Phylogeny and systematic revision of the family Pseudokeronopsidae (Protista, Ciliophora, Hypotricha), with description of a new estuarine species of Pseudokeronopsis

Xumiao Chen, John C. Clamp & Weibo Song

Submitted: 24 April 2011
Accepted: 16 August 2011


The family Pseudokeronopsidae is a taxon of hypotrich ciliates with a history of uncertainty regarding its systematic and phylogenetic relationships to other members of the order Urostylida. Phylogenetic analyses of pseudokeronopsids were made using all available molecular and morphological information, and the patterns of morphogenesis of the group were reinvestigated. Results clearly demonstrated that the genera Thigmokeronopsis and Apokeronopsis are not confamilial with Pseudokeronopsis and Nothoholosticha but, instead, must be transferred to the family Urostylida. A new estuarine species, Pseudokeronopsis erythrina sp. n. was discovered and described using a combination of morphological and molecular characters. Species of Pseudokeronopsis are difficult to characterize but can be distinguished from one another by a combination of morphological and morphogenetic characters. Pseudokeronopsis similis is a freshwater species that has significant morphogenetic differences with other species of the genus and may not be congeneric with them.

Corresponding author: Weibo Song, Laboratory of Protozoology, Institute of Evolution and Marine Biodiversity, Ocean University of China, 5 Yushan Road, Qingdao 266003, China. E-mail: wsong@ouc.edu.cn
Xumiao Chen, Laboratory of Protozoology, Institute of Evolution and Marine Biodiversity, Ocean University of China, 5 Yushan Road, Qingdao 266003, China. E-mail: chenxumiao@163.com
John C. Clamp, Department of Biology, North Carolina Central University, Durham, NC 27710, USA. E-mail: jclamp@NCCU.EDU

Introduction

The order Urostylida is a species-rich group of ciliates in the subclass Hypotricha that has proven to be much more diverse than originally thought, and its morphogenetic patterns and molecular phylogeny have drawn much attention (Foissner 1996; Berger 2006; Chen et al. 2010a,c). The urostylid genus Pseudokeronopsis was established by Borror & Wicklow (1983) with P. rubra as the type species. The genus is characterized by a continuous adoral zone of membranelles, a bicorona of frontal cirri, a typical urostylid midventral complex, one marginal row of cirri on each side of the cell, presence of buccal and transverse cirri, and absence of caudal cirri. In addition, most species have characteristically pigmented cortical granules. Eight species of Pseudokeronopsis are known, most of them large forms that were isolated from marine or brackish habitats (Berger 2006; Song et al. 2006). Defining these species using morphological characters has been challenging because many potentially diagnostic characters are overlapping or similar (Song et al. 2006). Morphogenesis has been described in only four species, P. rubra, P. carnea, P. flava and P. similis, and was found to be similar, indicating a discrete assemblage (Hu & Song 2001; Song et al. 2002, 2004; Hu et al. 2004; Sun & Song 2005; Shi et al. 2007). Recently, we discovered an undescribed species of Pseudokeronopsis in the estuary of the Pearl River south of Guangzhou, China. Several studies of hypotrichs (Foissner & Stoeck 2008; Foissner et al. 2008, 2010; Hu et al. 2009a,b; Paiva & Silva-Neto 2009, Paiva et al. 2009; Huaing et al. 2010) have shown that analyses combining morphological and morphogenetic characters with molecular characters are more likely to yield reliable results, especially in some taxonomically confused groups. In the present paper, we used molecular methods combined with the examination of morphology and morphogenesis to investigate the undescribed species of Pseudokeronopsis and its
morphological relatives with the aim of accomplishing the following objectives: (i) make a new analysis of the phylogeny and systematics of the family Pseudokeronopsidae, (ii) add information about biodiversity of this taxonomically difficult genus, (iii) understand the conservative mode of morphogenesis seen in *Pseudokeronopsis*, and (iv) evaluate the consistency of cirral and ciliary patterns used for the definition of species in the genus.

**Materials and methods**

**Collection, isolation, and cytological methods**

*Pseudokeronopsis erythrina* sp. n. was collected from the estuary of the Pearl River (113°41’E; 22°38’N) south of Guangzhou, China, on 9 November 2008. The water temperature was 25.2 °C and salinity was 6.3‰.

Specimens were maintained in Petri dishes for several weeks at room temperature (approximately 22 °C) using rice grains to stimulate growth of bacteria as food for the ciliates. Isolated cells were observed *in vivo* using bright field and differential interference contrast microscopy at different magnifications. Staining with protargol (Wilbert 1975) and other morphological and morphogenetic observations were performed according to the methods of Chen et al. (2010b). Terminology is according to Berger (2006). The systematic classification mainly follows Lynn (2008).

**DNA extraction, PCR amplification, and sequencing**

Genomic DNA was extracted using a REDExtract-N-Amp Tissue PCR kit (Sigma, St. Louis, MO, USA). The gene coding for the ribosomal small subunit (SSU rRNA) was amplified with the eukaryotic universal SSU rRNA primers EuA (5’-AACCTGGTGTGATCCTGGCAGT-3’) and EuB (5’-TGATCCTTCTGGCAGGTTGCTTAC-3’) (Medlin et al. 1988) using the PCR cycling parameters of Yi et al. (2010). The PCR product of approximately 1.8 kb in length was purified by cloning into the pUCm-T vector (Sangon, Toronto, ON, Canada). Sequencing in both directions was carried out using an ABI Prism 377 Automated DNA Sequencer (Applied Biosystems Inc., Foster City, CA, USA) (Miao et al. 2009).

**Phylogenetic analyses**

The SSU rRNA sequence of the new species was aligned with sequences of 46 other taxa obtained from the GenBank database (see Fig. 1 for accession numbers). *Protocruzia contrax* was selected as the outgroup taxon. All

**Fig. 1.** Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian (BI) phylogenetic trees constructed with SSU rRNA gene sequences showing the position of *Pseudokeronopsis erythrina* sp. n. (boldface). Nodal support for branches in the ML, MP, and BI trees are marked in order (ML/MSP/BI). Support values for nodes that were higher than 50% are provided (“*” reflects statistical support below 50%), and “-” indicates disagreement in topology that could not be represented on the consensus tree. All branches are drawn to scale. The scale bar corresponds to two substitutions per 100 nucleotide positions. GenBank accession numbers are given for each species.
sequences were aligned using Clustal W implemented in BioEdit 7.0 (Hall 1999). Highly variable regions, in which alignment could not be determined unambiguously, were excluded prior to phylogenetic analyses.

The program Modeltest (Posada & Crandall 1998) selected the GTR + I (=0.4768) +G (=0.4763) model under the Akaike information criterion. Using these parameters, a maximum likelihood (ML) tree was constructed with PimML V2.4.4 (Guindon & Gascuel 2003) incorporating bootstrapping with 1000 replicates. Gaps were treated as missing data. Maximum parsimony (MP) analysis was performed with PAUP* 4.0b10 (Swofford 2002) using the tree-bisection–reconnection algorithm and bootstrapping with 1000 replicates. A Bayesian (BI) analysis was performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), with 1000 replicates. A Bayesian (BI) analysis was performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), with a run of 1,500,000 generations at a sampling frequency of 100 and a burn-in of 3750 trees (25%). Topologies of all trees were nearly identical; therefore, they were merged as a run of 1,500,000 generations at a sampling frequency of 100 and a burn-in of 3750 trees (25%). Topologies of all trees were nearly identical; therefore, they were merged into a single consensus tree for purposes of illustration.

To further examine relationships between pseudokeronopsids and other urostylid taxa, putative secondary structures of the SSU rRNA molecules of representative species were predicted with MFOLD (Zuker 2003; http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3). Default settings of MFOLD were used to produce the putative secondary structure of variable region 2 (V2 region) for each species. The structures were edited with RNA Viz 2.0 (Rijk & Wachter 1997) to produce acceptable illustrations.

Results

Molecular data and phylogenetic analyses

The SSU rRNA sequence of Pseudokeronopsis erythrina sp. n. was deposited in GenBank with accession number FJ775723. The length and GC content of the SSU rRNA gene were 1725 bp and 44.87%, respectively. The new species differs from P. rubra by 10 bp, from P. flava by 13 bp, and from P. carnea by 30 bp (Figs 1 and 2).

Two major clades made up the core Urostylida in our analyses (Fig. 1). One moderately supported major clade (ML bootstrap, 68%; MP bootstrap, 67%; BI posterior probability, 1.0) consists of Pseudourostyla cristata associated with the four species of Pseudokeronopsis in a maximally supported, monophyletic subclade accompanied by Notobolostichia, also with maximal support. All of the other urostylids cluster in a maximally supported clade in which Bergeriella occupies an isolated, basal position. Within this cluster, one assemblage consists of Apokeronopsis and Thigmokeronopsis in a maximally supported clade that is a sister group to a clade consisting of Apourostylopsis sinica and two species of Metaurostylopsis. Species of Diaxonella, Antebolostichia and Urostyla form a smaller, well-supported clade (ML, 93%/MP, 80%/BI, 1.0) that diverges from this assemblage (Fig. 1).

A putative model of secondary structure (Fig. 2A) consisting of a multi-branched loop and four paired regions (helices 9, 10, E10-1, and 11) was predicted based on the interpretation of the V2 region in major groups of ciliates by Strüder-Kypke et al. (2000). Structures of helices 9, 10, and 11 were more conserved than that of helix E10-1 in the seventeen species of core urostylids. Helix 9 contained two median bulges and a terminal loop, helix 10 had a median bulge and a terminal loop, and helix 11 had a terminal loop only. Helix 10 of Notobolostichia fasciata and four species of Pseudourostyla differed markedly from that of Thigmokeronopsis stoecki and three species of Apokeronopsis (Fig. 2A). The former had a single, large, median bulge, but the latter, as well as other urostylids in the same clade, had two, relatively small, median bulges (Fig. 2A, arrows). There was some slight variation in the size of the median bulges among the species of Apokeronopsis (Fig. 2A).

The four species of Pseudourostyla and Notobolostichia fasciata shared unique nucleotides at 22 sites in their SSU sequences (Fig. 2B, shaded boxes). By contrast, the three species of Apokeronopsis and Thigmokeronopsis stoecki shared unique nucleotides at 12 sites (Fig. 2B, shaded boxes) with other urostylids in their clade (Fig. 1) and Pseudoaurostyla cristata.

Description of Pseudokeronopsis erythrina sp. n.


Pseudokeronopsis erythrina sp. n. (Figs 3, 4, and 5A–D).

Etymology. The specific epithet is derived from erythros (Greek: red) and refers to the brick-red colour of the cell at lower magnifications (<100x).

**Fig. 2.** —A. Putative secondary structures of variable region 2 of the SSU rRNA gene comprising helices 9, 10, E10-1, and 11 for five species belonging to the emended family Pseudokeronopsidae and twelve species of urostylids in other families. Note the single, large median bulge of helix E10-1 in *Pseudokeronopsis* and *Nothoholosticha* compared to the two, smaller median bulges of helix E10-1 in *Thigmokeronopsis*, *Apokeronopsis*, and other members of the Urostylidae, *Bergeriella*, and *Pseudourostyla* (arrows). —B. Fourteen regions of the alignment of SSU rRNA gene sequences for 17 species belonging to the clade comprised of core urostylids in the tree shown in Fig. 1. Regions are separated by double lines, and positions of nucleotides at the beginning and end of each region are given at the top of each column. Homologous groups of nucleotides are indicated by shading.

**Type locality.** Estuary of Pearl River (113°41'E; 22°38'N) at Nansha, Longxue Island, Guangzhou, Guangdong Province, China.

**Deposition of type specimens.** The holotype slide (protargol preparation; registry no. CXM08110906) has been deposited in the collection of the Laboratory of Protozoology, Institute of Evolution and Marine Biodiversity, OUC, Qingdao, China. One paratype slide (protargol preparation) has been deposited in the Natural History Museum, London, UK (registry no. 2010:11:8:1).

**Morphological description**
Live specimens freshly isolated from samples relatively uniform in size, measuring mostly 150–200 × 30–40 μm; cells flexible in shape yet generally slender, elongate and elliptical with both ends tapering (Table 1 and Figs 3, 4 and 5A–D). Ratio of body length-to-width, 4–6:1;
dorsoventrally flattened with thickness/width ratio of 1.2–2.3. Buccal field approximately 1/3 of body length (Figs 3A and 5A). After living in culture for a few weeks, some cells (about 1/10 of population) variable in shape, ranging from long and band-like to plump; length/width ratio up to 8–10:1 in some extreme cases (Fig. 3B). More than 50 macronuclear segments, each measuring 5–15 μm in length (Fig. 3F, arrows). Micronuclei not seen. Two CVs usually present, measuring 10–20 μm in diameter at diastole, each with two inconspicuous collecting canals: one CV located in anterior 1/3 body and another in posterior 1/3 (Figs 3A and 4G, H, arrows). Contractile vacuoles discharge alternately at rather long intervals of 5–10 min. Sometimes, only one CV evident and located slightly posterior to middle of body (Fig. 4F, arrow). Pigmented cortical granules spherical and generally dark reddish-brown or brick-red (Figs 3C, D and 4B–G, I, K), approximately 1 μm in diameter, mainly in regular groups around cirri on ventral side (Fig. 4A, I) and around bristles dorsally (Fig. 4K); some granules not grouped but sparsely scattered at random throughout cortex. Aside from pigment granules, cytoplasm colourless, usually with several lipid droplets (ca. 5–7 μm across; Fig. 4J) and food vacuoles containing flagellates, diatoms, small ciliates or bacteria.

Locomotion typical for subclass Hypotricha; slowly crawling on substrate using cirri or rotating around longitudinal axis of body when swimming.

Ciliature as shown in Fig. 3E, F. Adoral zone (AZM) extending for 1/3 of body length from anterior end composed of 36–61 membranelles with distal end strongly curved. Paroral membrane (PM) short, approximately one-half as long as endoral membrane (EM). Frontal cirri (FC) numbering 8–12, arranged in two arcs forming a ‘bicorona’ (Fig. 3E, BiC, highlighted in grey) that con-

Fig. 3. A–F. Morphology of *Pseudokeronopsis erythrina* sp. n. from life (A–D) and after staining with protargol (E, F). —A. Ventral view of a typical individual, arrows mark the contractile vacuoles; —B. Variation in shape of the body. —C, D. Ventral (C) and dorsal (D) views, showing distribution of the cortical granules, arrows mark the sparsely distributed ones. —E, F. Ventral (E) and dorsal (F) views, arrowheads and arrow in E mark the two frontoterminal cirri and the buccal cirrus, respectively; arrows in F indicate the macronuclear nodules. AZM, adoral zone of membranelles; BiC, bicorona of frontal cirri; DK, dorsal kineties; EM, endoral membrane; LMR, left marginal cirral row; MVC, midventral complex; PM, paroral membrane; RMR, right marginal cirral row; TC, transverse cirri. Scale bars: 50 μm (in A, C–F); 100 μm (in B).
nects to midventral complex (MVC) consisting of 19–38 pairs of obliquely oriented cirri, with three cirri sometimes making up one ‘pair’ (Fig. 5C, arrows); MVC extending posteriad to 2–4 transverse cirri (TC; Figs 3E and 5A). No conspicuous gap between posterior end of MVC and TC. Two frontoterminal cirri (FTC; Figs 3E and 5B, arrowheads) always located between distal end of AZM and anterior end of right marginal row. Single buccal cirrus (BC; Figs 3E and 5B, arrows) located close to endoral membrane at point 1/3 PM length from anterior end. Left and right marginal rows comprising 38–57 and 41–64 cirri, respectively. Three dorsal kineties (DK) extending almost entire length of cell (Figs 3F DK and 5D, arrowheads).

Comments on the number of contractile vacuoles in *Pseudokeronopsis erythrina* sp. n.

Most cells of *Pseudokeronopsis erythrina* had two CVs yet some had only one. One possibility is that the latter actually had two CVs, but one was inactive for unknown reasons (perhaps elevated salinity in the wet mount), especially given the fact that CVs in cells in which two were visible had rather long periodicities of filling and discharge (5–10 min). Location of the single CV in the posterior half of the cell supports this hypothesis because the single CV of other hypotrichs is located in the middle of the cell (Song et al. 2009).

Morphogenesis during binary fission

The morphogenetic process is described briefly because the main events correspond with those of other members of *Pseudokeronopsis* (Figs 5E–N and 6, 7).

The proter’s oral primordium (1OP) appears apokinetically as a single anarchic field of closely spaced basal bodies to the right of the buccal field (Fig. 6A, C). Concurrent with this, basal bodies appear to the left of the parental MVC just below the level of the cytostome and form a narrow, longitudinal streak (the anlage of the proter’s fronto-ventral-transverse cirri, FVTA; Figs 5I and 6A, C). The opisthe’s oral primordium (2OP) forms close to the left side of its MVC, posterior to the parental equator (Figs 5E, F and 6A, C).

Next, the new membranelles of the AZM organize in the oral primordia of each daughter (Figs 5G, J and 6D, E, arrowheads) and differentiate (Fig. 6G, arrowheads), as is usual for the genus. The anlagen of the undulating membrane (UMA) develop to the right of the oral primordia of each daughter and then separate into two sections in each one to give rise to the paroral and endoral membranes (Figs 5J, L and 6E; arrowheads in Figs 5L and 7C). The first FC also develops from the UMA in both daughters (Figs 5K and 7A, double-arrowheads). Meanwhile, the anlage of the fronto-ventral-transverse cirri (FVTA) develops as a series of oblique lines of basal bodies to the right of the OP in
each daughter (Figs 5G and 6D, FVTA; Figs 5J and 6E, arrows) that then differentiate into cirri (Fig. 7A, C, arrows). The BC originates from the anterior basal bodies of the FVTA in each daughter (Figs 5L and 7C, double-arrowheads).

In the last stages of division, formation of the new oral apparatus is almost complete in each daughter, and the new ciliary structures move farther apart as they migrate towards their final positions (Fig. 5N). Marginal cirri and DK develop in the usual way for ciliates of this group by having the anlagen appear within the parental structures and stretch towards both ends of the dividing cell to form new ones for the daughters (Figs 5M, DK 6E–H, and 7 DKA, LMA, RMA). The macronuclear segments divide.

Fig. 5. Morphogenesis of Pseudokeronopsis erythrina sp. n. in cells stained with protargol. Parental cirri in shown in outline whereas new ones are shown in solid black. —A, B. Ventral (A) and dorsal (B) views of the same cell in early division, arrows indicate the macronuclear nodules. —C, D. Ventral views of cells in early division, arrowheads indicate the newly formed membranelles; insets show details of the oral primordia in the proters of both cells. —E, G. Ventral views of individuals in the middle stage of morphogenesis, arrowheads indicate the newly formed membranelles; arrows mark the oblique streaks; double-arrowheads show the frontal cirrus separating from the UMA; inset to G depicts the newly formed oral apparatus of the proter. —F, H. Dorsal views of the middle stage of morphogenesis showing division of macronuclear nodules. DKA, anlagen of dorsal kinetics; FVTA, fronto-ventral-transverse anlagen; LMA, left marginal cirral anlagen; 1OP, oral primordium of proter; 2OP, oral primordium of opisthe; RMA, right marginal cirral anlagen; UMA, anlagen of undulating membrane. Scale bars: 50 μm.
Table 1 Morphometric characterization of *Pseudokeronopsis erythrina* sp. n. All data are based on protargol-stained specimens.

<table>
<thead>
<tr>
<th>Characters</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>170</td>
<td>260</td>
<td>208.4</td>
<td>24.95</td>
<td>11.97</td>
<td>25</td>
</tr>
<tr>
<td>Body, width</td>
<td>33</td>
<td>70</td>
<td>48.8</td>
<td>8.53</td>
<td>17.48</td>
<td>25</td>
</tr>
<tr>
<td>Buccal field, length</td>
<td>60</td>
<td>90</td>
<td>71.1</td>
<td>8.76</td>
<td>12.32</td>
<td>25</td>
</tr>
<tr>
<td>Adoral membranelles, number</td>
<td>36</td>
<td>61</td>
<td>46.0</td>
<td>5.20</td>
<td>11.31</td>
<td>25</td>
</tr>
<tr>
<td>Buccal cirri, number</td>
<td>1</td>
<td>1</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Frontal cirri, number</td>
<td>8</td>
<td>12</td>
<td>10.1</td>
<td>1.22</td>
<td>12.12</td>
<td>25</td>
</tr>
<tr>
<td>Frontoterminal cirri, number</td>
<td>2</td>
<td>2</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Midventral complex pairs, number</td>
<td>19</td>
<td>38</td>
<td>27.4</td>
<td>3.95</td>
<td>14.41</td>
<td>25</td>
</tr>
<tr>
<td>Left marginal cirri, number</td>
<td>38</td>
<td>57</td>
<td>44.7</td>
<td>4.84</td>
<td>10.83</td>
<td>25</td>
</tr>
<tr>
<td>Right marginal cirri, number</td>
<td>41</td>
<td>64</td>
<td>49.9</td>
<td>5.52</td>
<td>11.06</td>
<td>25</td>
</tr>
<tr>
<td>Transverse cirri, number</td>
<td>2</td>
<td>4</td>
<td>2.5</td>
<td>0.59</td>
<td>23.25</td>
<td>25</td>
</tr>
<tr>
<td>Dorsal kinetics, number</td>
<td>3</td>
<td>3</td>
<td>3.0</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Macronuclear nodules, number</td>
<td>53</td>
<td>80</td>
<td>67.5</td>
<td>7.91</td>
<td>11.72</td>
<td>25</td>
</tr>
</tbody>
</table>

Measurements in μm. CV, coefficient of variation in %; Max, maximum; Mean, arithmetic mean; Min, minimum; n, sample size; SD, standard deviation.

Discussion

Phylogeny of the family Pseudokeronopsidae

In all of our analyses, the family Pseudokeronopsidae sensu Berger (2006) and sensu Lynn (2008) was a polyphyletic assemblage consisting of two widely separated and strongly supported clades, one consisting of *Pseudokeronopsis* and *Nothobolosticha* and the other of *Thigmokeronopsis* and *Apokeronopsis* (Fig. 1). Based on this, we remove *Thigmokeronopsis* and *Apokeronopsis* from the family Pseudokeronopsidae and transfer them to the family Urostylidae sensu Lynn (2008). Even though our analyses were based on the sequences of just the SSU rRNA gene, it is one that provides many conserved and semi-conserved characters. Sequences of *Pseudokeronopsis* and *Nothobolosticha* species contained unique molecular characters in 14 semi-conserved regions of the alignment (Fig. 2B) that clearly differentiate them from other core urostylids, including *Pseudourostyla cristata*. These differences are reflected in the secondary structure of the V2 region of the SSU rRNA molecule (Fig. 2A); therefore, it is clear that *Pseudokeronopsis* and *Nothobolosticha* represent a distinct taxon at the family level that is sharply divergent from other core urostylids, and there is no reason to suppose that additional molecular information will contradict results obtained with SSU rRNA sequences.

*Pseudokeronopsis* and *Nothobolosticha* are more closely related to one another than to other core urostylids with respect to morphological and morphogenetic evidence. They have a similar body shape, arrangement of cortical granules, and cirral pattern but differ in the presence (vs. absence) of a frontal bicorona and presence (vs. absence) of frontoterminal cirri. The morphogenesis of *Nothobolosticha* has not been described completely; however, the main features can be inferred from its reorganization process (Li et al., 2009), and there are no conspicuous differences between the two genera. Moreover, *Nothobolosticha* was a sister to the *Pseudokeronopsis* clade with maximal support in all of our molecular phylogenies (Fig. 1), which was consistent with the studies of Li et al. (2009) and Yi et al. (2010). Finally, the putative secondary structures of the V2 region of the SSU rRNA molecules in *Pseudokeronopsis* and *Nothobolosticha* and their unique nucleotide signatures in the SSU rRNA alignment are markedly different from those of other core urostylids (Fig. 2).

By contrast, *Apokeronopsis* and *Thigmokeronopsis* differ from both *Pseudokeronopsis* and *Nothobolosticha* by having distinctly separated midventral rows, anlagen for marginal rows and dorsal kinetics formed de novo, and development of transverse cirri in a long row (in *Apokeronopsis*) or thigmotactic cirri present (in *Thigmokeronopsis*). *Apokeronopsis* and *Thigmokeronopsis* are grouped together in a single, maximally supported clade that nests within the clade comprising the family Urostylidae in all molecular phylogenies (Fig. 1). Furthermore, they have the distinctive secondary structure of the V2 region of the SSU rRNA molecules and unique SSU rRNA nucleotide signatures that characterize members of the Urostylidae, Bergeriellidae, and Pseudourostylidae (Fig. 2). Thus, there is no reason to doubt the polyphyly of the Pseudokeronopsidae as formerly constituted and the assignment of *Apokeronopsis* and *Thigmokeronopsis* to the Urostylidae.

The similarities with respect to the pattern of cirri and dorsal ciliary rows and their morphogenesis that are shared between *Apokeronopsis* and *Thigmokeronopsis*, on the one hand, and *Pseudokeronopsis* and *Nothobolosticha*, on the other, could be attributed either to convergent evolution or to preservation of an ancestral pattern (Shao et al., 2007). The former hypothesis seems more likely because neither of the clades that share the pattern of ciliature formerly used to characterize the Pseudokeronopsidae occupy a basal position (Fig. 1). The similarities in morphogenesis might appear to support the latter hypothesis; however, they actually support neither hypothesis because taxa with convergent morphological features also would be expected to share morphogenetic features, owing to the evolution of one being linked to the other.

Berger (2006) defined the family Pseudokeronopsidae as follows: Urostylida with distal end of the adoral zone of membranelles extending far towards the posterior and lacking caudal cirri; during cell division, many macronuclear nodules do not fuse into a mass but divide individually, and the parental adoral zone of membranelles totally replaced. To this, we can add the following molecular...
Fig. 6. A–J. Middle and late stages in morphogenesis of *Pseudokeronopsis erythrina* sp. n. in cells stained with protargol. Parental cirri are shown in outline whereas new ones are shown in solid black. —A, C. Ventral views, arrows indicate the anlage of the fronto-ventral-transverse cirri (FVTA) in each daughter; the anlage of the undulating membrane gives rise to the first frontal cirrus in both daughters (double-arrowheads in A), the paroral and endoral membranes (arrowheads in C); double-arrowheads in C show the buccal cirrus. —B, D. Dorsal views, arrows mark the macronuclear nodules. —E, G, H, I. Ventral views, arrows indicate that two frontoterminal cirri have separated from the last streak and then migrated anteriad (H, I). —F, J. Dorsal views of late division. The cirri connected by lines are from the same anlage (double-arrowheads in H). DK, dorsal kineties; DKA, anlagen of dorsal kineties; LMA, anlagen of left marginal cirral; MVC, midventral complex; RMA, anlagen of right marginal cirral; TC, transverse cirri. Scale bars: 50 μm.
characterization of the family: Helix 10 of the SSU rRNA molecule with one large, median bulge (Fig. 2A) and unique nucleotide identities at 20 positions in an alignment of SSU rRNA sequences (Fig. 2B). The complete alignment is available on request from the Laboratory of Protozoology, Institute of Evolution and Marine Biodiversity, OUC, Qingdao, China.

Morphological comparison of Pseudokeronopsis erythrina with its congeners

Pseudokeronopsis remains one of the least resolved taxa of hypotrichs because major morphological characters, including form of the cell body, colour, and pattern of ciliation overlap among most reported forms (Foissner 1982; Wirnsberger et al. 1987; Song et al. 2006). In addition to P. erythrina, eight valid species have been reported in the genus, P. rubra (type species), P. flavica, P. carneae, P. sepetibensis, P. simillis, P. decolor, and P. multinucleata (Borror & Wicklow 1983; Hu & Song 2001; Hu et al. 2004; Song et al. 2004, 2006; Wanick & Silva-Neto 2004; Sun & Song 2005; Berger 2006; Shi et al. 2007; Shao et al. 2008a). In addition, P. pararubra Hu et al. 2004 was made a synonym of P. carneae by Song et al. (2006), and P. ovalis

Fig. 7. A–N. Photomicrographs of Pseudokeronopsis erythrina sp. n. after protargol impregnation. —A, B. Ventral views of general infraciliature (A) and the anterior portion (B), the frontoterminal cirri (arrowheads) and the buccal cirrus (arrow). —C. Arrows mark three cirri among zig-zag structure in midventral complex. —D. Dorsal view of the dorsal kinetics (arrowheads) —E, F, G. Ventral views, to show the 2OP and FVTA in the opisthe, arrowheads mark the newly formed membranelles in the 2OP. —H. The dividing macronuclear segments (arrows). —I. Ventral views, to show the FVTA in the proter. —J, K. Ventral views of middle stage dividers, arrows show the FVTA, arrowheads mark the newly formed membranelles, and double-arrowheads indicate that the first frontal cirrus is generated from the UMA. —L. Ventral view of a late divider, arrow indicates the newly segmented cirri in midventral complex, double-arrowheads mark the buccal cirrus, while arrowhead indicates the new UM. —M. Dorsal view, showing the new dorsal kinetics (DK). —N. Ventral view of a very late divider, to show the proter and the opisthe, arrows mark the two frontoterminal cirri migrating to their final positions. DKA, dorsal kinetics anlagen; FVTA, fronto-ventral-transverse cirral anlagen; LMA, left marginal cirral anlagen; 2OP, oral primordium of opisthe; TC, transverse cirri; UMA, undulating membranes anlagen. Scale bars: 50 μm (A, B), 20 μm (C–M).
was transferred to Apokeronopsis by Shao et al. (2008a) (Table 2).

**Pseudokeronopsis similis** (Stokes, 1886) Borror & Wicklow 1983 can be distinguished easily from *P. erythrina* and all other congener by its moniliform macronucleus and freshwater habitat (Berger 2006). *Pseudokeronopsis decolor* (Wallengren, 1900) Borror & Wicklow 1983 differs from all other congeners by having a colourless cortex (Berger 2006).

The pigmented species of *Pseudokeronopsis*, including *P. erythrina*, are shown in Table 2. *Pseudokeronopsis rubra* (Ehrenberg, 1838) Borror & Wicklow 1983 most closely resembles *P. erythrina*, being similar in size and shape of the cell body, colour, and basic infraciliature. However, it differs from the latter in having ellipsoid granules (vs. spherical), one contractile vacuole located in the posterior 1/3 of the body (vs. 2), and usually five dorsal kineties (vs. consistently three) (Wirnsberger et al. 1987; Hu & Song 2001; Berger 2006; Song et al. 2006).

**Pseudokeronopsis carnea** (Cohn, 1866) Wirnsberger et al. 1987 also is red-like *P. erythrina* but differs from it by having more dorsal kineties (5–8 vs. 3) and transverse cirri (7–11 vs. 2–4), characteristic discoid pigment granules, and one contractile vacuole (Table 2; Wirnsberger et al. 1987; Hu et al. 2004; Song et al. 2006).

**Pseudokeronopsis flavicans** (Cohn, 1866) Wirnsberger et al. 1987 can be separated from *P. erythrina* by its yellowish colour, ellipsoid pigment granules, and single contractile vacuole located in the posterior 1/4–1/6 of the body (Table 2) (Wirnsberger et al. 1987; Song et al. 2004, 2006; Berger 2006). *Pseudokeronopsis flava* (Kahl, 1932) Borror & Wicklow 1983; as redefined by Song et al. (2006), also has ellipsoid, brownish-yellow pigment granules rather than spherical, red ones and can be further separated from *P. erythrina* by its larger size (200–300 × 40–55 μm vs. 120–220 × 20–50 μm), more slender shape, number of contractile vacuoles (one at anterior 1/3 vs. almost always two), and greater number of dorsal kineties (4–5 vs. 3) (Table 2; Song et al. 2002, 2006).

**Pseudokeronopsis sepetibensis** Wanick & Silva-Neto 2004 can be distinguished from *P. erythrina* by its smaller size (100–140 × 20–26 vs. 120–220 × 20–50 μm), light greenish-yellow colour (vs. dark red to brick-red), and 4–6 dorsal kineties (vs. 3) (Wanick & Silva-Neto 2004; Berger 2006). The infraciliature of *Pseudokeronopsis multiciliatea* (Maupas, 1883) Borror & Wicklow 1983 has not been described; thus, it can be compared only with the living cell of *P. erythrina*. The former is characterized by having yellowish cytoplasm with brick-red cortical granules arranged in five longitudinal bands, which suggests that it might have five dorsal kineties. It possesses 12–13 transverse cirri and is therefore clearly different from *P. erythrina* in this respect (Table 2). In addition, its two distinctly sep-

### Table 2. Comparison of seven morphologically similar species of *Pseudokeronopsis*

<table>
<thead>
<tr>
<th>Characters</th>
<th><em>P. rubra</em></th>
<th><em>P. flavicans</em></th>
<th><em>P. flava</em></th>
<th><em>P. carnea</em></th>
<th><em>P. sepetibensis</em></th>
<th><em>P. multinucleata</em></th>
<th><em>P. erythrina</em> sp. n.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body shape</td>
<td>Slender, both ends tapered</td>
<td>Slender, often twisted, both cell ends rounded</td>
<td>Slender and band-like, cell size extremely variable</td>
<td>Plump, frontal area wide, both cell ends rounded</td>
<td>Both cell ends round</td>
<td>Slender and band-like to plump</td>
<td>Slender, both ends tapered</td>
</tr>
<tr>
<td>Color of cell at higher magnification</td>
<td>Brick-red to yellowish orange</td>
<td>Yellow-brown</td>
<td>Dark brown-brown</td>
<td>Light greenish-yellow</td>
<td>Brick-red</td>
<td>Dark brownish-red</td>
<td>Brick-red</td>
</tr>
<tr>
<td>Granules, shape</td>
<td>Elliptical</td>
<td>Elliptical</td>
<td>Elliptical</td>
<td>Spherical</td>
<td>Not given</td>
<td>Elliptical</td>
<td>Absent</td>
</tr>
<tr>
<td>Contractile vacuoles, number</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>2 (occasionally only 1 visible)</td>
</tr>
<tr>
<td>Contractile vacuoles, position</td>
<td>Posterior 1/3</td>
<td>Posterior 1/3</td>
<td>Posterior 1/3</td>
<td>Posterior 2/3</td>
<td>Posterior 1/3</td>
<td>Arranged longitudinally</td>
<td>Posterior 1/3</td>
</tr>
<tr>
<td>Macronuclear nodules, number</td>
<td>&gt;100</td>
<td>72–97</td>
<td>72–78</td>
<td>Numerous</td>
<td>&gt;100</td>
<td>Not given</td>
<td>Not given</td>
</tr>
<tr>
<td>Adoral membranelles, number</td>
<td>ca. 50</td>
<td>ca. 55</td>
<td>ca. 55</td>
<td>ca. 45</td>
<td>ca. 45</td>
<td>ca. 45</td>
<td>ca. 45</td>
</tr>
<tr>
<td>Cilia pairs in bicornate number</td>
<td>5–7</td>
<td>5–9</td>
<td>5–9</td>
<td>5–9</td>
<td>5–9</td>
<td>5–9</td>
<td>5–9</td>
</tr>
<tr>
<td>Transverse cirri, number</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Measurements in micrometer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Measurements in micrometer.**

© 2011 The Authors • Zoologica Scripta © 2011 The Norwegian Academy of Science and Letters, 40, 6, November 2011, pp 659–671

669
arated ventral rows, as redescribed by Borror & Wicklow (1983), are atypical for Pseudokeronopsis, suggesting it may belong to a different genus.

**Morphogenetic comparison of Pseudokeronopsis erythrina with congers**

Morphogenesis is known in only four of the eight previously described species of *Pseudokeronopsis*, *P. rubra*, *P. carnea*, *P. flava*, and *P. similis* (Hu & Song 2001; Hu et al. 2004; Sun & Song 2005; Shi et al. 2007). In general, morphogenetic events in *P. erythrina* closely resemble those of *P. rubra*, *P. carnea* and *P. flava*, which can be summarized as follows: (i) in the proter, the 1OP originates de novo beneath the surface of the buccal cavity and replaces the old oral apparatus completely; (ii) in both daughters, the FVTA originates as a single set of streaks contrast, in the freshwater species, *P. similis* (i) no new 1OP is formed in the proter, and it retains the parental AZM; (ii) the FVTA originates as a single set of streaks in the early stages of morphogenesis and then divides into two sets, one for each daughter; and (iii) the macro-nucleary segments fuse into a single mass in the middle of division (Shi et al. 2007). These clear differences between *P. similis* and its congeners suggest that it may not be a valid member of *Pseudokeronopsis*, but its taxonomic placement cannot be determined with certainty at the present time because there are (i) no clear infraciliary differences between it and other members of the genus and (ii) molecular data for *P. similis* are lacking.

**Acknowledgements**

Our deep thanks are expressed to Dr Zhenzhen Yi, post-doctoral scholar in the Laboratory of Protozoology, OUC, for her help in the treatment of molecular materials and in the phylogenetic analyses used in the present study. We are grateful to Dr Chen Shao for kindly reading the first drafts and offering advice. Research sponsored by the Natural Science Foundation of China (project number: 31030059). We also thank the anonymous reviewers and editors for their helpful criticism of the first version of this manuscript.

**References**


