Morphology, morphogenesis and small-subunit rRNA gene sequence of the novel brackish-water ciliate Strongylidium orientale sp. nov. (Ciliophora, Hypotrichia)

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A novel stichotrich ciliate, Strongylidium orientale sp. nov., was discovered from a mangrove river in Hong Kong, southern China, and its morphology was investigated through observations in vivo and after protargol impregnation. Cells are 80–120 \( \times \) 35–50 \( \mu \text{m} \) in vivo and fusiform in shape, with rounded anterior and tapered posterior ends. It is characterized by its brackish habitat and by the presence of two types of cortical granules arranged irregularly throughout the cortex. Morphogenetic events of cell division and physiological reorganization are described. The main ontogenetic features were: (i) only the posterior portion of the parental adoral zone of membranelles was renewed by dedifferentiation of the old structures; (ii) the oral primordium in the opisthe occurred apokinetically; (iii) the left and right ventral rows originated intrakinetically and the final left ventral row was spliced from two cirri from the frontoventral cirral anlage, a short cirral row from the anlage for the right ventral row and a long cirral row which was formed from the whole anlage of the left ventral row; (iv) the marginal rows developed intrakinetically; (v) the dorsal kineties replicated entirely de novo and did not fragment; and (vi) the two macronuclear nodules fused into a mass and then divided. Based on small-subunit rRNA gene sequences, phylogenetic analyses showed a close relationship with its congener Strongylidium pseudocrassum and with the genus Pseudouroleptus.

INTRODUCTION

Since the genus Strongylidium Sterki, 1878 was established, several species from different habitats (marine, brackish and fresh water and soil) have been included in it. However, most of them need redescription based on detailed observation in vivo and the infraciliature from modern protargol impregnation. Paiva & Silva-Neto (2007) published an important paper in which they combined a redescription of Strongylidium pseudocrassum Wang & Nie, 1935 and a description of its ontogenetic events for the first time. Using the literature, they scrutinized the 22 morphological species assigned to Strongylidium (Berger, 2001), dividing them into five basic groups. Based on their redefinition, only the 14 species of group 1 (congeners that possess two long marginal and two long ventral cirral rows, including the type species) were included in this genus; the two species of group 2 (having a single long ventral row) possibly belong to Hemiamphisiella, and the other six species from groups 3 (two species with one long right and one short left ventral rows), 4 (two species that possess short ventral rows) and 5 (two species with more than two long marginal or ventral rows) were excluded. Although not based on the type species, the work of Paiva & Silva-Neto (2007) also provided a clear and very useful revision and redefinition that is helpful for further investigation of Strongylidium.
In the present paper, we describe morphological, morphogenetic and small-subunit (SSU) rRNA gene sequence-based phylogenetic analyses of Strongylium orientale sp. nov., isolated from a mangrove river in Hong Kong, southern China. Its ontogenetic characters and the results of our phylogenetic analysis both show a close relationship with its congener S. pseudocrassum and with the genus Pseudouroleptus.

METHODS

Sample collection and identification. Samples were collected on 3 December 2009 from a small river connecting a mangrove to the sea in Hong Kong (22° 29' 50" N 114° 01' 48" E; water temperature 20 °C, salinity 15.7‰). Water (total volume 200–300 ml) from the sampling site was maintained in Petri dishes at room temperature (about 23 °C) with rice grains as a food source to promote the growth of bacteria as food for the ciliates. Cells were examined in vivo using bright-field and differential interference contrast microscopy (Shao et al., 2011) and were impregnated with protargol following the method of Wilbert (1975) in order to reveal the infraciliature. Counts and measurements of the morphological characteristics of stained specimens were performed at a magnification of ×1000. Drawings were made with the help of a camera lucida. Terminology mainly follows Berger (2008).

DNA extraction and sequencing. Genomic DNA was extracted from cells washed with filtered habitat water using a REDExtract-N-Amp Tissue PCR kit (Sigma) according to the manufacturer’s instructions. The SSU rRNA gene was amplified using the universal primers EuKA and EuKB (Medlin et al., 1988). Cloning and sequencing were performed according to Yi & Song (2008).

Sequence availability and phylogenetic analyses. The SSU rRNA gene sequence of the novel species was aligned with sequences of 44 other ciliates downloaded from the GenBank database (see Fig. 5 for accession numbers) using CLUSTAL W implemented in BioEdit 7.0 (Hall, 1999). Novistrombidium testaceum, Strombidinopsis acuminata, Strombidium purpureum and Tintinnidium mucicola were selected as the outgroup taxa. Highly variable regions in which alignment could not be determined unambiguously were excluded before phylogenetic analysis. Modeltest (Posada & Crandall, 1998) was used to select the GTR + I (0.5193) + G (0.4788) evolutionary model under the AIC criterion. Using these parameters, a maximum-likelihood (ML) tree was reconstructed with PhyML version 2.4.4 (Guindon & Gascuel, 1998), and a Bayesian inference (BI) analysis was performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) after using MrModeltest 2.2 (Nylander, 2004) to select the GTR + I (0.5142) + G (0.4762) evolutionary model under the AIC criterion. The program was run for 1 500 000 generations with a sampling frequency of 100 and a burn-in of 3750 trees (25%). A single consensus tree was created from these trees for analyses. TreeView version 1.6.6 (Page, 1996) and MEGA 4.0 (Tamura et al., 2011) were used to view and edit trees for publication. Systematic classification mainly follows Lynn (2008).

RESULTS

Strongylium orientale sp. nov.

Diagnosis

Body measuring 80–120 × 35–50 μm in vivo, flexible and slightly contractile. Fusiform in shape, with rounded anterior and tapered posterior ends. Two types of cortical granules; both colourless and spherical. Larger ones are approx. 1.5–2 μm across, while smaller ones are approx. 0.2 μm across, with both kinds arranged irregularly throughout the cortex, rendering the cell grey. Adoral zone bipartite with 5–6 distal and 18–22 proximal membranelles. Single buccal, three frontal and on average one (0–2) post-peristomial cirri; 33–45 left and 23–42 right ventral cirri; 26–36 left and 28–42 right marginal cirri. Three dorsal kinetics with one caudal cirrus at end of each one. Two macronuclear nodules and one to three micronuclei. Brackish habitat.

Type locality

A mangrove river in Hong Kong, southern China (22° 29' 50" N 114° 01' 48" E; water temperature 20 °C, salinity 15.7‰).

Type specimens

The protargol slide containing the holotype specimen (see Fig. 2); registration no. CXM09120301/1), and four para-type (registration no. CXM09120301/2–5) have been deposited in the Laboratory of Protozoology, Ocean University of China, PR China.

Etymology

L. neut. adj. orientale or belonging to the East, eastern. Named to reflect the location where the type specimen was discovered.

Morphological description (Table 1 and Figs 1 and 2)

Cell in vivo about 80–120 × 35–50 μm, flexible and slightly contractile, somewhat fusiform in shape, with rounded anterior and tapered posterior ends, a ratio of length to width about 3:1 and dorsoventrally flattened about 3:1 (Figs 1a–c and 2a–c). Adoral zone comprises nearly 30 % of body length. Pellicle thin and soft, two types of cortical granules colourless and spherical. Larger ones approx. 1.5–2 μm across, with smaller ones approx. 0.2 μm across, both arranged irregularly throughout cortex, rendering the cell grey (Figs 1d, e and 2d, f). Cytoplasm colourless, usually with many lipid droplets (approx. 3 μm across) and food vacuoles containing diatoms and bacteria. Contractile vacuole about 15–20 μm across, located near left margin in mid-body region, pulsating at intervals of 1–2 min.

Locomotion by slow crawling on the bottom of Petri dishes and among debris, and by rotating around longitudinal axis when swimming.

Infraciliature as shown in Fig. 1(f–h). A conspicuous gap separating the adoral zone into two parts, with distal and proximal parts comprising 5–6 and 18–22 membranelles, respectively. Paroral membrane a little longer than endoral one, slightly curved inwards. Single buccal cirrus located at anterior quarter of undulating membranes. Three clearly differentiated frontal cirri and single cirrus III/2. One post-
**Table 1.** Morphological characterization of *Strongylidium orientale* sp. nov.

Data are based on observations of 25 protargol-impregnated specimens.

<table>
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<tr>
<th>Character</th>
<th>Min.</th>
<th>Max.</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
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<td>(μm)</td>
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peristomial cirrus on average (in 25 random specimens, 18 cells with one, two cells with two and five cells with none), located behind proximal end of adoral zone (Figs 1f and 2h, j). Two long and oblique sigmoid ventral cirral rows. Left ventral row beginning approximately near rightmost frontal cirrus and comprising 33–45 cirri. Right ventral row starting about at the level of posterior end of undulating membranes, with 23–42 cirri. Left and right marginal rows comprise 26–36 and 28–42 cirri, respectively, and reach to posterior end of cell (Figs 1g and 2i), with cilia 10–15 μm long in vivo. After protargol impregnation, some fibres appeared associated with cirri in left and right ventral rows (Figs 1g and 2g). Three dorsal kinetics (with dorsal bristles 2–4 μm long in vivo) extending almost the entire length of the cell with one caudal cirrus (15–20 μm long in vivo) at the end of each one (Fig. 1h). Invariably two macronuclear nodules and one to three micronuclei located at the left side of body (Figs 1h and 2e, h–k).

**Morphogenesis during binary fission (Figs 3 and 4)**

Stomatogenesis commenced with the apokinetal formation of a longitudinal field of closely spaced basal bodies behind the parental adoral zone of membranelles, which formed the oral primordium of the opisthe (Figs 3a and 4a). With the proliferation of basal bodies, the longitudinal field of basal bodies differentiated new membranelles posteriorad. Simultaneously, in the proter, the frontoventral cirral anlagen originated de novo close to the old undulating membranes. There were some anarchic primordia formed

of the adoral zone of membranelles arched to the right. In both dividers, the anlagen of undulating membranes split into paroral and endoral membranes and gave rise to the leftmost frontal cirri. The frontoventral cirral anlagen differentiated into one III/2, one buccal, one post-peristomial, three frontal cirri and two cirri for the left ventral row (Figs 3h and 4f, h).

In the middle stage, the oral primordium of the opisthe developed and continued to form new membranelles posteriorad. In the proter, the anlagen of undulating membranes were formed from the old structures, and the most posterior part of the old adoral zone of membranelles also dedifferentiated. At the same time, the frontoventral cirral anlagen developed into three streaks. The left and right ventral rows dedifferentiated intrakinetically into anlagen (Figs 3e, f and 4d).

In the late stage, the old adoral zone was retained by the proter, with only the most posterior part renewed by dedifferentiation, and, in the opisthe, the anterior part of the adoral zone of membranelles arched to the right. In both dividers, the anlagen of undulating membranes split into paroral and endoral membranes and gave rise to the leftmost frontal cirri. The frontoventral cirral anlagen differentiated into one III/2, one buccal, one post-peristomial, three frontal cirri and two cirri for the left ventral row (Figs 3h and 4f, h).

The marginal rows developed intrakinetically and the dorsal kinetics anlagen (DKA) originated de novo; one caudal cirrus was formed at the posterior end of each DKA (Figs 3c, h, i and 4c–e, g).

The two macronuclear nodules fused into a single mass and then divided. The divisional process of the micronuclei was unclear, although they were recognizable in some of the dividers (Figs 3c, g, i and 4b–h).

One stage of physiological regeneration was evident and showed similar processes of cortical development to those in the proter (Fig. 4i).

**Molecular data and phylogenetic analyses**

The SSU rRNA gene sequence of *Strongylidium orientale* sp. nov. has been deposited in GenBank/EMBL/DDBJ with the accession number KC153532. The length and G+C content of the SSU rRNA gene were 1728 bp and 45.25 mol%.

We included a broad selection of SSU rRNA gene sequences from 45 species in the phylogenetic analyses. The topologies of the ML and BI trees were basically congruent. *S. orientale* sp. nov. clustered first with *S. pseudocrassum* (ML/BI, 99.7/1.00) and they then together formed a well-supported branch with *Pseudouroleptus caudatus* (ML/BI, 99.8/1.00) (Fig. 5).

**DISCUSSION**

**Comparison with related congeners**

In the detailed and clear revision of the genus *Strongylidium* presented by Paiva & Silva-Neto (2007),
the 22 morphological species assigned to Strongylidium were divided into five groups. S. orientale sp. nov. should be included in group 1, the members of which are characterized by the possession of two long ventral and two marginal cirral rows. Fourteen congeners were originally assigned to this group by Paiva & Silva-Neto (2007), including Strongylidium crassum, the type species.

Strongylidium lentum (Biernacka, 1963) Paiva & Silva-Neto, 2007, Strongylidium muscorum Kahl, 1932 and the marine form Strongylidium sp. (Kahl, 1932) have multiple macronuclear nodules and thus clearly differ from other members of group I and also our novel species.

Strongylidium contortus (Gelei, 1954) may be conspecific with the freshwater form Strongylidium sp. (Kahl, 1932) because they have similar body size and cirral number (Paiva & Silva-Neto, 2007). Our novel species can be distinguished from them by its smaller body size (80–120 × 35–50 μm vs 140–170 × 50–60 μm).

Strongylidium caudatum Kahl, 1928 has a distinctly long tail and conspicuously elongated adoral crown. Strongylidium coronatum Vuxanovici, 1963 and Strongylidium labiatum Kahl, 1932 have long peristomes that occupy about half of the cell length (Paiva & Silva-Neto, 2007); in addition, S. labiatum has a noticeably wider peristome and

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**Fig. 1.** Strongylidium orientale sp. nov. observed in vivo (a–e) and after protargol impregnation (f–h). (a) Ventral view of a representative individual. (b, c) Lateral views of cells of variable shapes. (d, e) Ventral (d) and dorsal (e) views, showing the distribution of cortical granules. (f) Ventral ciliature of anterior portion of cell, to show the buccal cirrus (arrowhead), post-peristomial cirrus (arrow), the gap between the two parts of the AZM (double arrowheads) and three enlarged frontal cirri (dashed box). (g, h) Ventral (g) and dorsal (h) views of the same individual, showing infraciliature and nuclear apparatus. Arrows point to the anterior region of the ventral cirral rows (g) and micronucleus (h), and arrowheads indicate the fibres associated with cirri (g) and caudal cirri (h). AZM, Adoral zone of membranelles; CV, contractile vacuole; DK1–3, dorsal kineties 1–3; EM, endoral membrane; III/2, cirrus III/2; LMR, left marginal row; LVR, left ventral row; Ma, macronuclear nodules; PM, paroral membrane; RMR, right marginal row; RVR, right ventral row. Bars, 50 μm.

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a prominent peristomial lip and paroral membrane, and its nuclear apparatus contains a single micronucleus located between two macronuclear nodules (Paiva & Silva-Neto, 2007). These characters distinguish these species from our novel species.

*Strongylidium lanceolatum* Kowalewskiego, 1882 differs from *S. orientale* sp. nov. by having an elongated, neck-like anterior constriction and lacking a post-peristomial cirrus (Paiva & Silva-Neto, 2007).

The type population of *Strongylidium mucicola* Kahl, 1932 is depicted with a much more evident frontal scutum (vs absent in *S. orientale* sp. nov.) and with macronuclear nodules located to the right margin of the cell (vs left in *S. orientale* sp. nov.).

In our novel species, the right ventral cirral row starts at the level at which the undulating membranes end, and the left ventral cirral row begins at the distal end of the adoral membranelles (near the rightmost frontal cirrus). These characteristics can help to distinguish it from the type

A novel stichotrich ciliate from mangrove

Fig. 2. Photomicrographs of *Strongylidium orientale* sp. nov. from life (a–f) and after protargol impregnation (g–k). (a) Ventral view of a representative specimen. (b, c) Different cells showing variable body shape. (d, f) Ventral (d) and dorsal (f) views, showing the distribution of cortical granules; arrows point to the larger ones, arrowheads mark the smaller ones and double arrowheads show the dorsal cilia. (e, k) Showing the nuclear apparatus (arrows mark micronuclei); the replication band of the macronuclear nodule appears in an interphase cell (k). (g) Part of a ventral view; arrowheads indicate the fibres associated with cirri. (h–j) Ventral views, to show the buccal cirrus (arrowheads), the gap between the two parts of the AZM (double arrowheads) and three enlarged frontal cirri (dashed box). Arrows point to the anterior region of the ventral cirral rows (h, i) and the post-peristomial cirrus (j; the holotype specimen). AZM, Adoral zone of membranelles; EM, endoral membrane; III/2, cirrus III/2; LMR, left marginal row; LVR, left ventral row; Ma, macronuclear nodules; PM, paroral membrane; RMR, right marginal row; RVR, right ventral row. Bars, 50 μm.
species *S. crassum* (wherein the two ventral cirral rows begin at the same level, at around the mid-way point of the undulating membranes; Sterki, 1878).

According to the original description of *Strongylidium microstoma* Dragesco & Dragesco-Kerneis, 1986, *S. orientale* sp. nov. has a shorter body length after protargol impregnation (107–138 vs 135–188 μm), fewer adoral membranelles (23–28, mean 26 vs 36–40, mean 37) and fewer cirri in the left (mean 38 vs 45) and right (mean 31 vs 43) ventral rows (Dragesco & Dragesco-Kerneis, 1986).

*S. pseudocrassum* Wang & Nie, 1935 has a similar body shape, size and cirral pattern to our isolate. However, the...
Brazilian population of *S. pseudocrassum* described by Paiva & Silva-Neto (2007) has fewer cirri than *S. orientale* in the left ventral row (22–36, mean 30 vs 33–45, mean 38), right ventral row (12–32, mean 25 vs 23–42, mean 31), left marginal row (17–33, mean 23 vs 26–36, mean 31) and right marginal row (18–35, mean 28 vs 28–42, mean 35).

*Strongylidium californicum* Kahl, 1932 has a slender and elongated body shape (with a ratio of length to width of about 5:1), which is different from *S. orientale* sp. nov., where the cell is fusiform with rounded anterior and tapered posterior ends and a ratio of length to width of about 3:1.

**Fig. 4.** Photomicrographs of *Strongylidium orientale* sp. nov. in morphogenesis after protargol impregnation. (a) Vinal view of an early divider, showing the appearance of the oral primordium of the opisthe (OP). (b, c) Vental (b) and dorsal (c) views of the same specimen; the arrow marks the FVA in the proter (b), the arrowhead points out an anarchic primordium (b) and double arrowheads (c) indicate the DKA. (d, e) Vental (d) and dorsal (e) views of the same divider in middle stage; arrows point to the FVA in the proter (d) and Mi (e), arrowheads show that the anlagen of the ventral rows form intrakinetally (d) and double arrowheads mark the DKA. (f, g) Ventral (f) and dorsal (g) views of the same divider in late stage; arrows point to the leftmost frontal cirri formed from the UMA (f), arrowheads mark the buccal cirri (f) and the forming caudal cirri from the DKA (g) and the double arrowhead shows the post-peristomial cirri in the proter. Dashed circles indicate the anterior segment of the left ventral cirral row and circles mark the median segment of the left ventral cirral row. (h) Ventral view of a newly formed cell; arrow points to the anterior end of the right ventral row. (i) Vental view of a reorganizer, marking the leftmost frontal cirrus formed from the UMA (arrow) and new buccal cirrus (arrowhead). DKA, Anlagen of dorsal kineties; FVA, anlagen of frontoventral cirri; LMA, anlagen of left marginal row; Ma, macronuclear nodules; Mi, micronuclei; OP, oral primordium; RMA, anlagen of right marginal row; UMA, anlagen of undulating membranes. Bars, 50 μm.
Special morphogenetic characters of *Strongylidium* and related genera

To our knowledge, morphogenetic processes have never been scrutinized in the type species *S. crassum*. Paiva & Silva-Neto (2007) described the detailed process in *S. pseudocrassum* for the first time in its congeners.

*S. orientale* sp. nov. exhibits similar ontogenetic features to *S. pseudocrassum*: (i) the oral primordium in the opisthe occurs apokinetally; (ii) the left and right ventral rows originate intrakinetally and the final left ventral row is formed from three parts, two cirri from the frontoventral cirral anlagen, a short cirral row from the anlagen of the right ventral row and the whole anlagen of the left ventral row (we do not have specimens of late stages; the postulated migration is shown in Figs 3h and 4f, h); (iii) the marginal rows develop intrakinetally; (iv) the dorsal kineties replicate entirely *de novo* and do not fragment; and (v) the two macronuclear nodules fuse into a mass and then divide.

The parental adoral zone of membranelles remains complete in *S. pseudocrassum*, but the posterior part is renewed by dedifferentiation in *S. orientale* sp. nov. Paiva & Silva-Neto (2007) stated that the posterior part of the adoral zone of *S. pseudocrassum* is renewed during the reorganization process. Perhaps this also happened during the ontogenetic process but was overlooked by Paiva & Silva-Neto (2007)? Because of the lack of some key stages during the ontogenetic process of *S. orientale* sp. nov., the origins of the frontoventral cirral anlagen in the opisthe are not clear and cannot compared with *S. pseudocrassum*.

*Pseudouroleptus* Hemberger, 1985 and *Hemiamphisiella* Foissner, 1988 are the most morphologically similar genera to *Strongylidium*, and they share the same manner of organization of the left ventral cirral row. The replicating patterns of their dorsal kineties, however, are totally different: the third dorsal kinety segments the fourth one in *Pseudouroleptus*, whereas it forms *de novo* without fragmentation in *Strongylidium*. Similarly, the pattern of formation of the right ventral row is different: it develops from the sixth frontoventral cirral anlagen in *Pseudouroleptus* and *Hemiamphisiella* compared with intrakinetel formation in *Strongylidium*.

Species of *Amphisiella* (the type genus of the family Amphisiellidae) share a similar mode of formation of their...
amphisiellid median cirral row with *Strongylidium*, *Pseudouroleptus* and *Hemiamphisiella* in their left ventral row, both of which are spliced from different anlagen. However, in *Amphisiella*, the anterior part of the FVTA-IV stays at the frontal portion and forms as a short cirral row instead of joining the final amphisiellid median cirral row. This differs from *Strongylidium*, *Pseudouroleptus* and *Hemiamphisiella*, in which the anterior part of the FVTA-IV migrates and merges into the final left ventral row.

**Phylogenetic position**

The overall topology of our phylogenetic tree is congruent with published papers (Dai & Xu, 2011; Foissner & Stoeck, 2011; Miao et al., 2011; He & Xu, 2011; Paiva et al., 2012). Our phylogenetic analysis also indicates a close relationship between *Pseudouroleptus* and *Strongylidium*, which tends to support the morphological (similar infraciliature patterns) and morphogenetic (same formation pattern of the left ventral row) data. The morphogenetic data, however, exhibit some unique features for *Strongylidium*: the right ventral row formed intrakinetally and the dorsal kinetics originated *de novo* without fragmentation, in contrast to *Pseudouroleptus*.

*Pseudouroleptus* was originally classified in the Amphisiellidae (Hemberger, 1982, 1985) and later transferred to the Kahlidiellidae by Tuffrau (1987). Eigner (1997) assigned it to the family Oxytrichidae under the order Sporadotrichida, because it shows 'neokinetal 3' anlagen development (Eigner & Foissner, 1999; Berger, 1999). According to Lynn (2008), *Strongylidium* was placed within the family Spirofilidae Gelei, 1929 and *Pseudouroleptus* was assigned as *incertae sedis* in the family Kahlidiellidae Tuffrau, 1979, both of which are under the order Stichotrichida Fauré-Fremiet, 1961. The two genera have different morphogenetic modes for their right ventral row and dorsal kinetics; however, this might not be enough to separate them at the order level, since they share many more similarities.

Ontogenetic data, together with features of the physiological reorganization, play important roles in defining relationships among the hypotrichous ciliates (Chen et al., 2011; Paiva et al., 2012). It is still hard to decide which characteristics should have the greater weight at different taxonomic levels. In the light of the lack of a more detailed morphogenetic redescription and morphogenetic information for *S. crassum* (type species), more information is required before it will be possible to arrive at a clearer classification for this genus.

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