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Phylogenetic analyses suggest that *Psammomitra* (Ciliophora, Urostylida) should represent an urostylid family, based on small subunit rRNA and alpha-tubulin gene sequence information

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The morphologically unique ciliate *Psammomitra* has long been considered as a systematically uncertain stichotrich. This is mainly because of its highly specialized morphology and a lack of either detailed information concerning its ontogenesis, or molecular data. Based on the small subunit rRNA (SSrRNA) gene and alpha-tubulin gene sequences, we re-evaluated the phylogenetic position of *Psammomitra retractilis* using multiple algorithms. Phylogenetic trees inferred from the SSrRNA gene sequences representing a total of 53 spirotrichs demonstrated the closest relationship of *Psammomitra* was with *Holosticha*-like taxa, with strong support, which clearly suggested that *Psammomitra* should be placed into the order Urostylida although it branched at a rather deep level, and is likely to be closely related to Holostichidae. With consideration to molecular evidence and morphological characters, *Psammomitra* should be a clearly outlined taxon at about the rank of family, i.e. Psammomitridae **stat. nov.**, within the order Urostylida. The improved diagnosis for this family is as follows: Urostylida possessing extremely contractile, elongated body which consists of three parts: head, trunk, and slender tail; midventral complex composed of midventral pairs only and restricted to about anterior 1/3 of ventral surface; frontal, frontoterminal, and transverse cirri present; one left and one right marginal rows which commence near proximal end of adoral zone and extend to near rear body end.

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INTRODUCTION

The subclass Stichotrichia Small & Lynn, 1985 appears to be an especially diverse and taxonomically confused group within the phylum Ciliophora (e.g. Foissner *et al.*, 2004; Schmidt *et al.*, 2007). Although molecular data, such as small subunit ribosome RNA (SSrRNA) gene sequences, have contributed significantly to the reconstruction of phylogenetic relationships within the Stichotrichia (e.g. Bernhard *et al.*, 2001; Hewitt *et al.*, 2003; Foissner *et al.*, 2004; Schmidt *et al.*, 2007), important questions still remain. For example, the phylogenetic positions of some taxa remain unclear because of the absence of either/both morphogenetical or/and molecular information.

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One such candidate is *Psammomitra retractilis*. This taxon is the sole species of the genus and has been frequently found in marine habitats (for a review, see Berger, 2006). It has an urostylid midventral complex composed of cirral pairs only, and a characteristic long tail (Song & Warren, 1996). Its systematic position is subject to a long and ongoing dispute (for a review, see Berger, 2006). Song & Warren (1996) described this species in detail and suggested its assignment to the urostylids based on its cirral pattern, which is composed of a few midventral pairs, two frontoterminal cirri, and some transverse cirri (Fig. 1). In Berger's (2006) review, *Psammomitra* was classified as a member of Holostichidae. Berger (2006) also stated that this genus is possibly an urostylid instead of a dorsomarginalian taxon because of the absence of dorsomarginal kineties, although he suspected that *P. retractilis* might be an oxytrichid, based on some morphological features, e.g. it forms one, two, or three additional cirral anlagen, a process which is likely to occur several times independently in 18-cirri oxytrichids. Despite a well-known morphology, the systematic position of *Psammomitra* within the Stichotrichia remains speculative.

We here re-evaluated the phylogenetic placement of *P. retractilis* by analysing its SSrRNA and alpha-tubulin gene sequences. In addition, we also sequenced and included the alpha-tubulin gene of *Thigmokeronopsis stoecki* Shao *et al.*, 2008, a typical urostylid taxon, to examine the relationship of *P. retractilis* with typical urostylids.

MATERIAL AND METHODS

Ciliate collection and identification

Psammomitra retractilis and *T. stoecki* were collected from the coast of the Yellow Sea in the vicinity of Qingdao (36°08′N, 120°43′E), using microscope slides immersed for one to several weeks as artificial substrates, in May 2005 and May 2007, respectively. Morphological studies and species identifications were according to Shao *et al.* (2008).

Terminology and systematic classification are according to Lynn & Small (2002). However, terminology and systematic classification of urostylids are according to Berger (2006), and those of oxytrichids are according to Berger (1999).

DNA extraction

Genomic DNA was extracted using REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) with modifications suggested by Gong *et al.* (2007). In brief, one or more cells of each population were isolated and transferred to a drop of autoclaved seawater, which was then washed in sterile seawater several times to remove other potential minute protists. The cell was subsequently transferred to a 1.5-ml microtube with a minimal volume of sterile seawater. The concentration and quality of the extracted genomic DNA was checked by a NanoDrop-ND1000 spectrophotometer (Gao *et al.*, 2008).

Amplification and sequencing

The PCR amplifications of the SSrRNA and alpha-tubulin genes were performed using a TaKaRa *ExTaq* DNA Polymerase Kit (TaKaRa Biomedicals, Japan). Primers used for SSrRNA gene amplification were Euk A (5′–AAC CTG GTT GAT CCT GCC AGT–3′) and Euk B (5′–TGA TCC TTC TGC AGG TTC ACC TAC–3′) (Medlin *et al.*, 1988) covering nearly the full-length of the gene. PCR conditions were as follows: 5 min initial denaturation (95 °C), followed by 35 cycles of 1 min at 95 °C, 1.5 min at 56 °C; 2.5 min at 72 °C, with a final extension of 15 min (72 °C). The partial alpha-tubulin gene was amplified using the forward primer Tub-1 (5′–AAG GCT CTC TTG GCG TAC AT–3′) and the reverse primer Tub-2 (5′–TGA TGC CTT CAA CAC CTT CTT–3′) (Miao *et al.*, 2007). PCR conditions were: 5 min initial denaturation (95 °C), followed by 35 cycles of 1 min at 95 °C, 1 min at 56 °C and 1.5 min at 72 °C, with a final extension of 15 min (72 °C).

After confirmation of the appropriate size of the amplified fragments (1.7 kb for the SSrRNA gene and 1.1 kb in case of the alpha-tubulin gene) on an agarose gel, each PCR product was purified using the TIANgel Midi Purification Kit (TIANGEN, Beijing, China). Subsequently, purified PCR fragments were inserted into a pUCm-T cloning vector (Shanghai Sangon Biological Engineering & Technical Service Company, Shanghai, China). Plasmids were extracted from transformed *Escherichia coli* overnight cultures using the plasmid mini-prep spin column kit (Shanghai Sangon Biological Engineering & Technical Service Company, Shanghai, China). SSrRNA and alpha-tubulin amplifications were sequenced bidirectionally (Invitrogen sequencing facility, Shanghai, China) using three forward and three reverse modified SSrRNA sequencing primers (Medlin *et al.*, 1988) and the RV-M and M13-20 primers.

Genealogical analyses

Three data sets were included in the analyses: (1) alpha-tubulin gene sequences including all the hypotrichous and stichotrichous sequences available; (2) alpha-tubulin amino acid sequences; and (3) SSrRNA gene sequences including 53 spirotrichs (see Table 1 for sequence availability). Following preliminary

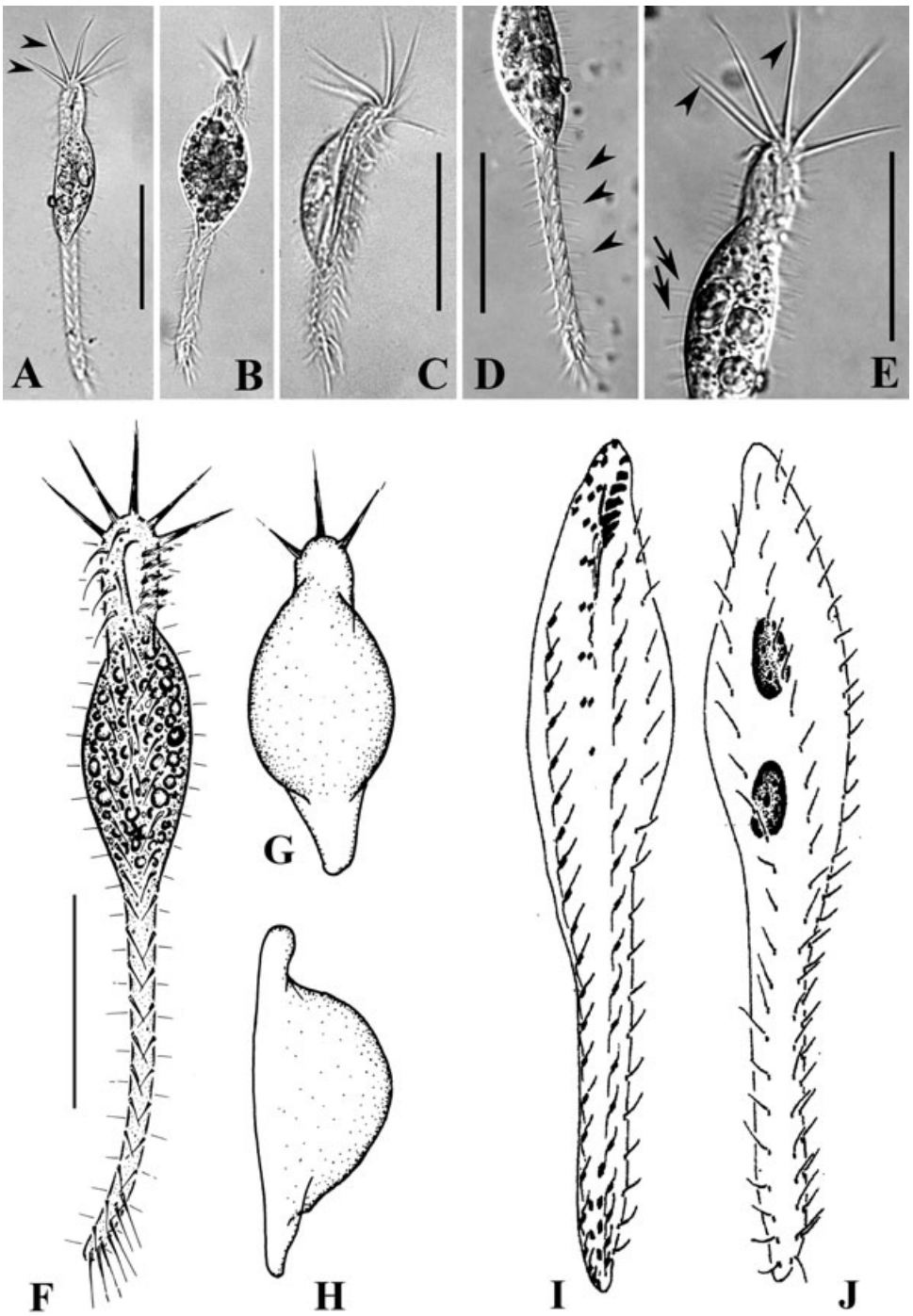

Figure 1. Morphology and infraciliature of *Psammomitra retractilis* (F–J, from Song & Warren, 1996). A, B, F, individuals in extended states to show the typical body shapes. Arrowheads in (A) mark the long, dominant membranelles. C, lateral view of a contracted specimen. D, posterior part, to demonstrate the long dorsal cilia. E, anterior part. Arrowheads indicate the long membranelles, whereas arrows mark the dorsal cilia. G, H, dorsal and lateral views of contracted cells. I, J, ventral and dorsal views to show the infraciliature and macronuclear nodules. Scale bars: A, C, D, F = 40 μm; E = 30 μm.

Table 1. List of the small subunit rRNA (SSrRNA) and alpha-tubulin gene sequences used in Figures 2–4

Species	Accession no.		Species	Accession no.	
	SSrRNA	α–tubulin		SSrRNA	α-tubulin
Amphisiella magnigranulosa	AM412774	–	*Parabirojimia similis*	DQ503584	–
Anteholosticha manca	DQ503578	–	*Paradiophrys irmgard*	EU189070	–
Apokeronopsis bergeri	DQ777742	–	*Paraurostyla weissei*	AF164127	–
Apokeronopsis crassa	DQ359728	–	*Pattersoniella vitiphila*	AJ310495	–
Aspidisca steini	AF305625	–	*Phacodinium metchnikoffi*	AJ277877	AY554040
Certesia quadrinucleata	DQ059581	–	*Plagiotoma lumbrici*	AY547545	–
Cyrtohymena citrina	AY498653	–	*Pleurotricha lanceolata*	AF508768	–
Diophryopsis hystrix	EF486861	–	*Prodiscocephalus borrori*	DQ646880	–
Diophrys appendiculata	AY004773	–	*Pseudoamphisiella alveolata*	DQ503583	–
Diophrys sp.	–	AY037857	*Pseudoamphisiella lacazei*	DQ777743	–
Engelmanniella mobilis	AF164134	–	*Pseudokeronopsis flava*	DQ227798	–
Euplotes focardii	EF094960	AF408404	*Pseudokeronopsis rubra*	EF535729	–
Euplotidium arenarium	Y19166	–	*Pseudourostyla cristata*	DQ019318	–
Euplotoides octocarinatus	AJ310489	X69466	*Rubrioxytricha ferruginea*	AF370027	–
Gastrocirrhus monilifer	DQ864734	–	*Steinia sphagnicola*	AJ310494	–
Gastrostyla steinii	AF164133	–	*Sterkiella histriomuscorum*	AF508770	AY883859
Gonostomum namibiense	AY498655	–			
Holosticha bradburyae	EF123706	–	*Styxophrya quadricornutus*	X53485	–
Holosticha diademata	DQ059583	–	*Stylonychia lemnae*	AF508773	–
Holosticha heterofoissneri	DQ059582	–	*Tetmemena pustulata*	AF50877	–
Laurentiella strenua	AJ310487	–	*Thigmokeronopsis stoecki*	EU220226	–
Metaurostylopsis sp.	EU220227	–	*Trachelostyla pediculiformis*	DQ057346	
Metaurostylopsis struederkypkeae	EU220228	–	*Uroleptus gallina*	AF164130	–
Moneuplotes crassus	AJ305255	AY041105	*Uroleptus lepisma*	AF508765	–
Moneuplotes vannus	AJ310488	Z11769	*Uroleptus pisces*	AF164131	–
Orthamphisiella breviseries	AY498654	–	*Uronychia transfuga*	AF260120	–
Oxytricha granulifera	X53486	Z11763	*Urostyla grandis*	AF164129	–
Sterkiella cavicola	–	Y10035			

–, No sequence available.

analyses, we omitted sequences of choreotrichs and oligotrichs from analyses, because their inclusion in alignments and trees in the present study resulted in unstable topologies, with *Holosticha* spp. falling into the clades of choreotrichs and oligotrichs in some trees.

We performed phylogenetic analyses of spirotrichs based on SSrRNA gene sequences. The sequences were aligned using the ClustalW implemented in BIOEDIT 7.0.0 (Hall, 1999), and further modified manually using BIOEDIT. The final alignment that was used for subsequent phylogenetic analyses included 1762 positions. The General Time reversible (GTR) + I (= 0.4304) + G (= 0.5067) was selected as the best model with Akaike information criterion by the program MrModeltest 2 (Nylander, 2004) was used for both Bayesian and maximum likelihood (ML) inference. Using these settings, a ML tree was constructed with the PhyML V2.4.4 program (Guindon & Gascuel, 2003). The reliability of internal branches was assessed using a nonparametric bootstrap method with 1000 replicates. A Bayesian inference (BI) analysis was performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Markov chain Monte Carlo (MCMC) simulations were then run with two sets of four chains using the default settings: chain length 1 500 000 generations, with trees sampled every 100 generations. The first 300 000 generations were discarded as burn-in. The remaining trees were used to generate a consensus tree and to calculate the posterior probabilities (PP) of all branches using a majority-rule consensus approach. Neighbor-joining (NJ) and maximum parsimony (MP) analyses were performed with PAUP* 4.0b10 (Swofford, 2002) and the reliability of the internal branches was estimated by using the bootstrap method with 1000 replicates (Felsenstein, 1985). For MP analyses, 500 parsimony-informative characters were included in our study. Parameters for the MP tree were as follows: 100 random-addition sequences, and tree bisection-reconnection branch swapping.

An alignment of 1073 positions of alpha-tubulin gene was used for tree constructions. Bayesian, ML, and MP trees were built as above. For the first two analyses, the GTR+I (= 0.5069) +G (= 1.1434) model was selected by MrModeltest and 279 parsimony informative characters were included in the MP analysis. Phylogenetic trees were visualized with TreeView v1.6.6 (Page, 1996) and MEGA 4 (Tamura *et al.*, 2007).

The alpha-tubulin amino acid sequences were translated using GeneDoc 2.6.002 (Nicholas & Nicholas, 1997). Three hundred and fifty-two positions were included in the final sequence alignment. The alpha-tubulin genealogies based on the amino acid sequences were constructed using the MP algorithm in the package PAUP* as above, and 21 parsimony informative characters were included. Amino acid alignments were also analysed in MrBayes and PhyML, where Blosum62 + G (= 0.186) was selected as the best amino acid model using ProtTest 1.4 (Abascal, Zardoya & Posada, 2005).

To explore the conflict between data sets, the partition homogeneity test (Farris *et al.*, 1994; Cunningham, 1997) was applied to the combined data matrix using the partition homogeneity test in PAUP* 4.0b10, with 1000 replications. SSrRNA and alpha-tubulin gene sequences were found to be significantly incongruent by using the partition homogeneity test ($P = 0.039$). We therefore did not use the combined data for analyses.

RESULTS

Deposition of sequences

All new sequences have been deposited in the GenBank data base with the following accession numbers: EF486865 (*P. retractilis* SSrRNA gene), EU678914 (*P. retractilis* alpha-tubulin gene), EU678915 (*T. stoecki* alpha-tubulin gene).

Genealogical analyses based on SSrRNA gene

Three of the four stichotrichous orders (*sensu* Lynn & Small, 2002), namely Sporadotrichida, Stichotrichida, and Urostylida did not emerge as monophyletic groups in any trees we recovered. Furthermore, Oxytrichidae and Holostichidae, two of four families into which *Psammomitra* was historically classified, were also shown to be polyphyletic (Figs 2, 3).

In all our analyses (Figs 2, 3), the representatives of Dorsomarginalia were split into four clades; however, the close relationship between uroleptids and oxytrichids was hinted. The family Oxytrichidae was never found as a monophyly, whereas the subfamily Stylonychinae seemed to be monophyletic.

Psammomitra clustered with *Holosticha* spp. with high/low supports (1.00 BI, 91% ML, 95% MP, 78% MP). The *Holosticha–Psammomitra* clade represented a basal group within the subclass Stichotrichia, but placed separately from other urostylids including *Urostyla* and *Pseudokeronopsis*, etc. *Parabirojimia similis* clustered with sporadotrichid species *Trachelostyla*, forming a group between the *Holosticha–Psammomitra* clade and other urostylids. All the remaining urostylids grouped together in all phylogenetic trees (Figs 2, 3). Within this group, the representatives of Acaudalia were split into two clades in our analyses: *Pseudokeronopsis* and *Pseudourostyla* grouped consistently together (1.00 BI, 80% ML, 76% NJ, 66% MP), whereas *Thigmokeronopsis* always clustered with *Apokeronopsis* and *Metaurostylopsis* (1.00 BI, 99% ML, 97% NJ, 90% MP).

Genealogical analyses based on alpha-tubulin gene

The relationships within the Stichotrichia were poorly resolved in the alpha-tubulin trees (Fig. 4). However, we found general consistent patterns between genealogical analyses of alpha-tubulin (Fig. 4) and SSrRNA (Figs 2, 3).

Analyses of alpha-tubulin nucleotide sequences (Fig. 4A) did not support the monophyly of Hypotrichia and Stichotrichia, but low support (0.57 BI, 63% ML, 57% MP) for the grouping of urostylids (viz. *Psammomitra* and *Thigmokeronopsis*). Surprisingly, these two urostylids had a closer relationship with *Diophrys*, a hypotrich genus, than with other stichotrichs, although supports for this clustering pattern were low in all analyses (0.85 BI, 48% ML, 57% MP).

Phylogenetic analyses based on alpha-tubulin amino acids (Fig. 4B) had low resolution with the exception of a few taxa; however, they showed that Stichotrichia seemed to be monophyletic, with *Psammomitra* and *Thigmokeronopsis* branching within this group. As shown in analyses inferred from SSrRNA gene sequences (Figs 2, 3), *Diophrys* sp. was apart from these two urostylids, although it did not fall into a clade with other hypotrichs.

DISCUSSION

To which order does *Psammomitra* belong?

The systematic placement of *Psammomitra* has been greatly argued over and has always been variable in previous researches (Kahl, 1932, 1935; Borror, 1972; Corliss, 1977; Jankowski, 1979; Small & Lynn, 1985; Tuffrau & Fleury, 1994; Dini, Lucchesi & Macchioni, 1995; Song & Warren, 1996), whereas according to the newly updated Lynn & Small's (2002) system, it was

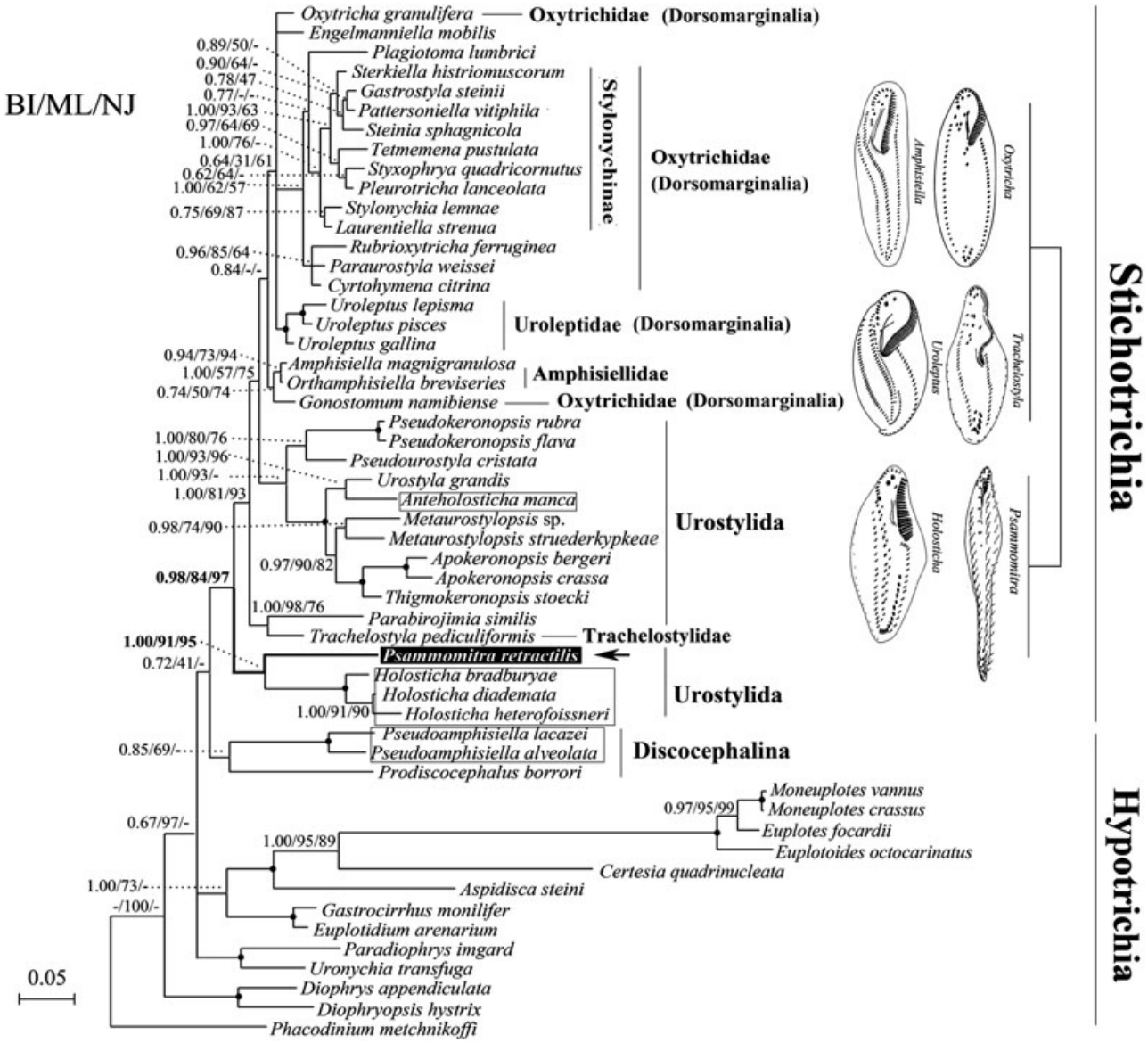

Figure 2. Phylogenetic tree based on small subunit rRNA sequences showing the position of *Psammomitra retractilis*, by Bayesian inferences applying the GTR + G + I model. '-' reflects disagreement between a method and the reference Bayesian tree at a given node. The fully supported (1.00/100%/100%) branches are marked with solid circles. *Psammomitra* is shaded black, and holostichids are enclosed in rectangles. Thick branches and arrows denote position of investigated species. The scale bar corresponds to five substitutions per 100 nucleotide positions. Infraciliature of *Oxytricha* and *Uroleptus* (from Foissner *et al.*, 2004), *Amphisiella* (from Li *et al.*, 2007), *Trachelostyla* (from Gong *et al.*, 2006), and *Holosticha* (from Hu & Song, 2001) are also shown.

tentatively assigned in Amphisiellidae, a family of the order Stichotrichida. According to the recent revision on urostylids (Berger, 2006), *Psammomitra* was placed in Holostichidae, a taxon belonging to the order Urostylida.

In our molecular trees constructed using the SSrRNA gene sequences of 53 spirotrichs (Figs 2, 3), *P. retractilis* is unambiguously identified as a sister group to *Holosticha,* hence supporting the idea that this taxon could be a member of the holostichids (*s.l.*) as suggested by many previous studies (Corliss, 1977, 1979; Tuffrau, 1979, 1987; Detcheva, 1992; Berger, 2006). The alpha-tubulin nucleotide sequences also indicated that *Psammomitra* could be related to another urostylid, *Thigmokeronopsis* (Fig. 4A), although this was only weakly supported. The low support might be caused by insufficient taxa sampling. Partly supporting the above results (Figs 2, 3,

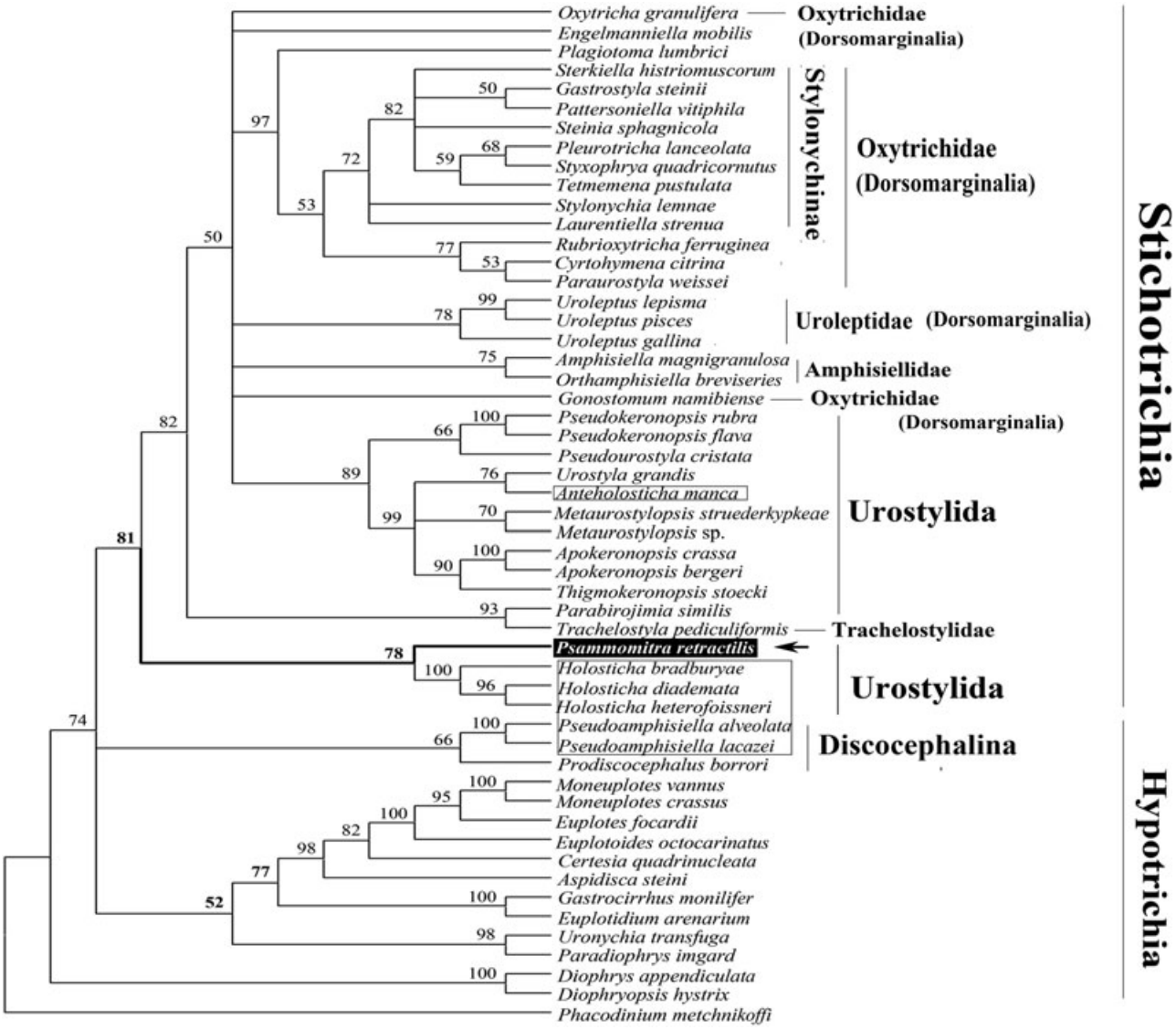

Figure 3. Maximum parsimony phylogeny of small subunit rRNA genes. *Psammomitra* is highlighted in black, and holostichids are enclosed in rectangles. Thick branches and arrows denote position of investigated species. Numbers on branches are values generated from 1000 bootstrap replicates.

4A), the alpha-tubulin amino acid data set only hinted that *Psammomitra* is a stichotrich (Fig. 4B), which might be caused by the lower resolution of the alpha-tubulin amino acid tree. This lack of resolution is a result of the few informative sites in the amino acid alignment.

Therefore, the placement based on molecular data corresponds to the morphological and the general formation process of all ciliature during morphogenesis although only partly revealed (Song & Warren, 1996), which indicates that *Psammomitra* should be an urostylid.

SUPPORTING *PSAMMOMITRA* AS THE RANK OF FAMILY

The conclusion that *Psammomitra* should be assigned to Urostylida challenged the arrangement of *Psammomitra* into either Trachelostylidae (Small & Lynn, 1985; Dini *et al.*, 1995), Amphisiellidae (Tuffrau & Fleury, 1994; Lynn & Small, 2002), or Oxytrichidae (Kahl, 1932, 1935; Borror, 1972; Jankowski, 1979) by many investigators. Our SSrRNA gene sequence data (Figs 2, 3) clearly also rejected the proposal of *Psammomitra* being a uroleptid species as suggested by Song & Warren (1996) (Figs 2, 3), as the latter

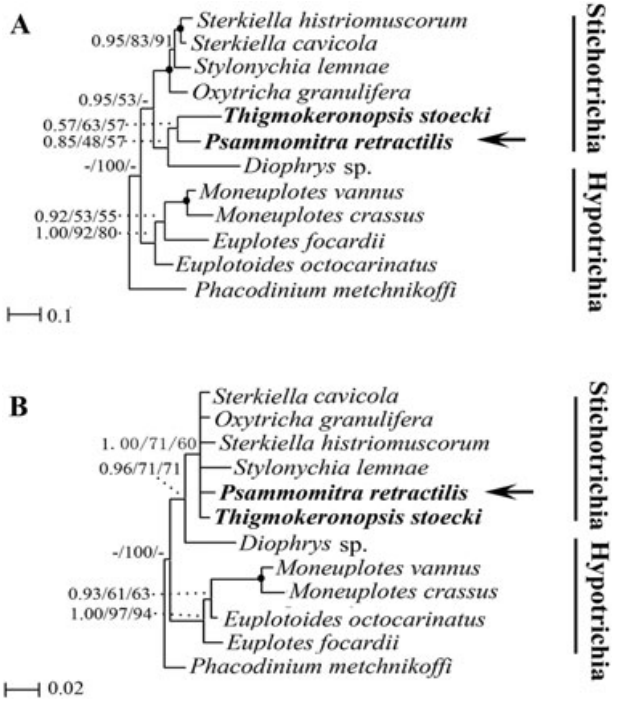

Figure 4. Bayesian trees based on different data sets showing phylogenetic relationships amongst Spirotrichea. '-' reflects disagreement between the maximum likelihood/maximum parsimony method and the reference Bayesian tree at a given node. The fully supported (1.00/100%/100%) branches are marked with solid circles. Species sequenced in the present study are shown in bold type. The scale bar corresponds to 10/2 substitutions per 100 nucleotide positions. A, phylogenetic analyses inferred from alpha-tubulin gene sequences data set. B, phylogenetic analyses inferred from alpha-tubulin amino acids data set.

branched as a sister clade to some stichotrichids and sporadotrichids (e.g. Foissner *et al.*, 2004; Schmidt *et al.*, 2007), whereas the clade *Holosticha–Psammomitra* branched first from the urostylids at a very deep level. In addition, *P. retractilis* lacks caudal cirri and dorsomarginal kineties, which both are conspicuous in most uroleptid species (Berger, 2006).

In our SSrRNA gene analyses (Figs 2, 3), an unexpected finding was that four genera in Holostichidae (*sensu* Berger, 2006) did not cluster together. Berger (2006) also considered that the diagnosis of Holostichidae (*sensu* Berger, 2006), because of the lack of apomorphies, only a combination of the most important plesiomorphies, indicated that the group is nonmonophyletic. Amongst these four genera, *Pseudoamphisiella* had a closer relationship with *Prodiscocephalus*, a species of Discocephalina, rather than with urostylids (Figs 2, 3), which is consistent with morphogenetic data (Song, Warren & Hu, 1997) and a previous molecular phylogenetic study (Yi *et al.*, 2008). Figures 2 and 3 also showed that *Anteholosticha* grouped with other urostylids instead of the clade of *Holosticha–Psammomitra*, which was discrepant with the morphological information that *Holosticha* is more closely related to *Anteholosticha* than to *Psammomitra*. Morphologically, *Psammomitra* differs from the traditional '*Holosticha*' (*s. l.*) in the following aspects (Song & Warren, 1996): (1) the former is conspicuously contractile, tripartite in head, trunk with a long tail relative to the body shape (vs. almost noncontractile, generally nonpartite in *Holosticha* and *Anteholosticha*); (2) midventral rows strongly shortened, i.e. extending only about half of the trunk length (vs. basically extend nearly the whole length of the body in *Holosticha* and *Anteholosticha*); (3) the anterior membranelles are highly differentiated, i.e. forming a corona-shaped structure with several long, rigid, spine-like membranelles (vs. nonspecialized in the latter two genera), and (4) thigmotactic in behaviour (vs. never thigmotactic in the latter two genera).

In conclusion, considering the information revealed by morphological and SSrRNA gene data (Fig. 2) that *Psammomitra* branched with *Holosticha* at a rather deep level, we believe that *Psammomitra* should be arranged in an isolated position near the Holostichidae but represent a taxon within the order Urostylida (*sensu* Berger, 2006) at the rank of family, i.e. Psammomitridae Jankowski, 1979 **stat. nov.**

Subclass Stichotrichia

Order Urostylida

Psammomitridae **stat. nov.**

*Improved diagnosis:*Urostylida possessing extremely contractile, elongated body that consists of three parts: head, trunk, and slender tail; midventral complex composed of midventral pairs only and restricted to about anterior 1/3 of ventral surface; frontal, frontoterminal, and transverse cirri present; one left and one right marginal row, which commence near proximal end of adoral zone and extend to near rear body end. Only one known genus.

*Remarks:*This family is recognized by the combination of features mentioned in the diagnoses, which outline it from other known families, e.g. Holostichidae, Bakuellidae, and Urostylidae (Berger, 2006).

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