Morphology, ontogenetic features and SSU rRNA gene-based phylogeny of a soil ciliate, *Bistichella cystiformans* spec. nov. (Protista, Ciliophora, Stichotrichia)

Yangbo Fan,¹ Xiaozhong Hu,¹ Feng Gao,¹ Saleh A. Al-Farraj² and Khaled A. S. Al-Rasheid²

¹Laboratory of Protozoology, Institute of Evolution & Marine Biodiversity, College of Fisheries, Ocean University of China, Qingdao 266003, PR China
²Zoology Department, King Saud University, Riyadh 11451, Saudi Arabia

The morphology, ontogeny and SSU rRNA gene-based phylogeny of *Bistichella cystiformans* spec. nov., isolated from the slightly saline soil of a mangrove wetland in Zhanjiang, southern China, were investigated. The novel species was characterized by having five to eight buccal cirri arranged in a row, three to five transverse cirri, four macronuclear nodules aligned, and 17–32 and 20–34 cirri in frontoventral rows V and VI, respectively, both extending to the transverse cirri. The main ontogenetic features of the novel species were as follows: (1) the parental adoral zone of the membranelles is completely inherited by the proter; (2) the frontoventral and transverse cirri are formed in a six-anlagen mode; (3) basically, the frontal-ventral-transverse cirral anlagen II–V generate one transverse cirrus each at their posterior ends, while anlage VI provides no transverse cirrus; (4) both marginal rows and dorsal kineties develop intrakinetally, no dorsal kinety fragment is formed; and (5) the macronuclear nodules fuse into a single mass at the middle stage. Phylogenetic analyses based on the SSU rRNA gene showed that the novel species groups with the clade containing *Bistichella variabilis*, *Parabistichella variabilis*, *Uroleptoides magnigranulosus* and two species of the genus *Orthoamphisiella*. Given present knowledge, it was considered to be still too early to come to a final conclusion regarding the familial classification of the genus *Bistichella*; further investigations of key taxa with additional molecular markers are required.

Introduction

Stichotrichian ciliates have proved to be one of the most important components of the microbial food web in almost all aquatic and terrestrial ecosystems worldwide, because they consume a significant portion of the bacterial productivity, enhancing nutrient cycles and energy flow to organisms at higher trophic levels (Foissner, 1987, 1998, 1999; Song et al., 2009).

Berger (2008) established the genus *Bistichella* as a taxon of unknown position in the Hypotricha (=Stichotrichia Small & Lynn 1985; Lynn, 2008), with four species transferred from the genus *Pseudouroleptus* and one from the genus *Amphisiella*. Based on its diagnostic features, e.g. the presence of two short frontal and two long frontoventral rows, the genus *Bistichella* should be classified in the order Stichotrichida Fauré-Fremiet 1961. Although the small subunit rRNA (SSU rRNA) gene sequence for *Bistichella variabilis* has recently become available (He & Xu, 2011), there remains a lack of morphogenetic data from which to determine the origin of the two long frontoventral rows. Given the present state of knowledge, therefore, it is premature to assign the genus *Bistichella* to any known family in the Stichotrichida.

During a recent survey of ciliates in mangrove wetland near Zhanjiang, southern China, a novel ciliate was isolated from slightly saline soil. The isolate was successfully maintained in raw cultures, giving the opportunity to study its morphology, morphogenesis and phylogeny based on SSU rRNA gene sequence data. We demonstrate here that the population represents a new member of the genus *Bistichella*, and discuss the familial classification of the genus.

Methods

**Sampling and cultivation.** *Bistichella cystiformans* spec. nov. was isolated from a soil sample collected from the Gaoqiao...
Mangrove National Nature Reserve in Zhanjiang (21° 31’ 30” N 110° 16’ 58” E), southern China. The wet soil sample was collected from the upper 0–5 cm on 8 April 2012 (Fig. 1a). After being air-dried, the sample was stored in a plastic bag prior to being studied on 18 April 2012. The samples were wetted using the non-flooded Petri dish method (Foisssner et al., 2002). Briefly, this method involves placing 50–500 g litter and mineral soil in a Petri dish (13–18 cm wide, 2–3 cm high) and saturating, but not flooding it, with distilled water (Fig. 1b). The species appeared several days after rewetting the sample in the Petri dishes with distilled water at a temperature of about 27 °C and salinity of 5%. Cells were maintained in the laboratory for about two weeks as raw cultures with smaller colpodids and scuticociliates, as well as bacteria, as food sources.

In order to investigate the cysts, about 20 specimens isolated from the raw culture were added to a large drop of filtered in situ water in a concave slide, which was kept in a wet chamber (Foisssner & Stoeck, 2011). All specimens had encysted within 12 h.

**Morphological and morphogenetic studies.** Living cells and cysts were picked out and observed using bright-field and differential interference contrast microscopy at ×100–1000 (Nikon Eclipse 80i, Japan). Protargol impregnation (Wilbert, 1975) was used to reveal the infraciliature and nuclear apparatus. Measurements of stained specimens were performed with an ocular micrometre. Drawings were made with the help of a camera lucida. To illustrate the changes which occurred during the morphogenetic process, parental cirri were depicted in contour lines, whereas new ones were shaded black. The terminology used is according to Berger (2008).

**DNA extraction, PCR amplification, and sequencing.** Genomic DNA was extracted from cells using a the DNeasy Tissue kit (Qiagen), following the manufacturer’s instructions. The PCR amplification of the SSU rRNA gene was performed according to the protocol of Huang et al. (2014) using the primers 82F (5’-GAACTGCGAAT-GGCTC-3’) and 18S-R (5’-TGATCCCTCTGCAAGGTTCACCTAC-3’) (Medlin et al., 1988). Purified PCR product of the appropriate size was directly sequenced in both directions on an ABI 3700 sequencer.

**Phylogenetic analyses.** The SSU rRNA gene sequence of the novel species, together with the sequences of 60 representative taxa downloaded from the GenBank database, were used in the present phylogenetic analyses. Two choreotrichs and two oligotrichs were selected as the outgroup species. Accession numbers are shown in Fig. 6, except for 13 urostyld species: Pseudokeronopsis flava (DQ227798), Uroleptopsis citrina (FJ870094), Nothoholosticha fasciola (FJ377548), Heterokeronopsis pulchra (JQ083600), Metaurostylopsis salina (EU220229), Monocoronella carnea (FJ775726), Urostyla grandis (EF535731), Anteholosticha manca (DQ503578), Diaxontella pseudorubra (GU942564), Apokeronopsis wrightii (EU417963), Thigmokeronopsis stoecki (EU220226), Hemicychoistyla sphagni (FJ361758) and Pseudourostyla cristata (DQ019318). Sequences were aligned using MUSCLE v3.7 (Penn et al., 2010) with default parameters. The resulting alignment was manually edited using the program BioEdit 7.0.5.2 (Hall, 1999). Ambiguously aligned regions and gaps were excluded prior to phylogenetic analysis, resulting in a matrix of 1727 characters (available from the authors upon request). Maximum-likelihood (ML) analysis was conducted using RAxML-HPC2 v7.3.2 on XSEDE (Stamatakis et al., 2008) on CIPRES Science Gateway (Miller et al., 2010), using the GTR+G model as the optimal choice. Support for the best ML tree came from 1000 bootstrap replicates with the GTR+CAT model. Bayesian inference (BI) analysis was performed with MrBayes v3.2.2 on XSEDE (Ronquist & Huelsenbeck, 2003) on the CIPRES Science Gateway, using the GTR+1 (=0.5278)+G (=0.4686) model as
selected by MrModeltest v.2.0 (Nylander, 2004). Markov chain Monte Carlo (MCMC) simulations were run with two sets of four chains for 2 000 000 generations with a sampling frequency of 100 and a burn-in of 5000 trees (25%). All remaining trees were used to calculate posterior probabilities using a 50% majority rule consensus. TreeView v.1.6.6 (Page, 1996) and MEGA 4.0 (Tamura et al., 2007) were used to visualize the tree topologies. The systematic classification follows Lynn (2008).

**Results**

*Bistichella cystiformans* spec. nov.

**Diagnosis.** Size about 120–200 μm × 40–80 μm; body elongate elliptical with both ends broadly rounded and 33–45 adoral membranelles, plus five to eight buccal cirri arranged in a row. Four to six cirri in frontal row III; a mean of seven cirri in frontal row IV usually extending to the same length as the adoral zone; 17–32 and 20–34 cirri in frontoventral rows V and VI, respectively, both extending to the transverse cirri; three to five transverse cirri, 20–35 left and 25–47 right marginal cirri; three bipolar dorsal kineties. Four macronuclear nodules and three to six micronuclei.

**Type locality.** Slightly saline soil from Gaoqiao Mangrove National Nature Reserve in Zhanjiang (21°31′30″N 110°16′58″E), southern China.

**Deposition of type specimens.** One protargol-impregnated slide with the holotype specimen (Fig. 2e, f) (registration number: FYB2012040801) and a paratype slide (registration number: FYB2012040801-1) have been deposited in the Laboratory of Protozoology, OUC, Qingdao, PR China.

**Etymology.** *cysti.*formans. [N.L. fem. n. *cystis* (from Gr. fem. n. *kustis*) the bladder and in biology, a cyst; L. part. adj. *formans* forming; N.L. part. adj. *cystiformans* cyst-forming]. This species-group name indicates that this species can form cysts in response to food starvation.

**Gene sequence.** The SSU rRNA gene sequence has been submitted to the GenBank database with the accession number KJ509196.

**Morphological description.** Morphology is described in Table 1 and depicted in Figs 2(a–f), 3(a–h) and 4(a–c). Size was 120–200 μm × 40–80 μm in vivo. Body elongate elliptical in outline, with the anterior and posterior end broadly rounded, dorsoventrally flattened about 3:1, highly flexible but not contractile (Figs 2a–c and 3a–b). Adoral zone approximately 2/5 of body length. Pellicle soft, without cortical granules underneath. Cytoplasm colourless, usually with many lipid droplets (approx. 3 μm across) and food vacuoles containing small colpodids, scuticociliates and bacteria, which were observed in both living and

---

**Fig. 2.** *Bistichella cystiformans* spec. nov. in vivo (a–c) and after protargol impregnation (d–f). (a) Ventral view of a representative individual; (b) left lateral view; (c) swimming movement; (d) detailed ventral view of the anterior portion with the frontal cirri and buccal cirri row shaded, while the broken lines connect cirri generated in the same anlage. (e, f) Ventral (e) and dorsal (f) view of the holotype to show the infraciliature and nuclear apparatus. AZM, adoral zone of membranelles; BC, buccal cirri; DK1–3, dorsal kineties; EM, endoral membrane; FC, frontal cirri; LMR, left marginal row; Ma, macronuclear nodules; Mi, micronuclei; PM, paroral membrane; RMR, right marginal row; TC, transverse cirri; III, IV, frontal rows; V, VI, frontoventral rows. Bars, 70 μm.
protargol-impregnated specimens. Contractile vacuole about 25 μm across, slightly above mid-body near left body margin (Fig. 2a), pulsating at intervals of about 30 s. Four macronuclear nodules, spherical to ellipsoid, conspicuous in vivo, arranged slightly to the left of the cell. Three to six spherical micronuclei, attached or near to the macronuclear nodules. Crawls slowly on substrates or rotates around the main body axis when swimming. Body-folding or twisting observed when crawling over debris. Cells easily encyst when food is scarce. Cysts usually globular and about 40 μm in diameter; cortex thin and indistinct, cortical granules absent (Fig. 3h). Cyst contents dominated by the nuclear apparatus and the cyst plasm with many globular inclusions 2–3 μm in diameter.

Infraciliature as shown in Figs 2(d–f) and 4(a–c). Adoral zone composed of 33–45 membranelles, with its distal portion located at the right anterior end of the body. Paroral and endoral membranes almost equal in length and parallel to each other, the former slightly longer than the latter. Cirral pattern and number of cirri usually variable, except for the three enlarged frontal cirri, with the rightmost one immediately behind the distal end of the adoral zone of membranelles. Five to eight buccal cirri arranged in a longitudinal row close to the paroral membrane. No frontoterminal cirri present. Four to six cirri in frontal row III, located at the same level as the row of buccal cirri. Frontal row IV composed of seven cirri on average, usually extending to the proximal end of the adoral zone; in eight out of 25 individuals this row comprised 16 cirri, extending nearly to the mid-body. Frontoventral row V composed of 17–32 cirri, starting from the anterior quarter of the body and extending slightly obliquely to the leftmost transverse cirrus. Frontoventral row VI composed of 20–34 cirri, starting near the anterior end of the right marginal row and terminating close to the right-most transverse cirrus. Usually, four transverse cirri, arranged in an oblique pseudorow, protruding beyond the posterior end of the body. In three out of 25 individuals, an additional frontoventral row and five transverse cirri were present. One left and one right marginal row confluent posteriorly, composed of 20–35 and 25–47 cirri, respectively. Three bipolar dorsal kineties with about 24 dikinetids in each row, slightly shortened anteriorly and posteriorly; dorsal cilia approximately 3 μm long in vivo. A short row with about four to six dikinetids was present on the anterior-left of the left-most dorsal kinety in four out of 25 individuals. Caudal cirri absent.

Table 1. Morphometric characterization of Bistichella cystiformans spec. nov.

All data are based on protargol-impregnated specimens. Measurements are all in μm. AZM, adoral zone of membranelles. CV, coefficient of variation (%); Max, maximum; Mean, arithmetic mean; Min, minimum; n, sample size; SD, standard deviation.

<table>
<thead>
<tr>
<th>Characteristic*</th>
<th>Min</th>
<th>Max</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length</td>
<td>86</td>
<td>146</td>
<td>106.3</td>
<td>16.43</td>
<td>15.5</td>
<td>25</td>
</tr>
<tr>
<td>Body width</td>
<td>26</td>
<td>86</td>
<td>41.3</td>
<td>15.08</td>
<td>36.5</td>
<td>25</td>
</tr>
<tr>
<td>AZM length</td>
<td>39</td>
<td>82</td>
<td>47.5</td>
<td>9.37</td>
<td>19.7</td>
<td>25</td>
</tr>
<tr>
<td>Number of adoral membranelles,</td>
<td>33</td>
<td>45</td>
<td>37.5</td>
<td>2.66</td>
<td>7.1</td>
<td>25</td>
</tr>
<tr>
<td>Distance between body anterior end and distal end of AZM</td>
<td>7</td>
<td>13</td>
<td>10.0</td>
<td>1.65</td>
<td>16.5</td>
<td>15</td>
</tr>
<tr>
<td>Number of frontal cirri</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Number of buccal cirri</td>
<td>5</td>
<td>8</td>
<td>6.5</td>
<td>0.77</td>
<td>11.9</td>
<td>25</td>
</tr>
<tr>
<td>Number of cirri in frontal row III</td>
<td>4</td>
<td>6</td>
<td>4.5</td>
<td>0.65</td>
<td>14.6</td>
<td>25</td>
</tr>
<tr>
<td>Number of cirri in frontal row IV</td>
<td>5</td>
<td>18</td>
<td>10.7</td>
<td>3.76</td>
<td>35.2</td>
<td>25</td>
</tr>
<tr>
<td>Distance between body anterior end and the anterior end of frontoventral row V</td>
<td>15</td>
<td>30</td>
<td>19.8</td>
<td>4.71</td>
<td>23.8</td>
<td>15</td>
</tr>
<tr>
<td>Number of cirri in frontoventral row V</td>
<td>17</td>
<td>42</td>
<td>24.3</td>
<td>4.84</td>
<td>19.9</td>
<td>25</td>
</tr>
<tr>
<td>Number of cirri in frontoventral row VI</td>
<td>20</td>
<td>34</td>
<td>27.1</td>
<td>3.29</td>
<td>12.2</td>
<td>25</td>
</tr>
<tr>
<td>Number of transverse cirri</td>
<td>3</td>
<td>5</td>
<td>4.0</td>
<td>0.50</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td>Distance between body posterior end and the rearmost transverse cirrus</td>
<td>6</td>
<td>25</td>
<td>14.1</td>
<td>5.57</td>
<td>39.4</td>
<td>15</td>
</tr>
<tr>
<td>Number of cirri in left marginal row</td>
<td>20</td>
<td>35</td>
<td>27.8</td>
<td>3.53</td>
<td>12.7</td>
<td>25</td>
</tr>
<tr>
<td>Number of cirri in right marginal row</td>
<td>25</td>
<td>47</td>
<td>33.2</td>
<td>4.99</td>
<td>15.0</td>
<td>25</td>
</tr>
<tr>
<td>Number of dorsal kineties</td>
<td>3</td>
<td>4</td>
<td>3.2</td>
<td>0.41</td>
<td>12.8</td>
<td>20</td>
</tr>
<tr>
<td>Number of dikinetids in dorsal kinety 1</td>
<td>20</td>
<td>32</td>
<td>24.0</td>
<td>3.42</td>
<td>14.3</td>
<td>15</td>
</tr>
<tr>
<td>Number of dikinetids in dorsal kinety 2</td>
<td>18</td>
<td>30</td>
<td>24.6</td>
<td>3.09</td>
<td>12.6</td>
<td>15</td>
</tr>
<tr>
<td>Number of dikinetids in dorsal kinety 3</td>
<td>20</td>
<td>29</td>
<td>24.9</td>
<td>2.39</td>
<td>9.6</td>
<td>15</td>
</tr>
<tr>
<td>Number of macronuclei nodules</td>
<td>4</td>
<td>4</td>
<td>4.0</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Length of anterior-most macronuclear nodule</td>
<td>17</td>
<td>28</td>
<td>21.9</td>
<td>3.37</td>
<td>15.4</td>
<td>15</td>
</tr>
<tr>
<td>Width of anterior-most macronuclear nodule</td>
<td>13</td>
<td>24</td>
<td>17.6</td>
<td>3.09</td>
<td>17.6</td>
<td>15</td>
</tr>
<tr>
<td>Number of micronuclei</td>
<td>3</td>
<td>6</td>
<td>4.1</td>
<td>0.83</td>
<td>20.2</td>
<td>25</td>
</tr>
<tr>
<td>Length of micronuclei</td>
<td>2</td>
<td>4</td>
<td>2.9</td>
<td>0.52</td>
<td>18.0</td>
<td>15</td>
</tr>
</tbody>
</table>
Morphogenesis during binary fission.

Stomatogenesis and frontal-ventral-transverse cirral anlagen. Morphogenesis during binary fission is depicted in Figs (5a–j), 6(a–d) and 4(d–j). Several critical morphogenetic stages were observed. Fig. 5(a) shows a relatively early divider demonstrating that new adoral membranelles begin to organize from anterior to posterior in the oral primordium of the opisthe and that six frontal-ventral-transverse cirral anlagen are recognizable; the parental undulating membranes are dedifferentiated to form the proter’s anlage I; while in the opisthe, anlage I is detached from the oral primordium (Fig. 4d, f).

Later, the new adoral membranelles continue to develop posteriorly in the opisthe; the anterior part of anlage I develops into the left-most frontal cirrus in both divider (Figs 5c, d and 4e, g); with the rest of it beginning to split longitudinally, giving rise to the paroral and endoral membranes.

Gradually, all cirral anlagen develop into new cirri. The anterior-most cirrus from anlage I, become three enlarged frontal cirri; the rear cirri derived from anlage II (except the rearmost one) migrate towards the newly formed paroral membrane and become a row of buccal cirri; anlage II to V (sometimes III to V) provide the rearmost cirri to form the transverse cirri, while anlage VI does not form a transverse cirrus at all; the remaining cirri from anlagen III to VI form the two frontal and two frontoventral rows (Figs 5f, g, i, j, 6a, b and 4i, h). Rarely, an additional anlage is present between the normal anlagen V and VI in the opisthe (Fig. 5c, d), which may contribute one transverse cirrus and an extra ventral row (Fig. 6c).

In a late divider, the anterior part of the new adoral zone curves to the right in the opisthe, while the parental adoral zone of membranelles remains completely intact throughout the morphogenetic process (Figs 6c, d and 4j).

Anlagen of marginal rows and dorsal kineties. The formation of the marginal rows and dorsal kineties proceeds intrakinetically in a conventional manner for hypotrichs. The
anlagen of the marginal rows are formed by the dedifferentiation of some cirri within each parental marginal row (Fig. 5a). These anlagen then stretch longitudinally and gradually replace the parental structures completely (Figs 5c, f, i, 6a, c and 4d, e, i). The dorsal kineties anlagen are also formed within the old rows in both dividers, such that with the proliferation of basal bodies, they elongate and supplant the parental structures (Figs 5b, e, h, j and 6b, d).

Nuclear apparatus. The macronuclear nodules fuse into a single mass in middle dividers and then divide to form four nodules for filial cells. The divisional process of the micronuclei was unclear, although they were recognizable in some dividers (Figs 5b, e, h, j, 6b, d and 4f, g, j).

**SSU rRNA gene sequence and phylogenetic analyses**

The SSU rRNA gene sequence of *Bistichella cystiformans* was deposited in the GenBank database with the accession number KJ509196. The length and DNA G + C content of the SSU rRNA gene were 1694 bp and 45.81 mol%, respectively. We included a broad selection of 61 species in the present phylogenetic analyses, containing almost all available SSU rRNA gene sequences from both the orders Stichotrichida and Sporadotrichida. The topologies of the ML and BI trees were basically congruent and, therefore, a single topology is presented based on the ML tree with support values from both algorithms indicated on branches (Fig. 7). In the
phylogenetic trees, B. cystiformans clusters in the clade containing B. variabilis, Parabistichella variabilis, Uroleptoides magnigranulosus and two species of the genus Orthoamphisiella (47% ML, 0.87 BI). Sequence comparisons indicated a similar result, with the sequence similarities between B. cystiformans and the species in the Bistichella–Parabistichella–Uroleptoides–Orthoamphisiella clade ranging from 99.2% to 99.4%.

Discussion

Morphological comparison with congeners


Fig. 5. Morphogenesis of Bistichella cystiformans spec. nov. after protargol impregnation. Ventral (a) and dorsal (b) view of an early middle divider, to show the formation of the frontal-ventral-transverse cirral anlagen and how the anterior end of the undulating membranes anlage contributes the leftmost frontal cirri (arrowhead); the arrow points to the formation of the left marginal anlage within the old cirral row. (c–e) Ventral (c, d) and dorsal (e) views of a mid-stage divider, showing formation of the leftmost frontal cirri (arrowheads) and the frontoventral cirri anlagen beginning to differentiate into cirri (d). Note the macronuclear nodules fuse into a single mass. (f–h) Ventral (f, g) and dorsal (h) views of a divider in the middle stage; arrowheads denote the separation of the undulating membranes. Note the macronuclear mass is about to divide. (i, j) Ventral (i) and dorsal (j) view of a late divider, showing the almost complete segmentation of the frontoventral cirri anlagen, arrows mark the new transverse cirri. DK, dorsal kineties; DKA, dorsal kineties anlagen; LMA, left marginal anlage; LMR, left marginal row; Ma, macronuclear nodules; Mi, micronuclei; OP, opisthe’s oral primordium; RMA, right marginal anlage; RMR, right marginal row. Bars, 70 μm.
without caudal cirri. Considering that the presence or absence of caudal cirri is widely used to separate hypotrich genera (e.g. the oxytrichid genus *Tachysoma* Stokes 1887 and the urostyloid genus *Caudiholosticha* Berger 2003) (Berger, 1999, 2006), the pisciform shape with a narrowed posterior end and the presence of caudal cirri in *Pseudouroleptus procerus* and *Pseudouroleptus terrestris*, make these semoresentable members of the genus *Metauroleptus* (Berger & Foissner, 1987; Hemberger, 1985; Berger, 2008). We, therefore, agree with the exclusion of these two species from the genus *Bistichella*, and accordingly, compare the novel species *B. cystiformans* with its four congeners (Table 2).

In terms of the infraciliature, *B. cystiformans* is closely related to *B. variabilis*. The former, however, can be recognized by the position of the rightmost frontal cirrus (immediately behind the distal end of the adoral zone of membranelles vs being positioned anteriorly at the same level as the other two frontal cirri) and the two rightmost frontoventral rows (V extending posteriorly to the leftmost transverse cirrus vs being distinctly away from transverse cirri; VI commencing near the anterior end of the right marginal row and terminating close to the rightmost transverse cirrus vs being mostly short and located in the posterior quarter of the body). Moreover, the novel species differs in having fewer adoral membranelles (33–45 vs 46–63), buccal cirri (5–8 vs 7–12), and fewer left (20–35 vs 37–63) and right (25–47 vs 44–78) marginal cirri (He & Xu, 2011).

*B. buitkampi* resembles *B. cystiformans* in body size and shape. However, the former can be separated from the latter due to having fewer buccal cirri (2–4 vs 5–8), fewer cirri in frontal rows III (2–3 vs 4–6) and IV (3–7 vs 5–18), a shorter frontoventral row VI (far away from transverse cirri vs close to transverse cirri), an absence of transverse cirri (vs presence) and the arrangement of the macronuclear nodules (in two separated pairs vs four in a row) (Foissner, 1982).

*B. namibiensis* is very similar to *B. cystiformans* in the arrangement of the two frontoventral rows, but differs from the latter in having two macronuclear nodules (vs four), fewer buccal cirri (2–5 vs 5–8), fewer cirri in frontal row III (approx. 1 vs 4–6) and IV (approx. 3 vs 5–18), as well as in having more cirri in frontoventral row VI (38–50 vs 20–34) and more left (44–58 vs 20–35) and right (42–61 vs 25–47) marginal cirri (Foissner *et al.*, 2002).

*B. humicola* was classified as a taxon incertae sedis in *Bistichella* by Berger (2008). It has been described mainly from fixed and opal blue-stained specimens and, as a result, the validity of the cirral pattern cannot be reliably ascertained. Nevertheless, it can be easily distinguished from *B. cystiformans* in having only one buccal cirrus (vs 5–8), the absence of transverse cirri (vs 3–5) and the presence of many (approx. 32) macronuclear nodules (vs four) (Gellért, 1956).

**Fig. 6.** Late stages of morphogenesis of *Bistichella cystiformans* spec. nov. after protargol impregnation. (a, b) Ventral (a) and dorsal (b) view of a late divider; note the dividing macronuclear nodules. (c, d) Ventral (c) and dorsal (d) view of a very late divider to show the cirri almost in their final positions; the arrowheads show the transverse cirri migrating to their final positions. DK, dorsal kineties; LMR, left marginal row; Ma, macronuclear nodules; Mi, micronuclei; RMR, right marginal row. Bars, 70 μm.
Morphogenetic pattern in the genus *Bistichella*

Prior to this investigation, no morphogenetic data were available for any species of the genus *Bistichella*. Our study of *B. cystiformans* was an attempt to remedy this defect; however, we were unable to fully determine the earliest stages of morphogenesis in our specimens and, therefore, the origin of the oral primordium and cirral anlagen is not available. Accordingly, morphogenesis in *B. cystiformans* can be summarized as follows: (1) in the proter, the parental adoral zone of membranelles is completely retained, while in the opisthe, the oral primordium is formed apokinetically in the posterior half of the cell; (2) the frontoventral and transverse cirri are formed by the conventional six-anlage mode; (3) the cirral streaks II–V (or III–V) generate one transverse cirrus each at their posterior ends, while streak VI provides no transverse cirrus; (4) the marginal rows occur within the old structures in a secondary mode; (5) the dorsal kineties develop intrakinetally and no fragments are formed; (6) all macronuclear nodules fuse into a single mass and then divide twice.

One of the most remarkable morphogenetic features in *B. cystiformans* is that the rightmost frontal-ventral-transverse cirral anlage (= VI) does not form a transverse cirrus at its rear end, which according to current knowledge, is unique in stichotrichs with six frontal-ventral-transverse cirral anlagen. Without exception in these species, the frontal-ventral-transverse anlage VI contributes one transverse cirrus (e.g. *Amphisiella*, *Gonostomum*, *Fragmocirrus*, *Trachelostyla*, *Oxytrichia*, *Notothylena*) (Berger, 1999, 2008, 2011; Chen et al., 2013a, b, c; Lv et al., 2013; Shao et al., 2013). Whether...
or not the absence of a special group of cirri is driven by environmental changes cannot be determined at present. Considering, however, that some taxa (e.g. *Apholosticha* and *Heterokeronopsis*) found in mangroves always exhibit a lack of specific cirral groups, the possibility that the difference observed is due to the effect of variable mangrove environments upon the phenotype cannot be excluded (Fan et al., 2014; Pan et al., 2013).

### Familial classification of the genus Bistichella

The genus *Bistichella* was established with species previously allocated to the genera *Pseudouroleptus* and *Amphisiella* (Berger, 2008). Unlike the oxytrichid genus *Pseudouroleptus*, *Bistichella* lacks dorsomarginal kinetics and dorsal kinety fragmentation, strongly indicating that it does not belong to the dorsomarginalian part of the Hypotricha, and should not be included in the family Oxytrichidae (Berger, 1999). Likewise, *Bistichella* lacks the amphisiellid cirral row, a critical character of the name-bearing type genus *Amphisiella*, and, therefore, neither can it be included in the family Amphisiellidae (Berger, 2008; Chen et al., 2013b). This position was also taken by Berger (2008) in his review of the amphisiellids, in which he classified *Bistichella* as a taxon of unknown position in the Hypotricha. The separation of the genus *Bistichella* from the genera *Pseudouroleptus* and *Amphisiella*

### Table 2. Comparison of the morphology of *Bistichella cystiformans* spec. nov. with its congeners

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body size <em>in vivo</em> (µm)</td>
<td>120–200 × 40–80</td>
<td>170–270 × 50–90</td>
<td>135–180 × 50–70</td>
<td>140–300 × 40–100</td>
<td>120*</td>
</tr>
<tr>
<td>Body shape</td>
<td>Elongate ellipsoid</td>
<td>Elongate ellipsoid</td>
<td>Dumbbell-shaped to sigmoidal</td>
<td>Elonge ellipsoid</td>
<td>Elongate ellipsoid</td>
</tr>
<tr>
<td>Number of adoral membranelles</td>
<td>33–45</td>
<td>46–63</td>
<td>34–40</td>
<td>39–48</td>
<td>31</td>
</tr>
<tr>
<td>Number of buccal cirri</td>
<td>5–8</td>
<td>7–12</td>
<td>2–4</td>
<td>2–5</td>
<td>1</td>
</tr>
<tr>
<td>Number of cirri in frontal row III</td>
<td>4–6</td>
<td>6–12</td>
<td>2–3</td>
<td>1†</td>
<td>9</td>
</tr>
<tr>
<td>Number of cirri in frontal row IV</td>
<td>5–18</td>
<td>7–18</td>
<td>3–7</td>
<td>3†</td>
<td>Possibly lacking</td>
</tr>
<tr>
<td>Number of cirri in frontoventral row V</td>
<td>17–42</td>
<td>35–62</td>
<td>25–37</td>
<td>33–46</td>
<td>24</td>
</tr>
<tr>
<td>Arrangement of frontoventral row V</td>
<td>Extending to the left-most transverse cirrus</td>
<td>Extending posteriorly to about 80% of body length</td>
<td>Extending posteriorly to about 75% of body length</td>
<td>Extending obliquely to transverse cirrus</td>
<td>Extending posteriorly to about 64% of body length</td>
</tr>
<tr>
<td>Number of cirri in frontoventral row VI</td>
<td>20–34</td>
<td>7–38</td>
<td>20–30</td>
<td>38–50</td>
<td>44</td>
</tr>
<tr>
<td>Arrangement of frontoventral row VI</td>
<td>Commencing behind distal end of the adoral zone and extending to the right-most transverse cirrus</td>
<td>Mostly short and located in the posterior quarter of body and extending to the right-most transverse cirrus</td>
<td>Commencing behind distal end of adoral zone and extending posteriorly to about 75% of body length</td>
<td>Commencing behind distal end of adoral zone and extending obliquely to transverse cirrus</td>
<td>Commencing behind distal end of adoral zone and extending posteriorly to rear cell end</td>
</tr>
<tr>
<td>Number of transverse cirri</td>
<td>3–5</td>
<td>5–7</td>
<td>6–15</td>
<td>4–6</td>
<td>Absent</td>
</tr>
<tr>
<td>Number of cirri in left marginal row</td>
<td>20–35</td>
<td>37–63</td>
<td>35–52</td>
<td>44–58</td>
<td>35</td>
</tr>
<tr>
<td>Number of cirri in right marginal row</td>
<td>25–47</td>
<td>44–78</td>
<td>39–49</td>
<td>42–61</td>
<td>41</td>
</tr>
<tr>
<td>Number of dorsal kineties</td>
<td>3–4</td>
<td>3–4</td>
<td>3</td>
<td>3</td>
<td>Data unavailable</td>
</tr>
<tr>
<td>Number of macronuclear nodules</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>Number of micronuclei, Data source</td>
<td>3–6</td>
<td>2–7</td>
<td>2</td>
<td>2–6</td>
<td>Data unavailable</td>
</tr>
</tbody>
</table>

*Method (*in vivo* or after fixation) not indicated.
†Data from a holotype specimen by Foissner et al. (2002).
and Amphisiella is also supported by molecular trees in which the former is quite distant from the latter two (He & Xu, 2011).

Present and previous phylogenetic studies based on SSU rRNA gene sequences show that the genus Orthamphisiella clusters into a clade including Bistichella with relatively low bootstrap values (He & Xu, 2011). The molecular data from the genus Orthamphisiella were only available for Orthamphisiella breviseries and an unidentified species and the assignment of both species into the genus Orthamphisiella is uncertain based on morphological data (Berger, 2008).

Considering that the SSU rRNA gene sequence of the type species Orthamphisiella stramenticola is unavailable, the systematic position of the genus Orthamphisiella remains uncertain. Moreover, members of the genus Orthamphisiella differs distinctly from those of the genus Bistichella in having no, or only one, long frontalventral row (vs two), and in that the frontalventral row of the proter is composed of parental cirri in the anterior portion and newly formed cirri in the posterior portion (vs only newly formed cirri), so both genera cannot have the same familial assignment from a morphological and morphogenetic perspective (Eigner & Foissner, 1991). Eigner (1997) established the family Orthoamphisiellidae to include Orthoamphisiella and some other genera, based primarily on the formation of within-anlagen in the two right-most ventral cirral rows. Actually, except for the type species Orthoamphisiella stramenticola, none of the taxa originally included corresponded with the familial classification (Berger, 2008). Thus, this family is redundant and not widely accepted (Berger, 2008; Lynn, 2008).

The genus Bistichella has some ontogenetic traits in common with kahlilliid genera, such as Kahlilli Corliss 1960, the type genus of the family Kahlillidae, e.g. the cirral anlagen are formed within the parental rows and dorsal kinety fragmentation is lacking (Berger, 2011). Kahlillids are also characterized by their tendency to preserve parental marginal rows and dorsal kineties in a variable number of divisions and in presenting dorsomarginal kineties. Members of the genus Bistichella, however, bear no parental marginal rows and/or dorsal kineties in divisions, and lack dorsomarginal kineties, and, therefore, this genus cannot be included in the family Kahlillidae.

The genus Metauroleptus Foissner et al. 2008 is very similar to the genus Bistichella in having three frontal cirri, two long and several short frontalventral rows, a row of buccal cirri, one left and one right marginal row (Foissner et al., 2008). In addition, the frontalventral-transverse cirral anlagen appear as the conventional six streaks, and the anlagen of marginal rows and dorsal kineties occur intrakinetically in both genera. Molecular data were unavailable, however, and, therefore, the systematic position of the genus Metauroleptus remains unknown at present.

The current study confirms that the genus Bistichella has a close relationship with the genus Parabistichella Jiang et al. 2013. This is in accordance with the morphological data, since both genera resemble each other in having three frontal cirri, long frontalventral rows, one left and one right marginal row and no caudal cirri (Jiang et al., 2013). It is still too early, however, to provide a definite familial assignment for these two genera, since the classification is based on only a limited number of taxa and is, in any case, far from settled given the low bootstrap values in the phylogenetic trees.

Acknowledgements

This work was supported by the Natural Science Foundation of China (project numbers: 41176119, 31030059 and 31111120437) and by King Saud University, Deanship of Scientific Research, International Research Group Program (project number: IREG14-22). Many thanks are due to Ms. Wenping Chen (SCNU) for sample collection and to Ms. An Liu (OUC) for gene sequencing. Our thanks are also given to Professor Xiaofeng Lin (SCNU) for ensuring institutional support for this research.

References


