Morphology and molecular phylogeny of *Pleuronema orientale* spec. nov. and *Pleuronema paucisaetosum* spec. nov. (Ciliophora, Scuticociliata) from Hangzhou Bay, China

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Two novel species, *Pleuronema orientale* spec. nov. and *Pleuronema paucisaetosum* spec. nov., isolated from coastal waters of Hangzhou Bay, China, were investigated with standard methods. *Pleuronema orientale* is characterized as follows: size *in vivo* 95–135 × 50–85 μm; usually one spherical macronucleus; 12–15 prolonged caudal cilia; two or three preoral kineties and 42–50 somatic kineties; membranelle 1 (M1) about 20 % of the anterior fragment of membranelle 2 (M2a) in length, consisting of three longitudinal rows of kinetosomes; posterior end of M2a hook-like; membranelle 3 (M3) three-rowed. *Pleuronema paucisaetosum* is characterized as follows: size *in vivo* about 55–85 × 25–55 μm; four or five preoral kineties and 21–23 somatic kineties; posterior end of M2a hook-like; M3 three-rowed. The small-subunit rRNA gene was sequenced for both species. Phylogenetic analyses revealed that *P. orientale* is most closely related to *Pleuronema puytoraci* and that *P. paucisaetosum* is sister to *Pleuronema grolierei* and *Pleuronema setigerum* (GenBank accession no. JX310015). With the inclusion of the two new sequences, the monophyly of the genus *Pleuronema* is not supported.

**INTRODUCTION**

Pleuronematida is one of four orders within the class Scuticociliatia. Its members are characterized by an expansive oral region with a prominent, curtain-like paroral membrane (Lynn, 2008). It is a diverse and ubiquitous group and its members have been reported in a wide range of habitats worldwide. The recent descriptions of several novel taxa indicate that the diversity of pleuronematids is underestimated (Fan et al., 2009, 2011a, b; Long et al., 2007; Miao et al., 2010; Wang et al., 2008b).

*Pleuronema* is a speciose genus in the order Pleuronematida, with 28 nominal species having been described.

Abbreviations: BI, Bayesian inference; M1, membranelle 1; M2a, anterior part of membranelle 2; M2b, posterior part of membranelle 2; M3, membranelle 3; ML, maximum-likelihood; SSU, small-subunit.

The GenBank/EMBL/DDBJ accession numbers for the small-subunit rRNA gene sequences of *Pleuronema orientale* and *P. paucisaetosum* are KF206429 and KF206430, respectively.

To date, the infraciliature of over 20 species has been revealed (Agatha et al., 1993; Dragesco, 1968; Dragesco & Dragesco-Kernéis, 1986; Fernandez-Leborans & Novillo, 1994; Wang et al., 2008a, c, 2009). However, like other scuticociliates, most of the descriptions in previous studies are insufficient, e.g. superficial descriptions of live cells and lacking details of the infraciliature. Recent work, based both on morphology and molecular data, indicates that misidentification and taxonomic confusion are common and that even apparently well-known species should be reinvestigated using modern methods (Pan et al., 2010, 2011; Wang et al., 2008c).

In past 20 years, surveys of marine ciliated protozoa in Chinese coastal waters of the Bohai and Yellow Seas have revealed six novel and two known species of *Pleuronema* (Pan et al., 2015; Song et al., 2009). In the present work, two novel species of the genus *Pleuronema* were collected from Hangzhou Bay, which is adjacent to the Yangtze estuary. Details of their morphology and molecular phylogeny are supplied.
METHODS

Sample collection, observation and identification. Pleuronema orientale spec. nov. was collected on 20 April 2012 from the intertidal region of a sandy beach on Dayang Island (30° 35’ 33.8”N 122° 04’ 59.1”E), where the water temperature was 19.5 °C and the salinity 6.8 ‰. Pleuronema pacificus (Dujardin, 1841) was dominant; the water temperature was 14.3 °C, salinity 13.4 ‰, pH 8.46 and dissolved oxygen concentration 5.12 mg l⁻¹. Samples of both water and surface sediment (<2 cm) were collected from puddles at low tide. The samples were kept at low temperature (<10 °C) while being transported to the laboratory and were examined within 4 h of collection.

Observations on living cells were carried out under bright-field and differential interference contrast microscopy. The infraciliature was revealed by the protargol staining method (Wilbert, 1975). Counts and measurements on stained specimens were performed at ×1000 magnification. Drawing of stained specimens was conducted with the help of a camera lucida. Terminology and systematics were mainly according to Lynn (2008) and Wang et al. (2008c).

DNA amplification and sequencing. Genomic DNA was extracted from three to five cells isolated from each sample using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer’s instructions, with the modification that one-tenth of the volume suggested for each reagent solution was used (Gao & Katz, 2014). A fragment of approximately 1700 bp comprising part of the small-subunit (SSU) rRNA gene was amplified through nested PCR using two sets of primers. The first set comprised universal forward and reverse primers, while the second comprised 8F forward and universal reverse primers. The first set comprised universal forward and reverse primers. The fragment was cloned into the competent cells of Escherichia coli DH5α following the manufacturer’s instructions, with the modification that one-tenth of the volume suggested for each reagent solution was used (Gao & Katz, 2014). A fragment of approximately 1700 bp comprising part of the small-subunit (SSU) rRNA gene was amplified through nested PCR using two sets of primers. The first set comprised universal forward and reverse primers, while the second comprised 8F forward and universal reverse primers. The fragment was cloned into the competent cells of Escherichia coli DH5α strain. Both strands of clones were sequenced on an ABI-PRISM 3730 automatic sequencer (Applied Biosystems).

Phylogenetic analyses. The SSU rRNA gene sequences of 43 other scuticociliates obtained from the NCBI GenBank database were used in addition to the newly characterized sequences of the two Pleuronematidae (for accession numbers, see Fig. 5). Phialodiscus armatilis and Uronema elegans were selected as outgroup species. Sequences were aligned using the GUIDANCE algorithm (Penn et al., 2010a) with the default parameters in GUIDANCE web server (Penn et al., 2010b) (http://guidance.tau.ac.il/ver2/). Ambiguous columns in the alignment were removed based on confidence scores calculated by GUIDANCE. The final alignment, including 1712 sites and 45 taxa, was used to reconstruct phylogenetic trees. The best-fit model for phylogenetic analyses was selected by both MrModeltest version 3.4 (Posada & Crandall, 1998) and MrModeltest version 2.2 (Nylander, 2004). Maximum-likelihood (ML) analysis was carried out using RAxML- HPC2 on XSEDE version 7.3.2 (Stamatakis, 2006; Stamatakis et al., 2008) on the CIPRES Science Gateway using the GTR+G+I model as the optimal choice. Support for the best ML tree came from 1000 bootstrap replicates. Bayesian inference (BI) analysis was performed using MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003) on the CIPRES Science Gateway with the same model as selected by the Akaile information criterion in MrModeltest version 2.0 (Nylander, 2004). The BI analysis was run with two sets of four chains for 1 000 000 generations and a sampling frequency of 100 generations. The first 25% of sampled trees were discarded as burn-in prior to tree reconstruction.

RESULTS AND DISCUSSION

Subclass Scuticociliata, 1867

Order Pleuronematida Faure-Fremiet in Corliss, 1956

Family Pleuronematidae Kent, 1881

Genus Pleuronema Dujardin, 1841

Pleonema orientale spec. nov. (Figs 1 and 2; Table 1)

Diagnosis. Cell about 95–135 × 50–85 µm in vivo, dorsoventrally flattened about 2:1; 12–15 prolonged caudal cilia; buccal area occupying 3/4 of body length, paroral membrane prominent; contractile vacuole positioned subcaudally; mostly one spherical macronucleus; two or three preoral kineties and 42–50 somatic kineties; membranelle 1 (M1) about 20% of anterior part of membranelle 2 (M2a) in length, consisting of three longitudinal rows of basal bodies; posterior end of M2a hook-like; membranelle 3 (M3) three-rowed.

Type locality. A puddle in the intertidal zone of a sandy beach on Dayang Island (30° 35’ 33.8” N 122° 04’ 59.1” E), Hangzhou Bay, China. The water temperature was 19.5 °C and the salinity 6.8 ‰.

Deposition of type material. A protargol slide with the holotype specimen is deposited in the Natural History Museum, London, UK, with registration number NHMUK 2015.4.9.1. A protargol slide with several paratype specimens is deposited in the Laboratory of Protozoology, Ocean University of China, Qingdao, China, with registration number PHB-12042001-1. Relevant specimens are all marked by black ink circles on the coverslip.

Etymology. The species-group name ‘orientale’ (o.ri.en.ta.le). L. neut. adj. eastern) indicates that this species was first discovered in China.

Description. Cell size about 95–135 × 50–85 µm in vivo, elliptical or oval in outline, with both ends broadly rounded (Figs 1a and 2a–d). Dorsoventrally flattened about 2:1 (Fig 2e). Buccal field occupying three-quarters of body length. Cytoplasm often colourless and transparent, containing numerous tiny granules (<3 µm) and several food vacuoles. Pellicle rigid and notched, beneath which extrusomes (approx. 6 µm long) are densely arranged (Fig. 2g, h). Usually one spherical macronucleus, centrally located, about 25 µm across; rarely four macronuclei (in one out of 20 individuals examined). One to three micronuclei, adjacent to macronucleus. Contractile vacuole positioned subterminally in dorsal side (Figs 1a and 2b, f). Somatic kineties about 10 µm long; 12–15 prolonged caudal cilia, about 25 µm long, projecting radially from

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posterior end of cell (Figs 1a and 2f). Locomotion by swimming rapidly while rotating about longitudinal axis of cell; sometimes lying motionless in water.

Forty-two to 50 somatic kineties terminating near anterior end of cell forming a small, glabrous apical plate (Fig. 1c, d). Kineties composed of dikinetids in anterior three-quarters of body, and monokinetids in posterior part. Two or three preoral kineties left of buccal field (Fig. 2l, m).

Oral apparatus of 'coronatum-type' (Figs 1b and 2i–m): M1 with one short and two longer rows of basal bodies; M2a posteriorly hooked-shaped, anteriorly and posteriorly two-rowed, middle section single-rowed in 'zigzag' pattern; M2b V-shaped; M3 three-rowed.

SSU rRNA gene sequence. The SSU rRNA gene sequence of Pleuronema orientale spec. nov. has been deposited in the GenBank database with the accession number, length and G+C content as follows: KF206429, 1679 bp, 43.95 mol%.

Comments on Pleuronema orientale spec. nov. Based on the structure of M2a, Wang et al. (2008c) divided Pleuronema into two groups: 'coronatum-type' (in which the posterior end of M2a is hooked-shaped) and 'marinum-type' (in which the posterior end of M2a is straight). In terms of the structure of M2a, body shape, cell size and the number of somatic kineties, P. orientale spec. nov. should be compared with at least six congeners, namely P. coronatum, P. salmastra, P. arctica, P. lynni, P. glaciale and Pleuronema puytoraci (Table 2).

Pleuronema orientale most closely resembles the well-known species P. coronatum (Fig. 3j, k). However, P. orientale possesses more somatic kineties (42–50 vs 35–43) and fewer preoral kineties (2–3 vs 4–8) and its M1 consists of three (vs two) basal body rows (Wang et al., 2008c). The SSU rRNA gene sequences differ by 98–110 bp.

The SSU rRNA gene sequences of P. orientale nov. spec. and P. puytoraci (Fig. 3e, f) differ from each other by only 2 bp. However, the former can be clearly separated from the latter by possessing significantly more somatic kineties (42–50 vs 28 or 29) (Pan et al., 2011).

Although P. lynni (Fig. 3i), P. salmastra (Fig. 3a, b), P. glaciale (Fig. 3l) and P. arctica (Fig. 3c, d) are similar to P. orientale spec. nov. in terms of cell size and the number of somatic kineties, they can be clearly separated from the latter by having more preoral kineties (6 or more vs 2 or 3) (Agatha et al., 1993; Corliss & Snyder, 1986; Dragesco & Dragesco-Kerneis, 1986; Fernandez-Leborans & Novillo, 1994).

Pleuronema paucisaetosum spec. nov. (Fig. 4; Table 1)

Diagnosis. Small Pleuronema, about 55–85 × 25–55 μm in vivo; ovoid or reniform in outline; buccal field

![Fig. 1. Pleuronema orientale spec. nov. in vivo (a) and after protargol staining (b–d). (a) Ventral view of a typical individual. (b) Detail of oral infraciliature. (c, d) Ventral (c) and dorsal (d) views of the holotype specimen, to show the infraciliature. M1, Membranelle 1; M2a, anterior part of membranelle 2; M2b, posterior part of membranelle 2; M3, membranelle 3; PM, paroral membrane. Bars, 40 μm.](image-url)
Fig. 2. Photomicrographs of *P. orientale* spec. nov. *in vivo* (a–h) and after protargol staining (i–m). (a–d) Ventral views of different individuals; arrowhead in (b) marks the contractile vacuole. (e) Lateral view. (f) To show the contractile vacuole (arrowhead) and caudal cilia (arrow) in posterior portion. (g) Detail of the cortex; arrowheads indicate extrusomes. (h) Cytoplasm; arrowheads point to extrusomes. (i) Anterior portion of buccal apparatus. (j) To show extrusomes (arrowheads) after protargol staining. (k) Anterior portion, to show M1. (l) Posterior portion of buccal field; arrowheads point to preoral kineties. (m) Posterior portion of buccal apparatus, arrowhead refers to a preoral kinety. See Fig. 1 for abbreviations. Bars, 40 μm.
occupying 3/4 of body length; usually one macronucleus; single contractile vacuole slightly dorsally positioned near posterior end; four or five preoral kineties, and 21–23 somatic kineties; M1 short, about 1/3 of M2a in length, posterior end of M2a hooked-like, M2b V-shaped, M3 three-rowed.

**Type locality.** Nanhui wetland (30°51′ 52.2″ N 121°56′ 13.9″ E), Shanghai, China. The water temperature was 14.3 °C, salinity 13.4 ‰, pH 8.46 and dissolved oxygen concentration 5.12 mg l⁻¹.

**Deposition of type material.** A protargol slide with the holotype specimen is deposited in the Natural History Museum, London, with registration number NHMUK 2015.4.9.2. A protargol slide with several paratype specimens is deposited in the Laboratory of Protozoology, Ocean University of China, Qingdao, China, with registration number PHB-12041702-1. Relevant specimens are all marked by black ink circles on the coverslip.

**Etymology.** The species-group name ‘paucisaetosum’ (pau.ci.sae.to.sum. N.L. neut. adj. having a small number of bristles) recalls the fact that this species possesses relatively few somatic kineties.

**Description.** Cell size about 55–85 × 25–55 μm in vivo, ovoid or reniform in outline with both end broadly rounded; in lateral view, ventral side somewhat concave, dorsal side convex (Fig. 4a, e, f). Buccal field occupying three-quarters of body length. Cilia of paroral membrane prominent, about 20 μm long, and forming a sail-like structure. Exusosomes 5 μm long, lying beneath notched pellicle. Cytoplasm colourless, containing several refringent globules (3–5 μm in diameter). One spherical macronucleus, about 13 μm across, positioned in anterior half of cell; rarely two macronuclei (in one out of 23 individuals). One to five micronuclei detected after staining. Single contractile vacuole, about 8 μm in diameter, located subterminally near dorsal margin (Fig. 4a). Somatic cilia 10 μm long; 12–15 prolonged caudal cilia, each about 25 μm long, projecting radially from posterior end of cell. Locomotion moderately fast while rotating about main body axis, sometimes motionless for short periods.

Twenty-one to 23 somatic kineties extending almost entire length of body, terminating anteriorly to form a small glabrous apical plate; each kinety consists of dikinetids in anterior two-thirds of body, and monokinetids in posterior third (Fig. 4c, d). Four or five preoral kineties left of buccal field (Fig. 4c, h).

Oral apparatus of ‘coronatum-type’ (Fig. 4b, g): M1 composed of one short and two longer rows of basal bodies, about one-third of M2a in length; M2a hook-like in posterior region, two-rowed in anterior and posterior portions, single-rowed in middle portion; M2b irregularly V-shaped; M3 three-rowed; paroral membrane about three-quarters of body in length.

**SSU rRNA gene sequence.** The SSU rRNA gene sequence of *P. orientale* spec. nov. has been deposited in the GenBank database with accession number, length and G + C content as follows: KF206430, 1681 bp and 43.37 mol%.

**Comments on Pleuronema paucisaetosum spec. nov.** In terms of cell shape, general infraciliature and the

<table>
<thead>
<tr>
<th>Character</th>
<th>Min.</th>
<th>Max.</th>
<th>Mean</th>
<th>Median</th>
<th>sd</th>
<th>cv (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length (μm)</td>
<td>97</td>
<td>133</td>
<td>113.2</td>
<td>110</td>
<td>11.51</td>
<td>10.2</td>
<td>23</td>
</tr>
<tr>
<td>Body width (μm)</td>
<td>60</td>
<td>82</td>
<td>70.2</td>
<td>69</td>
<td>5.37</td>
<td>7.7</td>
<td>23</td>
</tr>
<tr>
<td>Length of buccal field (μm)</td>
<td>51</td>
<td>84</td>
<td>65.2</td>
<td>67</td>
<td>8.88</td>
<td>13.6</td>
<td>23</td>
</tr>
<tr>
<td>Width of buccal field (μm)</td>
<td>36</td>
<td>58</td>
<td>45.3</td>
<td>45</td>
<td>6.32</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>No. of somatic kineties</td>
<td>42</td>
<td>50</td>
<td>47.4</td>
<td>48</td>
<td>2.27</td>
<td>4.8</td>
<td>23</td>
</tr>
<tr>
<td>No. of preoral kineties</td>
<td>2</td>
<td>3</td>
<td>2.4</td>
<td>2</td>
<td>0.51</td>
<td>21.3</td>
<td>23</td>
</tr>
<tr>
<td>No. of kinetosome rows in M3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>No. of macronuclei</td>
<td>1</td>
<td>4</td>
<td>1.2</td>
<td>1</td>
<td>0.67</td>
<td>55.8</td>
<td>20</td>
</tr>
<tr>
<td>No. of micronuclei</td>
<td>1</td>
<td>3</td>
<td>1.3</td>
<td>1</td>
<td>0.65</td>
<td>50</td>
<td>11</td>
</tr>
<tr>
<td>n: number of specimens</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
number of macronuclei, three congeners should be compared with the new form, namely *Pleuronema setigerum*, *P. coronatum* and *P. puytoraci*. 

*Pleuronema paucisaetosum* spec. nov. most closely resembles *P. setigerum* (Fig. 3g, h) in terms of the number of somatic kineties; however, it can be identified by its larger size (55–85 μm vs 30–50 μm *in vivo*) and the detailed structure of M2a (hook-like posterior end and one-rowed middle portion vs ring-like posterior end and middle section in zigzag pattern) (Pan et al., 2010). In addition, its SSU rRNA gene sequence differs from that of three *P. setigerum* isolates by 19–126 bp.

Although *P. paucisaetosum* spec. nov. is similar to *P. coronatum* and *P. puytoraci* in the shape of M2a, it differs from the latter two by its smaller body size (55–85 μm long vs 55–170 μm, 70–120 μm *in vivo*), fewer somatic kineties (21–23 vs 35–43, 28–29) and the structure of M1 (three-rowed vs two-rowed) (Pan et al., 2011; Wang et al., 2008c).

<table>
<thead>
<tr>
<th>Character</th>
<th><em>P. orientale</em></th>
<th><em>P. coronatum</em></th>
<th><em>P. puytoraci</em></th>
<th><em>P. lynni</em></th>
<th><em>P. arctica</em></th>
<th><em>P. glaciale</em></th>
<th><em>P. salmastra</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length <em>in vivo</em> (μm)</td>
<td>95–135</td>
<td>50–170</td>
<td>70–120</td>
<td>96–114*</td>
<td>131–242*</td>
<td>125–168*</td>
<td>50–116*</td>
</tr>
<tr>
<td>Somatic kineties (n)</td>
<td>42–50</td>
<td>35–43</td>
<td>28 or 29</td>
<td>34–40</td>
<td>39–61</td>
<td>44–58</td>
<td>43–63</td>
</tr>
<tr>
<td>Preoral kineties (n)</td>
<td>2–3</td>
<td>4–8</td>
<td>1–3</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>6–10</td>
</tr>
<tr>
<td>Ciliary rows in M3 (n)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

*Data are possibly from fixed specimens.*

Fig. 3. Species of *Pleuronema* that are similar to *P. orientale* spec. nov. or *P. paucisaetosum* spec. nov. (a, b) *P. salmastra* (from Dragesco & Dragesco-Kerneis, 1986). (c, d) *P. arctica* (from Agatha et al., 1993). (e, f) *P. puytoraci* (from Pan et al., 2011). (g, h) *P. setigerum* (from Pan et al., 2010). (i) *P. lynni* (from Fernandez-Lebrans & Novillo, 1994). (j, k) *P. coronatum* (from Song, 2000). (l) *P. glaciale* (from Corliss & Snyder, 1986). All images reproduced with permission.
Phylogenetic analyses based on SSU rRNA gene sequence data (Fig. 5)

Phylogenetic trees inferred from SSU rRNA gene sequences using BI and ML analyses generated similar topologies. Therefore only the ML tree is presented here, with support values from both algorithms (Fig. 5). The main findings from these analyses can be summarized as follows: (i) the order Pleuronematida is not monophyletic, with members of the order Thigmotrichida nested within it; (ii) four families of the order Pleuronematida, i.e. Pleuronematidae, Histiobalantiidae, Eurystomateliidae and Ctedoctematidae, are monophyletic with moderate or high support; (iii) the family Cyclidiidae is paraphyletic, with thigmotrichids nested within it (Gao et al., 2014).

In previous analyses, members of the genus Pleuronema either formed one clade or fell into two clades (Fan et al., 2009; Gao et al., 2013; Pan et al., 2015). With two new sequences added in the present work, Pleuronema is not monophyletic in the ML tree, as species of the genus Schizocalyptra are nested within it, although support for this branch is low (26%). In the BI tree, species of Pleuronema group into two clades, forming branches parallel to the Schizocalyptra clade. Greater taxon sampling and data from more gene markers are therefore needed in order to determine the phylogeny and systematics of the genus Pleuronema.

A ‘core’ Pleuronema lineage is recognized in both the ML and BI trees with poor to strong support (57/0.98). This ‘core’ clade comprises two lineages. The first clade consists of Pleuronema sp. 2 and Pleuronema sinica, with full support. The second clade is a large assemblage (94/1.00) consisting of one well-supported clade, in which P. paucisaetosum sp. nov. groups with Pleuronema grolierei and P. setigerum isolate 2, and then with P. cf. setigerum, and a poorly supported sister assemblage that comprises three subclades, (i) Pleuronema elegans and P. coronatum isolate 2, (ii) P. coronatum isolates 1 and 3 and (iii) P. orientale sp. nov., P. puytoraci and P. setigerum isolate 1. Greater taxon sampling along with data for more gene markers are needed in order to resolve the branching order within the core Pleuronema lineage.

The structure of the oral apparatus has traditionally been considered as an important diagnostic character in scuticociliates (de Castro et al., 2014; Fan et al., 2014; Foissner et al., 2014; Lynn, 2008). Morphologically, members of the genus Pleuronema can be divided into two types, ‘coronatum-type’ and ‘marinum-type’, based on the structure of M2a (Wang et al., 2008c). However, the two types intermingled with each other in the SSU rRNA gene trees, casting doubt on the value of this character for determining evolutionary relationships among species of the genus Pleuronema.
Two novel species of Pleuronema from China

Pleuronema, although it remains a critical feature for species identification.

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Fig. 5. Phylogenetic tree inferred from SSU rRNA gene sequences, showing the positions of P. orientale spec. nov. and P. paucisaetosum spec. nov. (in bold). Numbers at nodes represent bootstrap percentages for ML analysis from 1000 replicates and the BI posterior probabilities. Fully supported (100 %/1.00) branches are marked with filled circles. Hypotheses (–) indicate disagreement between ML and BI. Bar, 5 substitutions per 100 nucleotide positions. Solid squares mark species of Pleuronema with the ‘marinum-type’ oral apparatus; dots mark species of Pleuronema with the ‘coronatum-type’ oral apparatus.


