Taxonomy, morphology and phylogeny of three new oligotrich ciliates (Protozoa, Ciliophora, Oligotrichia) from southern China

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Oligotrich ciliates are common members of marine microplankton. However, their biodiversity is not well documented. In this study, the morphology and phylogenetic positions of three new oligotrich species, Spirostrombidium apourceolare spec. nov., Spirostrombidium subtropicum spec. nov. and Parallelostrombidium conicum spec. nov., collected from coastal habitats of southern China, were investigated. Spirostrombidium apourceolare is characterized by the girdle kinety which encircles the cell twice as two dextrally oriented whorls with some undulations and by the presence of several macronuclear nodules. Spirostrombidium subtropicum is recognized by the girdle kinety encircling the cell as two dextrally oriented whorls and extrusomes arranged in a stripe along the girdle kinety. Parallelostrombidium conicum differs from its congeners by the obconic body shape and the posterior portion of the girdle kinety extending downwards on the left ventral side to reach the posterior pole. In small-subunit rRNA gene trees, S. subtropicum clusters with Omegastrombidium elegans and Varistrombidium kielum, and Parallelostrombidium conicum is sister to the clade containing Novistrombidium sinicum, Novistrombidium orientale and Parallelostrombidium sp.

INTRODUCTION

Oligotrich ciliates are important components of the pelagic microbial food web and may episodically dominate the marine microzooplankton (Dale & Dahl, 1987; Fenchel, 2008; Pierce & Turner, 1992). However, the scale of oligotrich species diversity is poorly understood and, based on ecophysiological and molecular data, it has been estimated that 83–89 % of aloricate oligotrich species are unknown (Agatha, 2011a).

The pattern of the somatic kineties is considered an important character for investigating the taxonomy of oligotrichs (Agatha, 2004a, b, 2011b; Agatha & Strüder-Kypke, 2012; Jiang et al., 2012; Liu et al., 2011a, b, 2012; Song et al., 1999a; Xu et al., 2009). Based on the patterns of the ventral and girdle kineties and the position of the oral primordium, Agatha (2004a) split the genus Spirostrombidium into Spirostrombidium (sensu stricto), in which the ventral kinety and the posterior portion of the girdle kinety are inversely oriented, and the genus Parallelostrombidium, in which the ventral kinety and the posterior portion of the girdle kinety have the same orientation. To date, a total of 11 species have been assigned to the genera Parallelostrombidium and Spirostrombidium (Agatha, 2004a; Lei et al., 1999; Petz et al., 1995; Song et al., 1999b, 2013; Xu & Song, 2006; Xu et al., 2006a, b).

In this study, we describe three new oligotrich ciliates, Spirostrombidium apourceolare spec. nov., Spirostrombidium subtropicum spec. nov. and Parallelostrombidium conicum spec. nov., based on their living morphology and infraciliature. In addition, the small-subunit (SSU) rRNA genes
of *S. subtropicum* and *P. conicum* are sequenced and their phylogenetic positions are investigated.

**METHODS**

**Ciliate collection and identification.** *S. apourceolare* spec. nov. was collected from Daya Bay (22° 43’ N 114° 32’ E), Guangdong Province, China, on 8 November 2007. The water temperature was 21.7 °C, salinity 33.3%, and pH 8.2. *S. subtropicum* spec. nov. was collected from a mangrove wetland near Zhanjiang (21° 22’ N 109° 26’ E), Guangdong Province, China, on 29 March 2010. The water temperature was 20.8 °C, salinity 21.3%, and pH 7.7. *Parallelostrombidium conicum* spec. nov. was collected from a different mangrove wetland near Zhanjiang (21° 31’ N 109° 45’ E), Guangdong Province, China, on 26 March 2010. The water temperature was 19.7 °C, salinity 23.9%, and pH 7.8.

Samples were collected using 20 μm mesh plankton nets from surface water (0–0.5 m deep) that contained some organic debris. The samples were then transferred to Petri dishes and specimens were immediately isolated for further study in the laboratory. No cultures were established. Observations on living cells were carried out using bright-field and differential interference contrast microscopy. The protargol impregnation method (Wilbert, 1975) was used to reveal the infraciliature and nuclear apparatus. Counts and measurements were performed at ×40–1000 magnification. Drawings of live specimens were based on *in vivo* observations and photomicrographs. Drawings of protargol-impregnated specimens were made with a camera lucida at ×1000 magnification. Terminology is mainly according to Agatha (2004b). Classification follows Lynn (2008).

**Extraction, amplification and sequencing of DNA.** Five cells each of *S. subtropicum* and *Parallelostrombidium conicum* were collected from a micropetette and rinsed three or four times with autoclaved seawater in order to remove other protists (unfortunately there were insufficient isolates of *S. apourceolare* for us to obtain genomic DNA). In each case the cells were then transferred into a 2 ml microfuge tube with the minimum possible volume of seawater. Genomic DNA was extracted with a REDExtract-N-Amp Tissue PCR kit (Sigma). The PCR was performed according to Li et al. (2013) with the use of universal eukaryotic primers EuK A (5’-ACCTCTGGTGATCCTGCGAGT-3’) and EuKB (5’-TGTACCTCTGGACGGTACCTAC-3’) (Medlin et al., 1988). The PCR product was purified using the TIAN gel Midi Purification kit (Tiangen Bio.) and inserted into a pUCm-T vector (Sangon Bio.). DNA from plasmids was harvested using a QiAprep Spin Miniprep kit (Qiagen) and sequenced (Invitrogen sequencing facility, Shanghai, China) using the RV-M and M13-20 primers.

**Phylogenetic analyses.** The SSU rRNA gene sequences of *S. subtropicum* and *Parallelostrombidium conicum*, along with another 77 reference sequences from GenBank databases (for accession numbers see Fig. 7), were used for phylogenetic analyses. The outgroup taxa were: *Coleps nolandi*, *Prorodon tener*, *Prorodon viridis* (*Prorostomatidae*); *Lacinularia lyngbycola*, *Lacinularia magalardii* (*Lacinulariidae*); *Diplhydris appendiculata*, *Diplhydris oligothrix*, *Uronychia transfuga*, *Aspidiscus aculeata* (*Euplotidae*); and *Phacodinium metchnikoffi* (*Phacodiniidae*).

Phylogenetic trees were reconstructed according to the methods reported by Song et al. (2013). The SSU rRNA gene sequences were aligned using CLUSTAL X 1.83 (Jeanmougin et al., 1998) and refined by manually trimming the ends and the ambiguous regions using BioEdit 7.0 (Hall, 1999). In the case of *S. subtropicum*, the ambiguous regions removed from the sequence were in positions 406–408 and 1591–1607. The program MrModeltest v2.1 (Nylander, 2004) selected the GTR + I + G as the best model with Akaike information criteria, which was then used for both Bayesian inference (BI) and maximum-likelihood (ML) analyses. The BI tree was reconstructed with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) using the Markov chain Monte Carlo algorithm. The program was run for 1 000 000 generations with a sample frequency of 100 and a burn-in of 2500. The ML tree was reconstructed with PhyML v2.4.4 (Guindon & Gascuel, 2003). The reliability of internal branches was assessed using a non-parametric bootstrap method with 1000 replicates. TreeView v1.6.6 (Page, 1996) and MEGA 4.0 (Tamura et al., 2007) were used to visualize tree topologies.

**RESULTS**

Order Strombidiida Petz & Foissner 1992

Family Strombidiidae Fauré-Fremiet 1970

Genus *Spirostrombidium* Jankowski 1978

*Spirostrombidium apourceolare* spec. nov. (Figs 1 and 2; Table 1)

**Diagnosis.** Body broadly ellipsoidal, usually ~60 μm × 40 μm *in vivo* and ~56 μm × 32 μm after protargol impregnation. Extrusomes attached to cell surface in three to eight rows along girdle kinety and in single row along ventral kinety. About 20 ovoid macronuclear nodules scattered in cytoplasm. Usually ~28 anterior, ~16 ventral and two thigmotactic membranes. Girdle kinety spiralling approximately twice around cell with two undulations, comprising ~140 dikinetids. Ventral kinety crossing right margin of cell and terminating on dorsal side, comprising ~34 dikinetids.

**Type locality.** Coastal waters off Daya Bay (22° 43’ N 114° 32’ E), Guangdong Province, China.

**Etymology.** Combining the Latin prefix *apo-* (meaning from or off) with the species epiphet *urceolare* indicates the superficial similarity in ciliary pattern between this species and *Spirostrombidium urceolare*.

**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 2010.11.9.3. One protargol slide with paratype specimens is deposited in the Laboratory of Protozoology, SCNU, with registration number LWVW07110804.

**Description.** Cell size *in vivo* 50–70 μm × 30–50 μm and 48–66 μm × 24–45 μm (usually ~56 μm × 32 μm) after protargol impregnation. Cell shape broadly ellipsoidal, anterior and posterior ends bluntly rounded, widest point slightly above equatorial region (Figs 1a and 2a). Apical protrusion inconspicuous *in vivo* and undetectable after fixation.

Hemitheca composed of polygonal platelets ~3 μm across, covering posterior 2/5 portion of cell, posterior to the posterior whorl of girdle kinety (Figs 1f, arrow; 1h and 2e).
Distended cell surface not recognizable in protargol-impregnated cells. Cytoplasm colourless, usually containing many food vacuoles (approx. 2 μm in diameter), ingested diatom frustules up to 25 μm long and mineral particles (approx. 2–5 μm across), which probably originate from the sediment (Figs 1a, e and 2a). Extrusomes conspicuous, acicular shaped, ~9 μm × 0.5 μm in vivo; their attachment sites evenly spaced in three rows above the girdle kinety forming a stripe that surrounds the body; on the right side of posterior portion of cell, extrusome attachment sites arranged in a single row along the ventral kinety (Figs 1a, b, d–f, arrowheads, and 2c, d, arrowheads, f, g). The stripe of extrusome attachment sites above the first whorl of girdle kinety on dorsal side obvious and wide, where extrusomes are arranged in five to eight rows (Figs 1f and 2c, f). About 15–27 ovoid macronuclear nodules scattered in body, each about ~5 μm × 8 μm containing several large nucleoli (Figs 1j and 2l). Contractile vacuole, cytopyge and micronucleus not recognized. Locomotion by crawling over debris using thigmotactic membranelles or by swimming in spirals (Fig. 1c).

Buccal cavity narrow and deep, extending obliquely to 2/5 of cell length (Figs 1a and 2a). Adoral zone comprises 14–19 ventral and 26–31 anterior membranelles, which are separated by two thigmotactic membranelles (Figs 1i and 2b). Cilia of anterior membranelles 18–20 μm long in vivo, directed laterally or even slightly posteriorly when swimming; cilia of ventral membranelles 4–6 μm long in vivo; thigmotactic membranelles inconspicuous as their cilia are only slightly longer than the anterior membranelles, i.e. about 20–22 μm long, directed posteriorly (Fig. 1a). Bases of thigmotactic membranelles about 9 μm wide, anterior membranelles about 7–8 μm wide, and ventral membranelles about 3–6 μm wide, decreasing in width towards the cytostome (Figs 1i and 2b, arrowheads); each membranelle composed of three rows of basal bodies except for the two posteriormost membranelles which probably comprise only two rows. Endoral membrane probably composed of a single row of kinetosomes, located on inner wall of buccal cavity, extending anteriorly to centre of apical protrusion; no cilia observed (Fig. 1i). Pharyngeal fibres not recognized.

Somatic cilia fusiform and ~2 μm long in vivo, arranged in a girdle and ventral kinety (Figs 1g, i, j and 2g, h, k, l). Girdle kinety commences in shoulder area on the right of buccal vertex, extends by spiralling approximately twice

Fig. 1. *Spirostrombidium apourceolare* spec. nov. from life (a–f, h) and after staining with protargol (g, i, j). (a) Ventral view of a typical specimen. (b) Detail of extrusomes attached above the girdle kinety, arrowhead notes the outer ends of extrusomes. (c) Swimming trace. (d) Resting extrusomes. (e, f) Dorsal views showing the distribution of extrusomes (e) and extrusome attachment sites (arrowhead in f) as well as the cortical platelets (arrow in f). (g) Pattern of somatic ciliature, arrowheads note the two undulations. (h) Pattern of cortical platelets. (i, j) Ventral (i) and dorsal (j) views of the same specimen showing the ciliary pattern and the macronuclear nodules. AM, anterior membranelles; E, endoral membrane; Ex, extrusomes; GK, girdle kinety; Ma, macronuclear nodules; TM, thigmotactic membranelles; VK, ventral kinety; VM, ventral membranelles. Bars, 30 μm (a, e, f, g, i, j); 5 μm (d).
around cell, crosses posterior pole inverting its orientation, and terminates in posterior right ventral area (Figs 1g, i, j and 2g, h, k). Girdle kinety with two undulations along its course, one on the dorsal and the other on left ventral side (Fig. 1g, arrowheads). Girdle kinety consisting of 117–164 densely arranged dikinetids, the left basal body of each bearing a cilium. Ventral kinety commences right of buccal vertex in posterior 2/5 of right dorsal side; crosses right margin of cell and curves onto ventral side, terminating near posterior end of cell; posterior region of ventral kinety parallel to posterior region of girdle kinety (Figs 1g, i, j and 2k, l). Ventral kinety comprises 28–41 densely arranged dikinetids, each with an anterior cilium. Dividing individuals with oral primordium not found.

**Spirostrombidium subtropicum** spec. nov. (Figs 3 and 4; Table 1)


**Type location.** Mangrove wetland (21° 22’ N 109° 26’ E) near Zhanjiang, Guangdong Province, China. Brackish water.

**Etymology.** The specific epithet *subtropicum* refers to the fact that this species was discovered in a subtropical biotope.

**Deposition of slides.** A protargol slide with the holotype specimen (marked with a black circle) is deposited in the Natural History Museum, London, with registration number NHMUK 2010.11.9.4. Four paratype slides are deposited in the Laboratory of Protozoology, SCNU, with registration numbers LWW2010032901-01, LWW2010032901-02, LWW2010032901-03 and LWW2010032901-04.

**Deposition of SSU rRNA gene sequence data.** The SSU rRNA gene sequence is deposited in GenBank with accession
number JN712658, and its length and DNA G+C content are 1641 bp and 48.4 mol%, respectively.

**Description.** Body 45–70 μm × 25–40 μm *in vivo*, obovoidal to elongate obconical, widest in shoulder region, posterior end narrowly rounded (Figs 3a, e and 4a, b). Cells slightly swollen after protargol impregnation, size about 46–74 μm × 27–39 μm, no distended cell surface recognizable. Anterior end transversely truncated with conspicuous apical protrusion about 5 μm high at right side of peristome, which disappeared after protargol impregnation (Figs 3a and 4b, arrow, h). Dorsoventrally flattened, thickness : width ratio about 2 : 3 (Fig. 3f).

Cortical platelets not recognizable. Cytoplasm colourless, packed with lipid droplets 2–3 μm across and some food vacuoles containing ingested diatoms up to 15 μm long (Figs 3a, e and 4a, e). Mineral particles (approx. 2–4 μm across), probably originating from sediment, usually clustered in the posterior portion of cytoplasm. Extrusomes evenly spaced, forming a stripe that surrounds body; extrusome attachment sites in shallow furrow above girdle kinety; most extrusomes

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oriented obliquely to cell surface, but some oriented perpendicular to cell surface along the posterior portion of girdle kinety (Figs 3a, e and 4c–f, arrowheads). Individual extrusomes acicular, ~8 μm × 0.5 μm in vivo (Fig. 3b, c). Single, ellipsoidal macronucleus, about 15 μm × 25 μm, centrally located and containing numerous chromatin granules (Figs 3h and 4I, m). Contractile vacuole, cytopyge and micronucleus not recognized. Locomotion usually by crawling over debris on ventral side using thigmotactic membranelles for attachment; when disturbed, swimming in spirals (Fig. 3d).

Buccal cavity conspicuous, extending obliquely to right and terminating about 2/5 of way down body (Figs 3a, g and 4a, b). Oral apparatus consists of an endoral membrane and an adoral zone of membranelles (Fig. 3g). Adoral zone bipartite with about 20–26 anterior and 10–13 ventral membranelles. Two thigmotactic membranelles located between anterior and ventral membranelles (Figs 3g and 4j). Cilia of anterior membranelles up to 15 μm in length and directed laterally when swimming; bases of anterior membranelles mostly about 8 μm wide. Cilia of thigmotactic membranelles conspicuously longer (28–30 μm) than anterior membranelles and always directed posteriorly (Figs 3a, f and 4g, i, arrows); thigmotactic membranelles less conspicuous in protargol impregnations because the width of their bases is the same as those of anterior membranelles (Figs 3g and 4c). Cilia of ventral membranelles mostly about 3–5 μm in length; bases of ventral membranelles about 3–6 μm wide, decreasing in width towards the cytostome. Each membranelle composed of three rows of basal bodies, except for two posterior-most membranelles which probably comprise only two rows. Endoral membrane about 15 μm long, probably composed of a single row of kinetosomes and located on inner wall of right buccal lip; no cilia recognized, (Fig. 3g). Pharyngeal fibres not observed.

Somatic ciliature as shown in Fig. 3(g, h, i), composed of dikinetids; cilia ~2 μm long in vivo. Girdle kinety consisting of about 89–112 dikinetids, the left basal body of each bearing a cilium. Girdle kinety commences in shoulder area on right of buccal vertex; extends by spiralling around cell twice with the first whorl distinctly separated from buccal vertex; posterior portion curved slightly onto right ventral side so that it lays parallel to
posterior portion of ventral kinety but with inverse orientation; terminates at posterior 10% of cell (Figs 3g, h, i and 4k–n, arrows). Ventral kinety composed of about 18–26 densely arranged dikinetids, each having a cilium associated with its anterior basal body. Ventral kinety commences on the right of buccal vertex, extends posteriorly along right margin of cell, and terminates near posterior end of cell (Figs 3g, i and 4l, n). Dividing individuals with oral primordium not observed.

Genus *Parallelostrombidium* Agatha 2004

*Parallelostrombidium conicum* spec. nov. (Figs 5 and 6; Table 1)

Diagnosis. Cell shape elongate obovoidal, usually ~55 μm × 25 μm *in vivo* and ~62 μm × 31 μm after protargol impregnation. Anterior end transversely truncated with conspicuous apical protrusion. Extrusome attachment sites

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**Fig. 4.** Photomicrographs of *Spirostrombidium subtropicum* spec. nov. from life (a–i) and after staining with protargol (j–n). (a) Ventral view of a normal individual. (b) A plump specimen, arrow marks the apical protrusion. (c) Ventral view showing the adoral zone of membranelles and extrusomes (arrowhead). (d) Dorsal view showing the distribution of extrusomes (arrowheads). (e, f) Ventral (e) and dorsal (f) views of posterior cell portion showing the distribution of extrusomes (arrowheads). (g, h) Ventral views of anterior cell portion, arrows mark the thigmotactic membranelles, arrowhead indicates the extrusomes. (i) Apical view showing the thigmotactic membranelles (arrow). (j) Detail of the buccal apparatus. (k, l, m) Dorsal (k), ventral (l) and left lateral (m) views showing the somatic kineties and the macronucleus, arrow marks the posterior end of the girdle kinety. (n) Ventral view of posterior cell portion showing the girdle and ventral kineties, arrow marks the posterior end of the girdle kinety. GK, girdle kinety; Ma, macronucleus; VK, ventral kinety; VM, ventral membranelles. Bars, 30 μm (a, b).
above girdle kinety. Single macronucleus, ellipsoidal to globular in shape. Girdle kinety spiralling once around cell, posterior portion extending downwards on left ventral side to posterior pole. Ventral kinety centrally located on ventral side, parallel to girdle kinety. Ventral and girdle kineties consisting of ~16 and ~36 dikinetids, respectively. Adoral zone consisting of ~17 anterior and ~8 ventral membranelles.

**Type location.** Mangrove wetland (21°34' N 109°45' E) near Zhanjiang, Guangdong Province, China. Brackish water.

**Deposition of slides.** A protargol slide with the holotype specimen (marked with a black circle) is deposited in the Natural History Museum, London, with registration number NHMUK 2010.11.9.1. Three paratype slides are deposited in the Laboratory of Protozoology, SCNU, with registration numbers LWW2010032602-01, LWW2010032602-02 and LWW2010032602-03.

**Deposition of SSU rRNA gene sequence data.** The SSU rRNA gene sequence is deposited in GenBank with accession number JN712657, and its length and DNA G + C content are 1656 bp and 49.1 mol%, respectively.

**Etymology.** The Latin word *conicum* refers to the conical body shape of the new species.

**Description.** Body 45–60 μm × 20–30 μm in vivo, usually elongate obconical or obovoidal, widest above the equatorial region where the cell surface bulges (Figs 5a, d and 6a, b). Cells slightly swollen after protargol impregnation, size about 47–76 μm × 19–39 μm, distended cell surface not recognizable. Anterior region narrowed with a transversely truncated anterior end; collar region domed to form a conspicuous apical protrusion about 4 μm high, which disappeared after protargol impregnation (Figs 5a and 6b, e, arrows). Posterior portion slightly bilaterally flattened with posterior end bluntly pointed (Figs 5a and 6a).

Pellicle delicate, no polygonal platelets observed. Cytoplasm colourless, usually with many lipid droplets 1–2 μm across and food vacuoles with ingested yellow algae about 3–4 μm across (Figs 5a, e, f and 6a). Extrusomes acicular with slightly pointed posterior end, about 9 μm × 0.5 μm in vivo, directly inserted into cytoplasm along course of girdle kinety (Figs 5a, b, e, f, i and 6c, f, i, arrowheads). Extrusome attachment sites located about 1 μm above girdle kinety (Fig. 5b). Single,
centrally located macronucleus, variable in shape but usually ellipsoidal to globular (Figs 5h, k and 6d). Contractile vacuole, cytopyge and micronucleus not recognized. Locomotion by swimming in a zig-zag path (Fig. 5c).

Buccal cavity narrow and shallow, extending obliquely about 1/4 of the way down body (Fig. 5a). Adoral zone consists of 15–18 anterior and 7–9 ventral membranelles, each composed of three rows of kinetosomes, except for the two posteriormost membranelles which probably comprise only two rows. (Figs 5g, j, k and 6h). Anterior and ventral portions of adoral zone continuous and bases of anterior membranelles wider (6 μm wide) than those of ventral membranelles (3–5 μm wide) which decrease in width towards cytostome (Fig. 5g). Cilia of anterior membranelles up to 16 μm long in vivo, directed laterally or slightly posteriorly when swimming. Cilia of ventral membranelles mostly about 3–5 μm in length. Endoral membrane about 10 μm long, probably composed of a single row of kinetosomes located on inner wall of right buccal lip; no cilia recognized (Fig. 5g). Pharyngeal fibres not observed.

Somatic kineties composed of dikinetids. Girdle kinety commences in anterior 1/3 of cell to left of buccal vertex and below proximal end of anterior membranelles; spiralles dextrally (when viewed from anterior) across ventral and dorsal sides, then curves downwards on left ventral side; terminates almost at posterior pole of cell (Figs 5g, h, j, k and 6d, g, k, l). Girdle kinety composed of 30–41 dikinetids, left basal body of each bearing a cilium, about ~2 μm in length. Ventral kinety commences to left of buccal vertex, below anterior end of girdle kinety, extends posteriorly and

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**Fig. 6.** Photomicrographs of *Parallelostrombidium conicum* spec. nov. from life (a–c, e, i, j) and after staining with protargol (d, g, h, k, l). (a) A slender individual. (b) A typical specimen, arrow marks the apical protrusion. (c) Dorsal view showing the distribution of extrusomes (arrowhead). (d) Ventral view showing the somatic kineties and the macronucleus. (e) Ventral view of anterior cell portion of cell, arrow notes the apical protrusion. (f) Dorsal view showing the extrusomes (arrowhead). (g) Ventral view showing the anterior portion of the girdle kinety. (h) The buccal apparatus. (i) Ventral view of posterior cell portion, arrowheads mark the distribution of extrusomes. (j) A middle-late divider. (k, l) Right lateral (k) and ventral (l) views of posterior cell portion showing the girdle and ventral kineties. AM, anterior membranelles; GK, girdle kinety; Ma, macronucleus; VK, ventral kinety; VM, ventral membranelles. Bars, 20 μm (a–c).
terminates near posterior end of girdle kinety. Posterior 2/3 portion of ventral kinety located parallel with and adjacent to posterior portion of girdle kinety (Figs 5g, j, k and 6d, k, l). Ventrall kinety composed of 13–19 dikinetids, each with a cilium associated with its anterior basal body (Fig. 5g, j, k). No oral primordium recognized in impregnated specimens.

**Fig. 7.** Maximum-likelihood tree inferred from small subunit rRNA gene sequences indicating the phylogenetic positions of species of the genera *Spirostrombidium* and *Parallelostrombidium*. Numbers at the nodes represent support values in the following order: ML bootstrap values, and BI posterior probabilities. Nodes absent from one of the three phylogenies are indicated by a hyphen instead of a support value. Bar, 5 substitutions per 100 nt positions. Species sequenced in the present study are shown in bold type.
SSU rRNA gene sequence analyses (Fig. 7)

Trees reconstructed using different algorithms had almost identical topologies, therefore only the ML tree is shown (Fig. 7). In all trees, *S. subtropicum* clusters with *Omegastrombidium elegans* and *Varistrombidium kielium*, which together form a clade with *Apostrombidium parakielium*, although this clade is not well supported (0.70 BI, 19% ML). Species of the genus *Novistrombidium* fall into two clades: one includes *Novistrombidium orientale* and *Novistrombidium sinicum*, which groups with species of the genus *Parallelostrombidium* followed by *P. conicum* (0.82 BI, 29% ML); the other, which includes *Novistrombidium testaceum* and *Novistrombidium asphericum*, falls outside the main oligotrich assemblage in the ML tree and forms a polytomy with the *Parallelostrombidium*-*Spirostrombidium*-*Omegastrombidium*-*Varistrombidium*-*Apostrombidium* group and the *Strombidium* group in the BI tree (not shown).

DISCUSSION

Generic assignments of *S. apourceolare* and *S. subtropicum*

In the generic diagnoses supplied by Agatha (2003, 2004a), *Spirostrombidium* was defined as ‘girdle kinety dextrally spiraled, posterior portion inversely orientated and parallel to longitudinal ventral kinety’, and *Novistrombidium* was defined as ‘Strombidiidae with left portion of dextrally spiraled, posterior portion inversely orientated and parallel to longitudinal ventral kinety’. According to these diagnoses, the most significant difference between these two genera is whether the posterior portion of the girdle kinety is inversely orientated and parallel to the ventral kinety.

Considering their general appearance, *S. subtropicum* and *S. apourceolare*, share several characteristics in common with species of both the genus *Spirostrombidium* and the genus *Novistrombidium*, such as *Spirostrombidium cinctum*, *Spirostrombidium agatha*, *N. orientale* and *N. sinicum*. For example, the broadly ellipsoidal cell shape, the extrusomes equidistantly arranged along the girdle kinety, the presence of thigmotactic membranelles, the ovoidal macronucleus and the anterior end of the ventral kinety located below the right portion of the girdle kinety (Agatha, 2003; Song & Bradbury, 1998; Xu & Song, 2006; Xu et al., 2006a, 2009).

However, in both *S. subtropicum* and *S. apourceolare*, there is a short overlap of the posterior regions of the girdle and ventral kineties, which suggests that they are parallel to each other and thus should be assigned to the genus *Spirostrombidium*. In addition, our phylogenetic analyses based on SSU rRNA gene sequence data also failed to group *S. subtropicum* with species of the genus *Novistrombidium*. Thus the morphological and molecular data suggest that both novel species should be assigned to the genus *Spirostrombidium* rather than the genus *Novistrombidium*. It is, nevertheless, noteworthy that the generic assignments of some species are ambiguous because of the considerable variation in their ciliary patterns. For example, in some individuals of *S. subtropicum* and *S. apourceolare*, the posterior end of the girdle kinety is located just to the left of the posterior end of the ventral kinety, making it difficult to determine whether or not the posterior portions of these kineties are parallel (Figs 1g, 2k, 3g and 4n). Therefore, further studies are needed in order to determine more precisely the morphological distinction between the genera *Spirostrombidium* and *Novistrombidium*.

Comparison with similar species

Comparison of *Spirostrombidium apourceolare* spec. nov. with similar species

To date, eight *Spirostrombidium* morphospecies have been reported: *S. agatha*, *S. cinctum*, *Spirostrombidium echini*, *Spirostrombidium pseudocinctum*, *Spirostrombidium platum*, *Spirostrombidium schizostomum*, *Spirostrombidium sauerbreyae* and *Spirostrombidium urceolare* (Lei et al., 1999; Liu et al., 2009; Petz et al., 1995; Song et al., 1999b; Xu & Song, 2006; Xu et al., 2006a). *S. apourceolare* can be easily distinguished from all other congeners by having numerous (vs only one) macronuclear nodules (Xu et al., 2009).

Apart from *S. apourceolare*, only one congener has two girdle kinety whorls, viz., *S. schizostomum*. The novel species differs from *S. schizostomum*: (1) the presence (vs absence) of thigmotactic membranelles; (2) the appearance of the girdle kinety with two undulations (vs with no undulations); (3) having more anterior (26–31 vs 16–19) and ventral (14–19 vs 10–12) membranelles; (4) having more dikenitids in the ventral (28–41 vs 12–18) and girdle (117–164 vs 46–67) kineties; (5) the arrangement of the extrusomes (along the girdle and ventral kineties vs in the equatorial area on the ventral side and along the margin of the hemitheca on the dorsal side) (Xu et al., 2006a).

Extrusomes rarely accompany the ventral kinety in members of the genus *Spirostrombidium*: *S. apourceolare* and *S. cinctum* are the only two congeners that share this feature. However, *S. apouceolare* can be easily separated from the latter by the girdle kinety, which spirals twice (vs only once) around the cell.

Comparison of *Spirostrombidium subtropicum* spec. nov. with similar species

Two other species of the genus *Spirostrombidium*, *S. apouceolare* and *S. schizostomum*, have a girdle kinety that spirals around the cell twice and so should be compared with *S. subtropicum* (Xu et al., 2006a).

*S. schizostomum* most closely resembles *S. subtropicum* in terms of its general ciliary pattern. However, *S. subtropicum* can be separated from *S. schizostomum*: (1) the arrangement of extrusomes (along the girdle kineties vs in the equatorial area on the ventral side and along the margin of the hemitheca on the dorsal side); (2) the presence (vs absence) of thigmotactic membranelles (Xu et al., 2006a).

*S. subtropicum* can be clearly separated from *S. apouceolare* by having only one (vs 15–27) macronuclear nodule.
Comparison of Parallelostrombidium conicum spec. nov. with similar species. Prior to the present study, three species were assigned to the genus Parallelostrombidium: P. rhyticollare, P. siculum and P. paralatum (Alekperov et al., 2007; Montagnes & Taylor, 1994; Petz et al., 1995; Xu et al., 2006b).

P. paralatum resembles P. conicum in that its ventral and girdle kineties are partially parallel. However, the former can be easily distinguished from P. conicum by having two conspicuous thigmotactic membranelles (vs thigmotactic membranelles absent) and a blunted rounded (vs bilaterally flattened and tapered) posterior end (Xu et al., 2006b).

P. siculum and P. rhyticollare both resemble P. conicum in having an elongate obconical body shape. However, P. conicum can be separated from both these species by the following combination of characters: (1) the anterior end of the girdle kinety is located just above the anterior end of the ventral kinety (vs the anterior end of girdle kinety conspicuously separated from the anterior end of ventral kinety); (2) the girdle kinety spirals once around the cell (vs about 1.5 whorls in P. siculum and P. rhyticollare); (3) the anterior portion of the ventral kinety is widely separated from the girdle kinety (vs the anterior portion of the ventral kinety lies closely adjacent and parallel to the girdle kinety) (Alekperov et al., 2007; Montagnes & Taylor, 1994; Petz et al., 1995).

Phylogeny

The morphological analyses are rather inconsistent with our phylogeographic results. In the SSU rRNA gene-based trees, P. conicum did not cluster with species of the genus Parallelostrombidium, which were first designated by Gao et al. (2009) as species of the genus Spirostrombidium, so the monophyly of the genus Parallelostrombidium was not recovered. Considering that all the support values for their placements in the tree are at best variable, the relationships among members of the subclass Oligotrichia remain largely unresolved, which is consistent with previous studies (Gao et al., 2009; Kim et al., 2010; Li et al., 2013; Zhang et al., 2010).

Molecular data for oligotrichs are relatively scant given the high level of morphological diversity within this group. Thus, molecular data from more taxa and more markers are needed in order to resolve the phylogenetic relationships among the oligotrichs.

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Three new oligotrich ciliates


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