC) in MrModeltest v2 (Nylander, 2004). Markov chain Monte Carlo (MCMC) simulations were then run with two sets of four chains for 4,000,000 generations at a sampling frequency of 100 and a burn-in of 10,000 trees (25%). All remaining trees were used to construct the consensus tree. MEGA 4.0 (Tamura et al., 2007) analyses were used to visualize the tree topologies.

Results

Taxonomy

Order: Strombidiida Petz & Foissner, 1992
Family: Strombidiidae Fauré-Fremiet, 1970
Genus: *Spirostrombidium* Jankowski in 1978

Spirostrombidium paraurceolare sp. nov.
(Figs 6–26; Table S1)

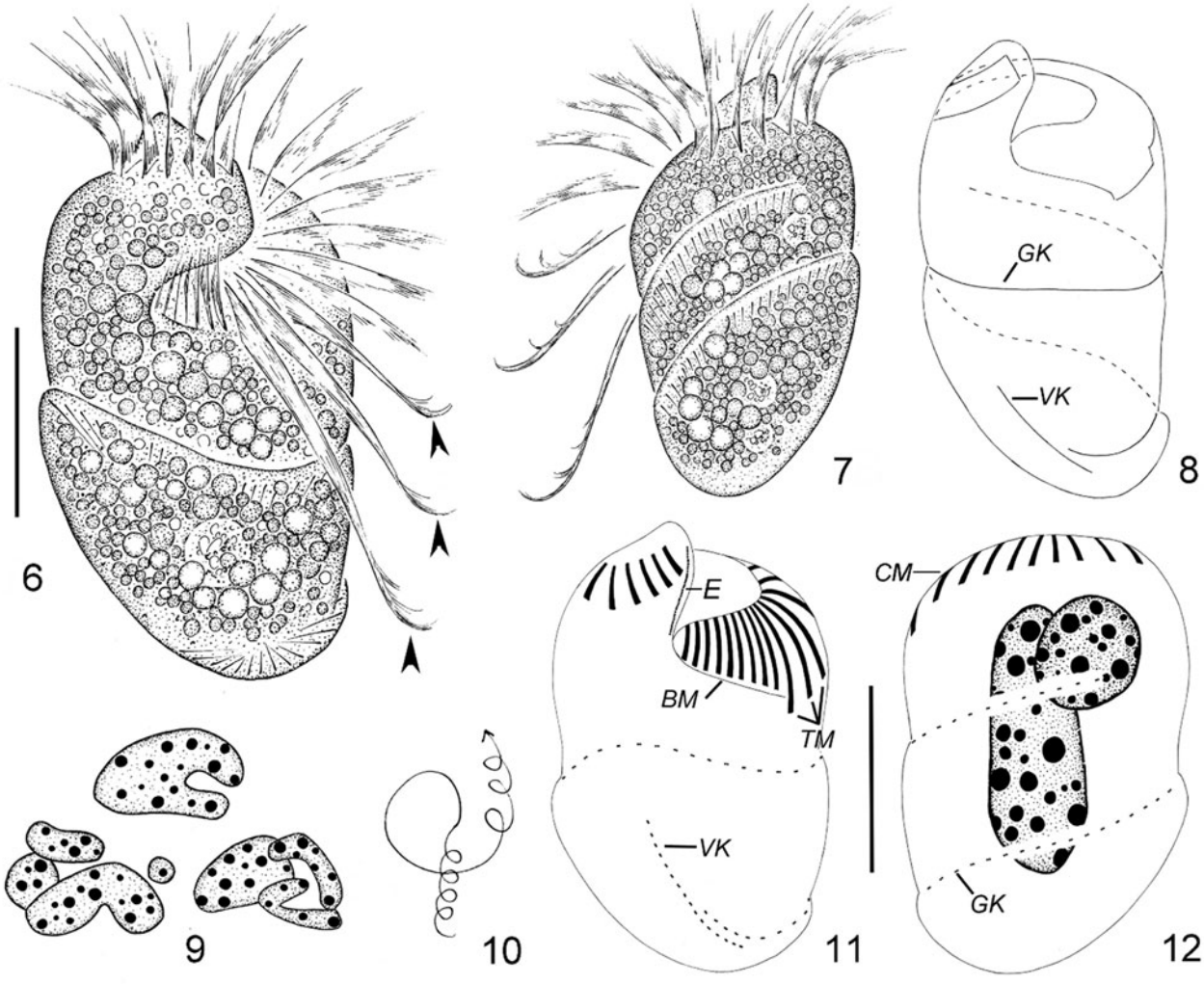
Diagnosis. *Spirostrombidium* with oblong asymmetrical cell shape, posterior end bluntly rounded and slanted slightly to the left; size about 40–60 × 20–40 μm *in vivo* and 23–43 × 16–25 μm after protargol staining; one to four macronuclear segments; 15–18 collar and 8–10 buccal membranelles, consistently three thigmotactic membranelles; girdle kinety composed of 47–63 dikinetids, spiralling around cell with one and a half whorls, and anterior end of girdle kinety located at right anterior third of dorsal side; ventral kinety composed of 12–17 dikinetids. Brackish habitat.

Ecological features. Brackish water, temperature 24.5 °C, salinity 17.0‰ and pH 7.2.

Type locality. Mangrove wetland near Zhanjiang (21°22′N, 110°25′E), Southern China.

Type deposition. A slide (registration number SW2013102403-1) containing the holotype specimen (Figs 11, 12) and two slides (registration numbers: SW2013102403-2 and SW2013102403-3) containing paratypes are deposited in the Laboratory of Protozoology, Ocean University of China.

Etymology. Combining the Latin prefix *para-* (meaning close) with the species epithet *urceolare* indicates the similarity in cell shape and oral membranelles between this species and *Spirostrombidium urceolare*.

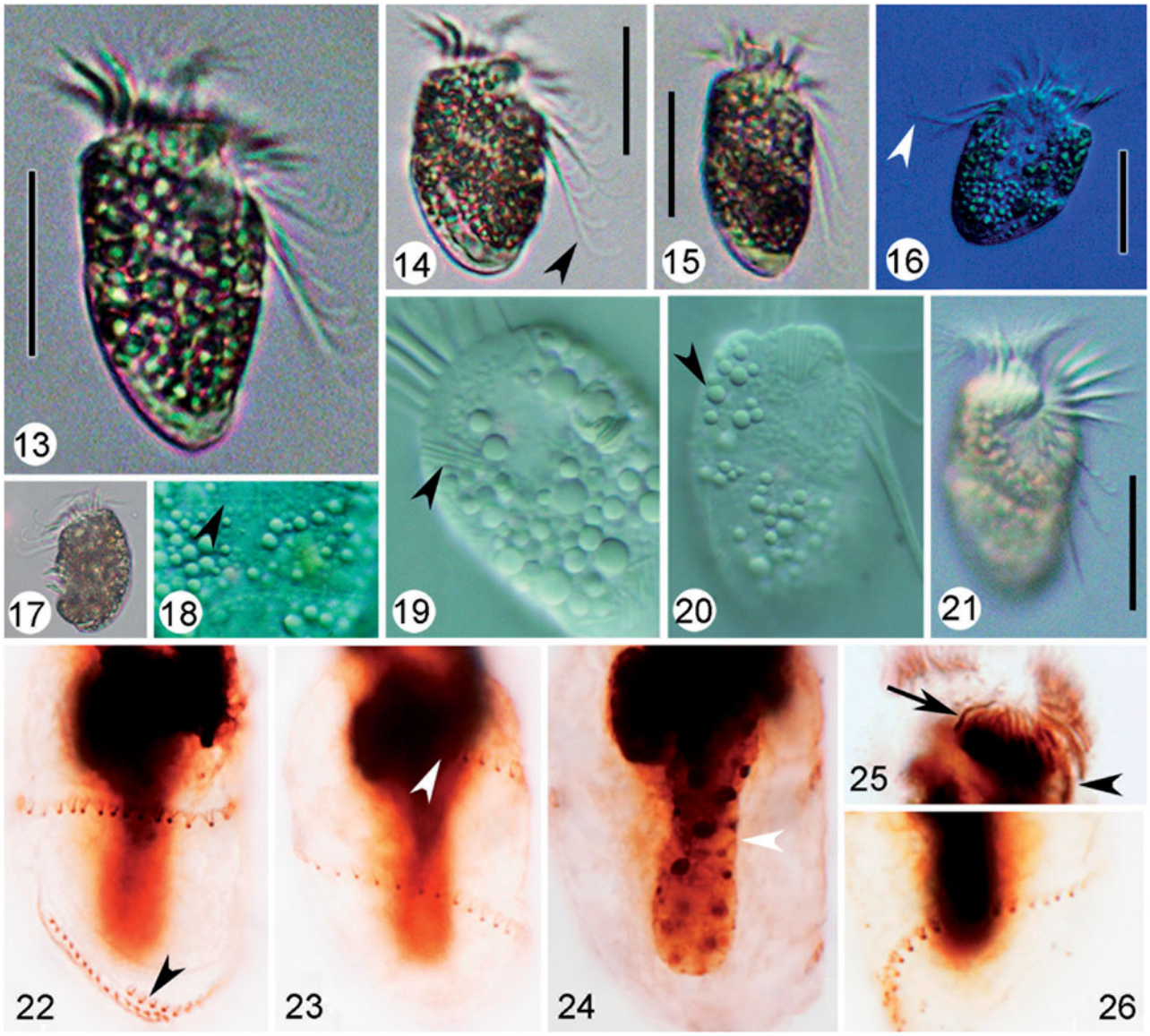


Figs. 6–12. *Spirostrombidium paraurceolare* sp. nov. from life (6, 7) and after staining with protargol (8–11); (6) Ventral view of a representative individual, arrowheads depict the three thigmotactic membranelles; (7) Dorsal view of a cell to show the distribution of extrusomes; (8) Pattern of somatic ciliature; (9) Variations of macronuclear segments; (10) Swimming trace; (11, 12) Ventral (11) and dorsal (12) views of the holotype specimen showing the ciliary pattern and macronuclear segments. BM, buccal membranelles; CM, collar membranelles; E, endoral membrane; GK, girdle kinety; TM, thigmotactic membranelles; VK, ventral kinety. Scale bars: 20 μm.

Description. Cell size ~40–60 × 20–40 μm *in vivo*, and 23–46 × 16–25 μm after protargol staining. Body oblong-shaped, posterior end bluntly rounded and slightly slanted leftwards, anterior end with an obvious apical protrusion, ~4 μm high, at right side of peristome; widest at mid-body of cell; dorsoventrally flattened with ratio of width: thickness ~3:2 (Figs 6, 7, 13, 21). Hemitheca absent.

Cytoplasm colourless, containing numerous lipid droplets about 2–5 μm across. Sometimes diatoms recognized in food vacuoles. Extrusomes rod-shaped, about 10 × 0.5 μm in size, arranged along the girdle kinety (Figs 18, 19). One to four, mostly two or three globular or sausage-like macronuclear segments centrally located in protargol-stained specimens (Figs 9, 12, 22–26), containing many roundish nucleoli (~1–2 μm across); micronucleus and cytopyge not recognized. Locomotion by lying on substratum, occasionally making anti-clockwise circles; sometimes crawling over debris using thigmotactic membranelles for attachment; when disturbed, swimming rapidly in spirals (Fig. 10).

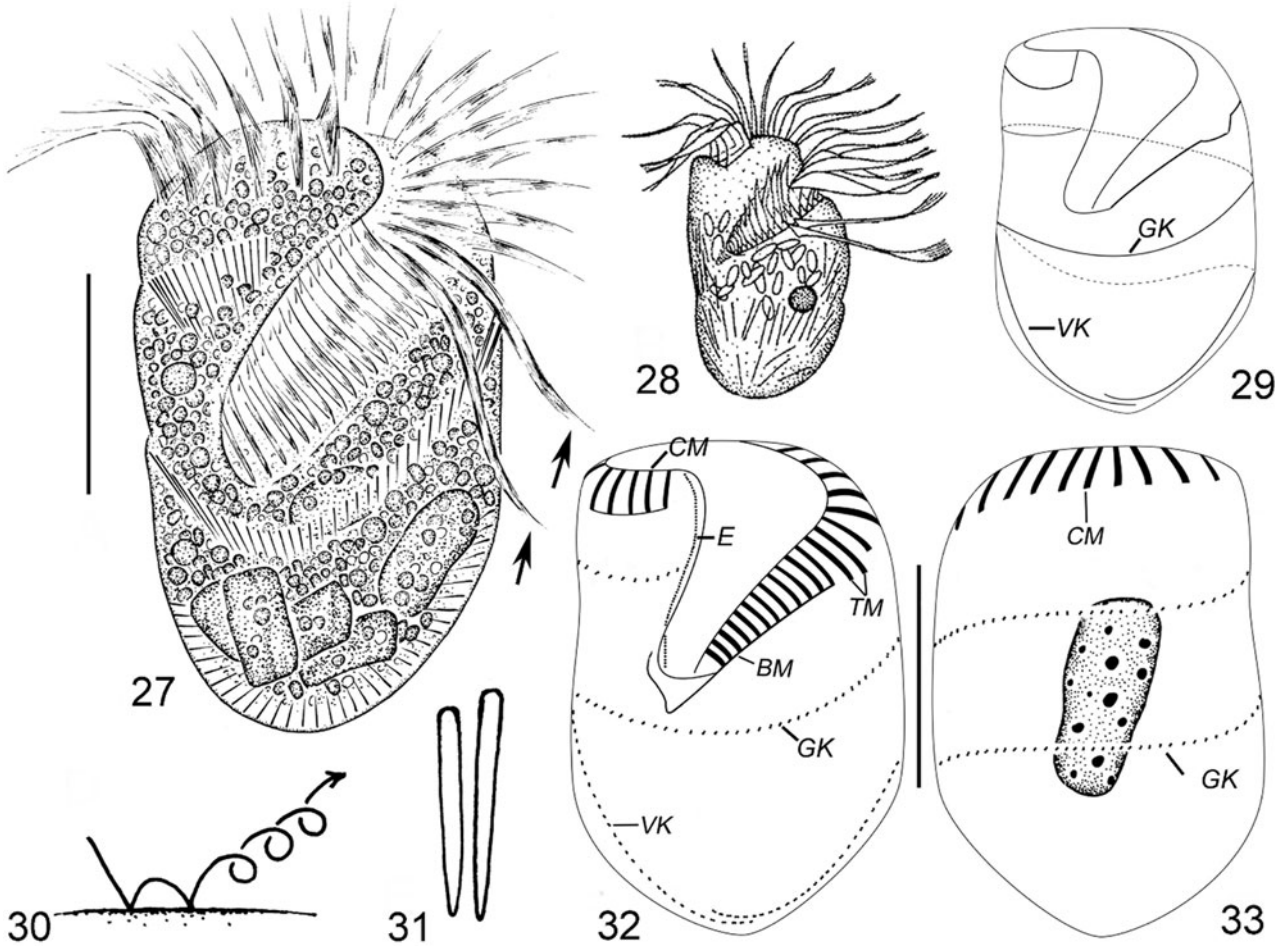
Buccal cavity prominent, extending obliquely rightward and terminating at the anterior third of the cell (Figs 6, 8, 21). Adoral zone of membranelles (AZM) continuous, composed of a collar portion with 15–18 membranelles, a buccal portion with 8–10 membranelles and three thigmotactic membranelles (Figs 6, 7, 13–16, 21). Each collar membranelle composed of three rows of basal bodies; bases of membranelles ~5 μm wide (Fig. 25), cilia about 12–16 μm long; bases of buccal



Figs. 13–26. Photomicrographs of *Spirostrombidium paraurceolare* sp. nov. from life (13–21) and after staining with protargol (22–26); (13) Ventral view of a representative individual; (14, 15) Ventral views of different cells, arrowhead shows thigmotactic membranelles; (16) Dorsal view of a live cell, arrowhead indicates the thigmotactic membranelles; (17) A divider; (18) Arrowhead shows extrusome girdle; (19) Dorsal view, arrowhead marks extrusomes; (20) Ventral view, arrowhead marks the endoplasmic granules; (21) Ventral views of anterior cell portion showing three thigmotactic membranelles; (22) Left lateral view of somatic ciliature, arrowhead marks the posterior end of the girdle kinety and ventral kinety; (23, 26) Dorsal view of somatic ciliature, arrowhead in K marks anterior end of the girdle kinety; (24) Macronuclear segments (arrowhead); (25) Overlooking of the buccal area, showing the endoral membrane (arrow) and the thigmotactic membranelle (arrowhead). Scale bars: 30 μm.

membranelles about 4 μm wide, cilia of buccal membranelles about 5 μm long. Three thigmotactic membranelles located between collar and buccal membranelles; the cilia length of the thigmotactic membranelles from distal to proximal one are 15, 20, 35 um, respectively; bases of membranelles ~7 μm wide (Figs 6, 13–16, 21). Endoral membrane positioned on the right inner wall of buccal cavity.

Somatic cilia ~1 μm long *in vivo*. Somatic ciliature comprises a girdle kinety and a ventral kinety. Girdle kinety consists of ~47–63 dikinetids, and commences in the posterior middle ventral area, extending by dextrally spiralling around cell with one and a half whorls, across dorsal and ventral sides to terminate at right anterior third of cell on dorsal side (Figs 8, 11, 12, 22, 23, 26). Ventral kinety consists of ~14 dikinetids, each



Figs. 27–33. *Spirostrombidium faurefremieti* sp. nov. from life (27, 28, 30) and after staining with protargol (29, 31, 32). (27) Ventral view of a representative individual, arrows mark thigmotactic membranelles; (28) Ventral view (original as *Strombidium sauerbreyae*, from Fauré-Fremiet, 1950); (29) Pattern of somatic ciliature; (30) Swimming trace; (31) Extrusomes; (32, 33) Ventral (32) and dorsal (33) views of the holotype specimen showing ciliary pattern and macronucleus. BM, buccal membranelles; CM, collar membranelles; E, endoral membrane; GK, girdle kinety; TM, thigmotactic membranelles; VK, ventral kinety. Scale bars: 20 μm.

having a cilium 1.5 μm in length. Ventral kinety located right of girdle kinety, commences at the posterior cell end; extends anteriorly, parallel to posterior end of girdle kinety and terminates in subequatorial region (Figs 8, 11, 22).

Spirostrombidium faurefremieti sp. nov.
(Figs 27–45; Table S1)

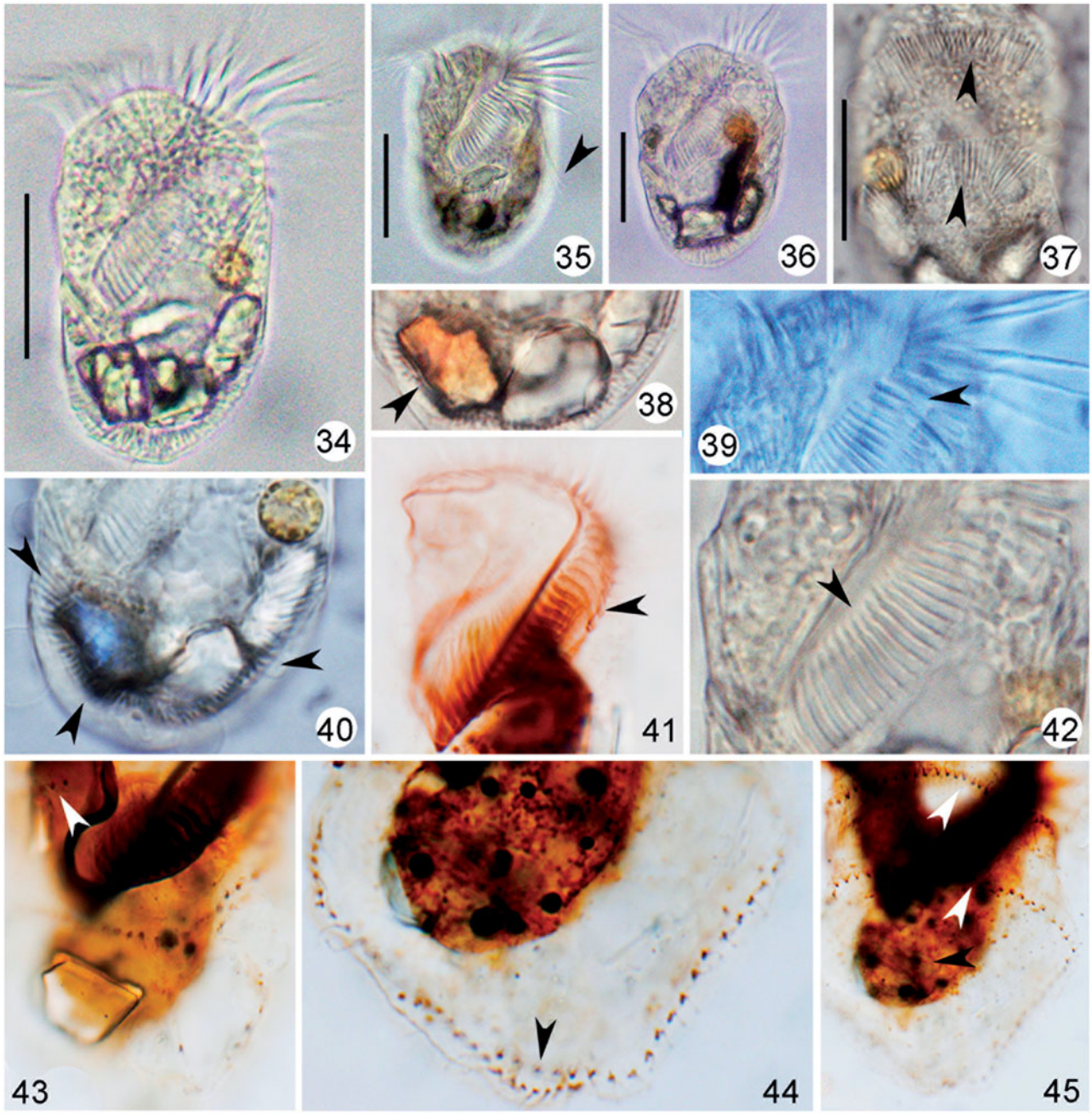
Diagnosis. Body broadly ellipsoidal with a deep buccal field extending to half of cell length. Size about 55–70 × 35–45 μm *in vivo* and 55–66 × 32–42 μm after protargol staining. Macronucleus ellipsoidal-shape, centrally located. Adoral zone composed of 23–28 collar, 15–19 buccal and two thigmotactic membranelles. Girdle kinety composed of 105–126 dikinetids, spiralling around cell with two whorls, and anterior end of girdle kinety located in shoulder region right of buccal field; ventral kinety composed of 25–29 dikinetids. Marine habitat.

Ecological features. Temperature 15 °C, salinity 29‰ and pH 7.9.

Type locality. Intertidal zone in Qingdao (36°05′N, 120°19′E), northern China.

Type deposition. A slide (registration number WR2016102002-2) containing the holotype specimen (Figs 32, 33) and two slides (registration numbers: WR2016102002-3 and WR2016102002-4) containing paratypes are deposited in the Laboratory of Protozoology, Ocean University of China.

Etymology. The specific epithet '*faurefremieti*' is named in honour of Emmanuel Fauré-Fremiet, a famous



Figs. 34–45. Photomicrographs of *Spirostrombidium faurefremieti* sp. nov. from life (34–40, 42) and after staining with protargol (41, 43–45); (34) Ventral view of a representative individual; (35, 36) Ventral view of two different individuals, arrowhead shows the thigmotactic membranelle; (37) Dorsal view showing the distribution of extrusomes (arrowheads); (38) Refractive particle in cytoplasm (arrowhead); (39) Part view of oral area, arrowhead shows the thigmotactic membranelles; (40) Ventral view of posterior cell portion. Arrowheads marks extrusomes; (41) Ventral view, arrowhead marks the thigmotactic membranelles; (42) Overlooking of the buccal area, arrowhead shows the buccal membranelles; (43) Ventral view of somatic ciliature, arrowhead marks the end of the girdle kinety; (44) Ventral view of somatic ciliature, arrowhead marks commencement of girdle and ventral kinety; (45) Girdle kinety (arrowheads). Scale bars: 25 µm.

ciliatologist who contributed extensively to the taxonomy and systematics of ciliates.

Description. Cell size about 55–70 × 40–45 µm *in vivo*, and 55–66 × 32–42 µm after protargol staining. Body shape generally constant, broadly ellipsoidal, widest at shoulder region, anterior and posterior ends rounded (Figs 27, 34–36). Apical protrusion inconspicuous *in vivo*. Dorsoventrally flattened, ratio of thickness: width about 2:3 (Figs 26, 34). Hemitheca absent.

Cytoplasm colourless (Fig. 34), usually containing many food vacuoles, which are about 2 μm in diameter and include diatoms. Usually 3–4 irregularly refractive particles, about 10–20 × 10–15 μm, present in posterior portion of cell (Figs 27, 38, 40). Extrusomes conspicuous, rod-shaped, 10 × 0.5 μm *in vivo*, arranged along girdle and ventral kineties (Figs 27, 31, 37, 40). Single, ellipsoidal macronucleus, about 25–35 × 13–17 μm in size after protargol staining, centrally located and containing numerous chromatin granules (Figs 33, 44, 45); micronucleus not recognized. Locomotion usually by crawling over debris using thigmotactic membranelles for attachment (Fig. 31); when disturbed, swimming rapidly in spirals.

Buccal cavity distinctly deep and prominent, extending obliquely towards right body side and terminating at about 1/2 of cell length (Figs 27, 29, 34–36, 41). Adoral zone composed of 23–28 collar, 15–19 buccal and two thigmotactic membranelles, each membranelle composed of three rows of basal bodies (Figs 27, 32, 34–36, 41). Two thigmotactic membranelles located between collar and buccal membranelles (Figs 27, 39, 41). Cilia of collar membranelles about 25 μm in length, bases of collar membranelles about 7 μm wide. Cilia of buccal membranelles about 3–4 μm long, bases of buccal membranelles about 7–10 μm wide, decreasing in width towards the cytostome. Cilia of thigmotactic membranelles conspicuously longer (40–45 μm) than those of collar membranelles and always directed posteriorly; bases of thigmotactic membranelles as wide as those of collar membranelles nearby. Endoral membrane located at the right inner wall of buccal cavity (Fig. 32).

Somatic ciliature composed of a girdle kinety and a ventral kinety. Girdle kinety consists of 105–126 densely arranged dikinetids, commencing in posterior ventral end and dextrally spiralling around cell twice, terminating in shoulder area on the right of buccal vertex (Figs 29, 32, 33, 43–45). Ventral kinety consists of ~25–29 densely arranged dikinetids. Ventral kinety commences at the posterior cell end, extends anteriorly, parallel to posterior end of girdle kinety and terminates at right side of cell and below the girdle kinety (Figs 29, 32, 44).

SSU rRNA gene sequences and phylogenetic analyses

The SSU rRNA gene sequences of *Spirostrombidium paraurceolare* sp. nov. and *S. faurefremieti* sp. nov. were deposited in GenBank with accession numbers, lengths, and guanine-cytosine (GC) content as follows: MG727704, 1729 bp, 45.98% and MG727703, 1670 bp, 48.80%, respectively (Fig. 54). A pairwise distance matrix of six *Spirostrombidium* species was generated, using the p-distance method (Table 1). Nucleotide pairwise distances ranged from 0.0043 to 0.0423. The highest nucleotide variation was between *S. schizostomum* and *S. paraurceolare* sp. nov., and the lowest genetic distance was found between *S. apourceolare* and *S. faurefremieti* sp. nov.

The topologies of the SSU rRNA gene trees constructed using ML and BI analyses are similar; therefore, only the ML tree is presented here, with support values from both methods at the branch nodes. As shown in the phylogenetic tree, the genus *Spirostrombidium* is polyphyletic as its members group into two separate clades including other strombidiids. Clade I includes five species of the genus viz. *S. schizostomum*, *S. agathae*, *S. apourceolare*, *S. subtropicum*, and *S. faurefremieti* sp. nov., and species of another four related genera (*Omegastrombidium*, *Antestrombidium*, *Novistrombidium*, *Parallelostrombidium*). *Spirostrombidium paraurceolare* sp. nov. clusters with *Varistrombidium kielum* and *Apostrombidium parakielum*, which together form clade II with *Strombidium paracalkinsi*; although this clade is not well supported (0.53 BI, 3% ML).

A key to the species in *Spirostrombidium*

Xu *et al*. (2006a) supplied a guide to the identification of seven *Spirostrombidium* spp. with ciliature data, and thereafter four new species were added. Here a new key is given below.

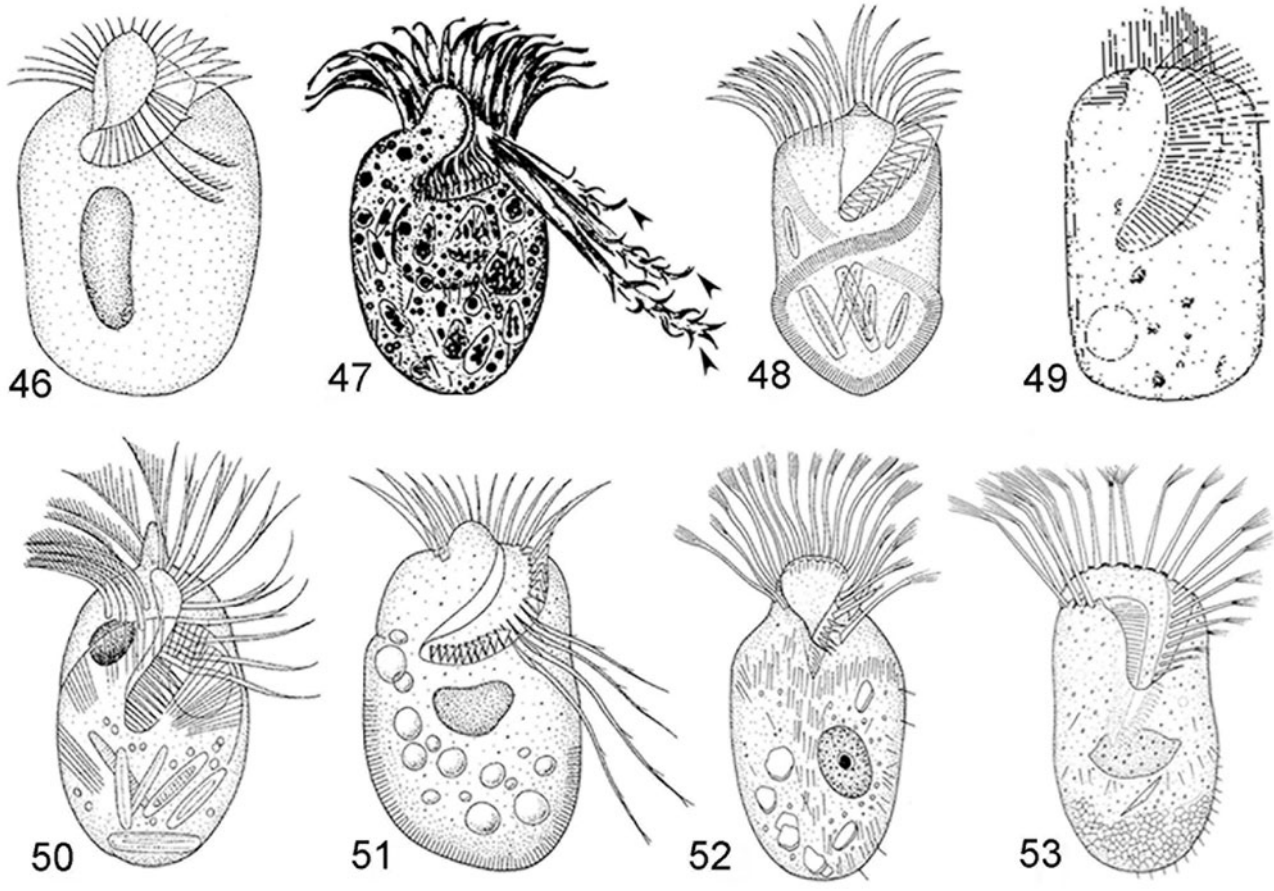
1 Free living forms 2
1′ Endocommensal in sea urchin ..... *S. echini*
2 Extra kinety present ..... *S. cinctum*
2′ Extra kinety absent 3
3 Three thigmotactic membranelles 4
3′ Two or without thigmotactic membranelles 5
4 13–16 buccal membranelles and more than 30 dikinetids in ventral kinety ..... *S. urceolare*
4′ 8–10 buccal membranelles and fewer than 20 dikinetids in ventral kinety ..... *S. paraurceolare* sp. nov.
5 Multiple macronuclear segments ..... *S. apourceolare*
5′ One macronuclear segment 6
6 Two thigmotactic membranelles 7
6′ No thigmotactic membranelles 8
7 Girdle kinety spiralling approximately once around cell ..... *S. agathae*
7′ Girdle kinety spiralling approximately twice around cell 9
8 Girdle kinety spiralling approximately twice around cell ..... *S. schizostomum*
8′ Girdle kinety spiralling approximately once around cell 10
9 < 14 buccal membranelles ..... *S. subtropicum*
9′ > 14 buccal membranelles *S. faurefremieti* sp. nov.
10 > 25 collar membranelles ..... *S. pseudocinctum*
10′ < 25 collar membranelles ..... *S. platum*

Discussion

Morphological comparison of *Spirostrombidium paraurceolare* sp. nov. with congeners

In terms of ciliature data, only 10 species can be compared with *Spirostrombidium paraurceolare* sp. nov. Among these, *S. urceolare* resembles the new species most in having three thigmotactic membranelles, and a girdle kinety spiralling around cell with one and a half whorls (Figs 46, 47), but can be easily separated from the latter by: (1) larger cell size *in vivo* (70–80 × 35–50 μm vs. 40–60 × 20–40 μm); (2) more buccal membranelles (13–16 vs. 8–10); and (3) a higher number of dikinetids in the ventral kinety (38–49 vs. 12–17) (Lei et al., 1999).

Apart from *S. urceolare*, there are four congeners with thigmotactic membranelles, viz. *S. apourceolare*, *S. agathae*, *S. cinctum*, and *S. subtropicum*. *Spirostrombidium paraurceolare* sp. nov. can be separated from *S. apourceolare* and *S. subtropicum* by having girdle kinety spiralling around cell with one and a half whorls (vs. two whorls; Liu *et al*., 2013). *Spirostrombidium paraurceolare* sp. nov. differs from *S. cinctum* by having: (1) fewer collar (15–18 vs. 23–28) and buccal (8–10 vs. 12–15) membranelles; (2) a lower number of dikinetids in the ventral kinety (12–17 vs. 18–29); (3) no extra kinety (vs. presence) (Liu *et al.* 2013). *Spirostrombidium agathae* is distinguished from the new species by: (1) the length of the cell *in vivo* (25–30 μm vs. 40–60 μm); (2) the number of thigmotactic membranelles (2 vs. 3); (3) the number of dikinetids in the girdle kinety (34–42 vs. 47–63); and (4) the commencement of the girdle kinety (on the ventral side vs. dorsal side) (Xu et al., 2006a). The new species can be separated from other congeners with ciliature though



Figs. 46–53. Living morphology of several strombidiids. (46) *Spirostrombidium urceolare* (Stein, 1867) Lei *et al.*, 1999 (from Fauré-Fremiet 1950); (47) *Spirostrombidium urceolare* (Stein, 1867) Lei *et al.*, 1999 (from Lei *et al.* 1999); (48) *Spirostrombidium sauerbreyae* (Kahl, 1932) Petz *et al.*, 1995 (from Kahl 1932); (49) *Strombidium cylindromorphum* Perejaslawzewa, 1886 (from Kahl 1932); (50) *Strombidium armatum* Burger, 1908 (from Song *et al*. 2000); (51) *Strombidium clavellinae* Buddenbrock, 1922 (from Buddenbrock 1922); (52) *Strombidium faurei* Dragesco, 1960 (from Dragesco 1960); (53) *Strombidium macronucleatum* Dragesco, 1960 (from Dragesco 1960).

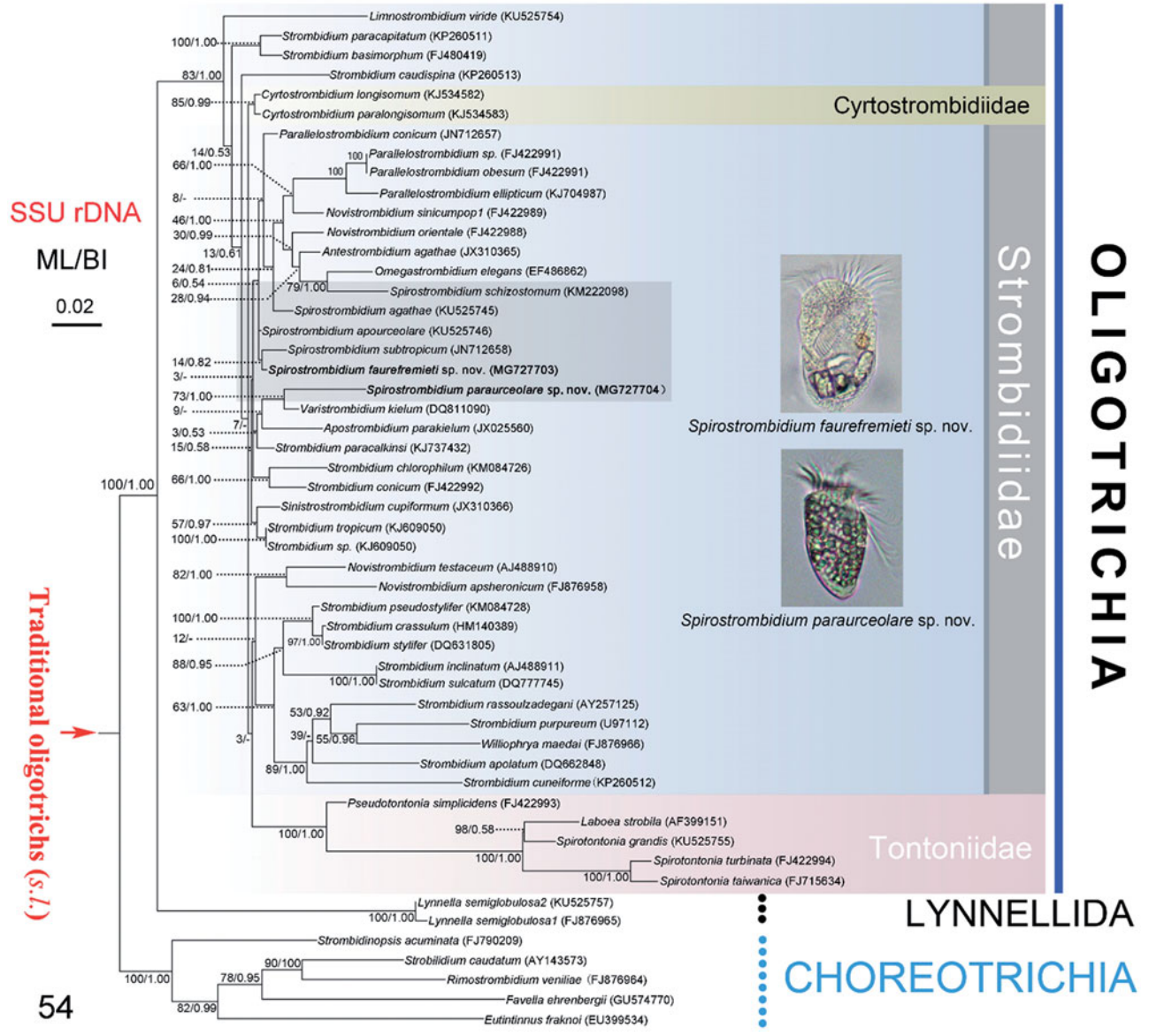


Fig. 54. Maximum likelihood (ML) tree inferred from SSU rDNA sequences, showing the position of *Spirostrombidium paraurceolare* sp. nov. and *Spirostrombidium faurefremieti* sp. nov. Numbers near branches denote ML bootstrap value/BI posterior probability. The scale bar corresponds to 2 substitutions per 100 nucleotide positions. All branches are drawn to scale. Systematic classification mainly follows Lynn (2008).

having thigmotactic membranelles (Lei et al., 1999; Xu et al., 2006a).

Spirostrombidium sauerbreyae lacks ciliature information (Fig. 48), but it differs from *S. paraurceolare* sp. nov. in the lack of thigmotactic membranelles (Entz, 1884; Kahl, 1932; Petz et al., 1995).

Intraspecific comparison of *Spirostrombidium faurefremieti* sp. nov.

Fauré-Fremiet (1950) described a French population of *Spirostrombidium sauerbreyae* (Kahl, 1932) (Petz et al., 1995). Based on the presence of thigmotactic membranelles, we can conclude that Fauré-Fremiet made a misidentification since *Spirostrombidium sauerbreyae* is more elliptical, and lacks thigmotactic membranelles (Kahl, 1932; Petz et al., 1995). Fauré-Fremiet's form corresponds well with *S. faurefremieti* sp. nov. regarding the basic morphology (viz. the size, dorsoventrally flattened body shape, the distribution of extrusomes, two thigmotactic membranelles, habitat), hence, both are very likely conspecific.

Table 1. Estimates of evolutionary divergence between SSU rRNA gene sequences of six *Spirostrombidium* species. The pairwise distance and base pairs differences of SSU rRNA gene between sequences are shown. All ambiguous positions were removed for each sequence pair. There were a total of 1617 positions in the final dataset. Evolutionary analyses were conducted in MEGA5, using the p-distance model.

		1	2	3	4	5	6
S. schizostomum	1						
S. agathae	2	0.0409 66					
S. apourceolare	3	0.0353 57	0.0118 19				
S. subtropicum	4	0.0328 53	0.0186 30	0.0099 16			
S. faurefremieti	5	0.0360 58	0.0112 18	0.0043 6	0.0105 16		
S. paraurceolare	6	0.0423 68	0.0373 60	0.0342 55	0.0317 51	0.0361 58	

Interspecific comparison of *Spirostrombidium faurefremieti* sp. nov.

Within the genus *Spirostrombidium*, three congeners have girdle kinety spiralling around cell with two whorls, viz. *S. apourceolare*, *S. schizostomum*, and *S. subtropicum*, and thus should be compared with our new form. *Spirostrombidium faurefremieti* sp. nov. can be separated from *S. apourceolare* by: (1) the absence (vs. presence) of apical protrusion; and (2) the number of macronuclear segments (one vs. 15–17) (Liu et al., 2013). The new species differs from *S. schizostomum* by: (1) the absence of apical protrusion (vs. presence); (2) having more collar (23–28 vs. 16–19) and buccal (15–19 vs. 10–12) membranelles; (3) the presence (vs. absence) of thigmotactic membranelles; (4) having more dikinetids in the girdle (105–126 vs. 46–67) and ventral (25–29 vs. 12–18) kineties (Xu et al., 2006a). Although *S. subtropicum* is very similar to *S. faurefremieti* sp. nov. in ciliature, the new species has a more pointed posterior end, a deeper and prominent buccal cavity, more buccal (15–19 vs. 10–13) membranelles, and lacks apical protrusion (vs. presence in *S. subtropicum*).

Spirostrombidium faurefremieti sp. nov. can be separated from *Spirostrombidium sauerbreyae*, a morphologically similar congener without ciliature (Fig. 48) by the lack of thigmotactic membranelles (Entz, 1884; Kahl, 1932; Petz et al., 1995).

Morphological comparison of two new species with *Strombidium*-species without ciliature

Considering the general morphology, comparisons should also be made with five *Strombidium* species: *S. cylindromorphum* Perejaslawzewa, 1886; *S. armatum* Burger, 1908; *Strombidium clavellinae* Buddenbrock, 1922; *Strombidium faurei* Dragesco, 1960 and *Strombidium macronucleatum* Dragesco, 1960, in which the ciliature data remain unknown (Table S2, see supplemental material online).

Strombidium cylindromorphum can be distinguished from *Spirostrombidium paraurceolare* sp. nov. and *Spirostrombidium faurefremieti* sp. nov. by its body shape (cylindrical vs. oblong, ellipsoidal), ratio of buccal field length to body length (∼ 1/2 vs. 1/4, 2/3) and absence of thigmotactic membranelles (vs. 3, 2) (Fig. 49; Kahl, 1932).

Compared with *Spirostrombidium paraurceolare* sp. nov., *Strombidium armatum* has a deeply extended buccal field (∼ 1/2 vs. 1/4 of body length) and a prominent apical protrusion (Fig. 49; Song et al., 2000). *Strombidium armatum* differs from *Spirostrombidium faurefremieti* sp. nov. in the number of thigmotactic membranelles (3 vs. 2) (Fig. 50; Song et al., 2000).

Strombidium clavellinae differs from two new species in the present work in: (1) cell size (70–80 μm vs. 40–60 μm, 55–70 μm), (2) the number of thigmotactic membranelles (4 vs. 3, 2), and (3) ratio of buccal field length to body length (∼ 1/3 vs. 1/4, 1/2) (Fig. 51; Buddenbrock, 1922).

Strombidium faurei can be separated from *Spirostrombidium paraurceolare* sp. nov. by the structure of its oral apparatus and the number of thigmotactic membranelles (2 vs. 3) (Fig. 51; Dragesco, 1960). *Strombidium faurei* differs from *Spirostrombidium faurefremieti* sp. nov. by the presence of an apical protrusion and a shallowly extended buccal field (Fig. 52; Dragesco, 1960).

Strombidium macronucleatum is different from *Spirostrombidium paraurceolare* sp. nov. and *Spirostrombidium faurefremieti* sp. nov. in its larger cell length (80–135 μm vs. 40–60 μm, 55–70 μm), and absence of thigmotactic membranelles (vs. 3, 2) (Fig. 53; Dragesco, 1960).

Phylogeny of *Spirostrombidium* species

In the phylogenetic trees, *Spirostrombidium faurefremieti* sp. nov. clusters with *S. subtropicum*, which corresponds well with their high similarity in morphology, viz. cell size (55–70 × 35–45 μm vs. 45–70 × 25–40 μm), bases of thigmotactic membranelles as wide as those of collar membranelles, and a girdle kinety spiralling around cell twice. On the other hand, *S. paraurceolare* sp. nov. clusters with *Varistrombidium*

kielum. *Varistrombidium kielum* has a barrel-shaped body, and a shallow buccal cavity but lacks thigmotactic membranelles, and thus is not like *Spirostrombidium paraurceolare* sp. nov. at all, which cannot explain the grouping of these two species in SSU rRNA gene trees. This kind of disagreement between phylogenies may result from the paucity of knowledge about molecular data of oligotrichs, e.g. 18S rRNA gene sequence data are only available for four species in the genus *Spirostrombidium* (Liu et al., 2013, 2015b, 2016, 2017). The present study confirms previous studies that *Spirostrombidium* is very likely polyphyletic.

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Supplemental data

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