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Molecular phylogeny of the scuticociliate, *Philaster* (Protozoa, Ciliophora), with a description of a new species, *P. apodigitiformis* sp. nov.

Abstract The systematic position of the scuticociliate, *Philaster*, has been highly ambiguous owing to a lack of either morphogenetic or molecular information. The small subunit rRNA gene of a new species, *Philaster apodigitiformis* sp. nov. was isolated from eggs of the puffer fish (*Takifugu rubripes*), was sequenced, and phylogenetic trees were constructed with different methods to assess its position and relationship among the scuticociliates. Results indicate that (1) *Philaster apodigitiformis* clearly falls into the order Philasterida and it is most closely related to the *Uronema–Paranophrys* assemblage, which groups with clusters consisting of families Uronematidae, Parauronematidae, Thyrophilacidae and Entorhipidiidae; (2) the two genera *Philaster* and *Philasterides* presently assigned to the family Philasteridae are in widely separated clades, indicating that the family is polyphyletic; and (3) the morphological similarity of *Philaster* and *Philasterides* is probably an example of evolutionary convergence. The new species *P. apodigitiformis* is recognised by a combination of the following features: body oval and slightly bilaterally flattened, cilia of paroral membrane conspicuously long and forming a sail-like shape, both membranelle 1 and 2 dominant, and approximately 40 somatic kineties present.

Key words interstitial ciliates, morphology, Scuticociliates, phylogeny

Introduction

For many years, the classification of scuticociliates has been largely based on characteristics of the infraciliature, mainly the structure of the buccal apparatus and its general pattern of morphology (Grolière, 1980; Foissner *et al.*, 1994; Song & Wilbert, 2000; Wang *et al.*, 2008a, 2008b). Such arrangements have been poorly supported by investigations based on other modern methods and classification often remains uncertain owing to disagreement at the familial or suprafamilial levels (Corliss, 1979; Jankowski, 1980, 2007; Puytorac, 1994; Lynn, 2008). The main reason is that the general pattern of stomatogenesis is very similar in scuticociliates (Small, 1967; Ma *et al.*, 2001, 2004, 2006; Hu *et al.*, 2008), and molecular data are largely unavailable in many groups (Miao *et al.*, 2008).

The genus *Philaster* Fabre-Domergue, 1885 was an ambiguous, poorly defined taxon until the infraciliature of the type species, *Philaster digitiformis*, was revealed (Mugard, 1948; Puytorac *et al.*, 1966; Grolière, 1974; Small & Lynn, 1985). Later, two further congeners, *P. bergeri* and *P. hiatti*, were reported (Thompson, 1969; Grolière *et al.* 1980). Since then, the systematic position of this genus has been highly confused and changeable. Corliss (1979) assigned it together with some other 'morphologically similar' forms (e.g. *Miamiensis*, *Philasterides* etc.) to the family Philasteridae. This arrangement is then changed from one to another in the systems suggested by Small and Lynn (1985), Puytorac (1994), Jankowski (2007) and Lynn (2008). In the last ten years, the systematic positions of many scuticociliates have been challenged by the addition of molecular data; however, *Philaster* remains untouched because of a lack of sequences. Recent studies on the marine ciliate fauna in north China have produced descriptions of many scuticociliates (Song, 2000; Wang *et al.*, 2008a, 2008b). In the summer of 2007, an undescribed species of parasitic *Philaster* was isolated from eggs of the puffer fish, *Takifugu rubripes*, giving us the opportunity to obtain the sequence of its small-subunit ribosomal RNA (SSU rRNA) gene and use it for phylogenetic analyses. The main aims of this paper were: to gain an understanding of the phylogenetic position of *Philaster*, and to describe the new species, which is named *Philaster apodigitiformis* sp. nov.

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Species	Accession number	Length (bp)	Species	Accession number	Length (bp)
Anophryoides haemophila	U51554	1763	*Metanophrys similis*	AY314803	1763
Bresslaua vorax	AF060453	1780	*Miamiensis avidus*	AY550080	1759
Cardiostomatella vermiformis	AY881632	1769	*Paramecium bursaria*	AF100314	1648
Cohnilembus verminus	Z22878	1717	*Paranophrys magna*	AY103191	1759
Coleps hirtus	U97109	1772	*Parauronema longum*	AY212807	1759
Colpoda inflata	M97908	1788	*Parauronema virginianum*	AY392128	1758
Cyclidium glaucoma	Z22879	1718	*Philaster* sp.- SZ-07041501	-	1755
Dexiotrichides pangi	AY212805	1764	*Philasterides dicentrarchi*	AY642280	1760
Entodiscus borealis	AY541687	1650	*Plagiopyliella pacifica*	AY541685	1759
Entorhipidium tenue	AY541688	1759	*Pleuronema coronatum*	AY103188	1759
Entorhipidium triangularis	AY541690	1645	*Prorodon teres*	X71140	1768
Epistylis wenrichi	AF335515	1723	*Pseudocohnilembus hargisi*	AY212806	1753
Eurystomatella sinicum	FJ012143	1757	*Pseudocohnilembus persalinus*	AY551906	1754
Glaucomides bromelicola	AJ810077	1745	*Schizocalyptra aeschtae*	DQ777744	1763
Histiobalantium natans	AB450957	1703	*Schizocaryum dogieli*	AF527756	1760
Homalogastra setosa	EF158848	1764	*Thyrophylax vorax*	AY541686	1758
Ichthyophthirius multifiliis	U17354	1747	*Uronema elegans*	AY103190	1757
Lembadion bullinum	AF255358	1746	*Uronema marinum*	Z22881	1680
Loxophyllum rostratum	DQ190465	1636	*Uronemella filificum*	EF486866	1676
Mesanophrys carcini	AY103189	1759	*Vorticella fusca*	DQ190468	1733

Table 1 List of the small subunit rRNA gene sequences used in the present work.

Materials and methods

Ciliate collection and identification

Philaster apodigitiformis sp. nov. was isolated from one-day old eggs of the puffer fish, *Takifugu rubripes*, in May 2007 in a fish hatchery (salinity 30‰) near Qingdao (36°08′N; 120°43′E), China. Each egg usually contained several ciliates, and they were released by rupturing the egg envelope with a fine needle. No cultures could be maintained, because the ciliates were able to survive for only one or two hours apart from the host. Observations on living cells were carried out using bright field and differential interference contrast microscopy (Lin *et al.*, 2008).

Another marine form (*Philaster* sp.-SZ-07041501), which is slender free-living with an *in vivo* inconspicuous paroral membrane and a larger buccal field (extending to about equatorial level; Fig. 3n, o) was isolated from the off-shore water near Shenzhen, south China (22°24′N, 113°54′E) by Yangang Wang. Since only several specimens were obtained, we were unable to perform further taxonomic studies. As an unidentified form, its SSU rRNA gene sequence was used in phylogenetic analyses.

The protargol silver-staining method according to Wilbert (1975) was used to reveal the infraciliature. Terminology and classification are according to Lynn and Small (2002).

DNA extraction, PCR amplification and sequencing

DNA was extracted using REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) as previously described in reference (Gong *et al.*, 2007). PCR amplification of SSU rRNA genes was performed with primers Euk A and Euk B (Medlin *et al.*, 1988). The typical amplification profile was programmed as follows: hold at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 2 min and extension at 72 °C for 3 min; and hold at 72 °C for 10 min. Purified PCR product was inserted into the pUCm-T vector and then sequenced (Yi *et al.*, 2008).

Sequence availability and phylogenetic analyses

A broad selection of SSU rRNA sequences of scuticociliates, other oligohymenophoreans, prostomes and copodids from GenBank (Table 1) were used to create an alignment by

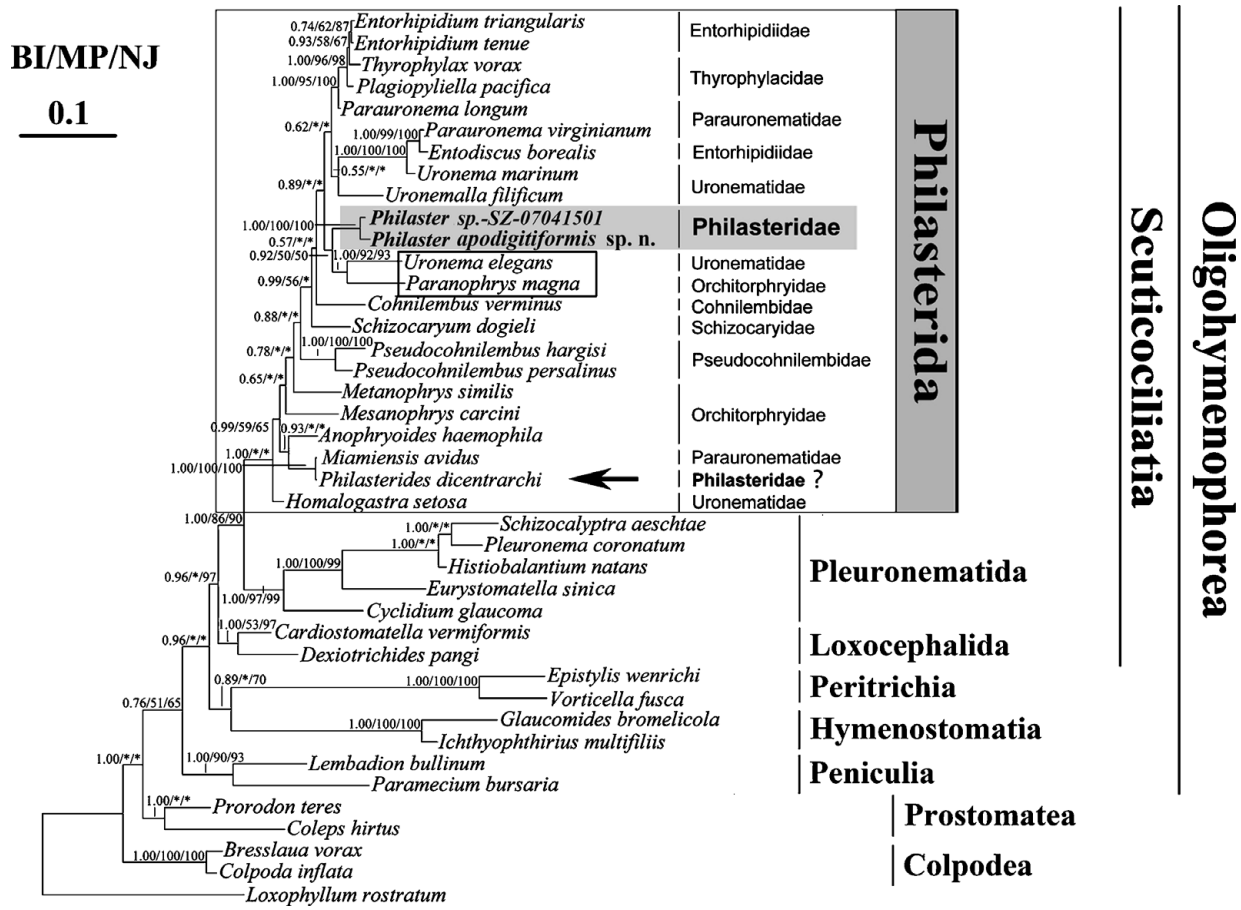

Figure 1 Phylogenetic tree of SSU rRNA gene sequences showing the positions of *Philaster apodigitiformis* sp. nov. by Bayesian inference (BI) applying the GTR+G+I model. Species sequenced in the present study are shown in bold type. Numbers near branches are as follows: BI posterior probability value/Maximum Parsimony bootstrap value/bootstrap value from neighbour-joining. The scale bar corresponds to ten substitutions per 100 nucleotide positions. Systematic classification follows Lynn (2008).

CLUSTAL W (Thompson *et al.*, 1994) that included 1493 sites and 41 taxa. This was used to construct a phylogenetic tree (Fig. 1) by the following methods: Bayesian Inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003), Maximum Parsimony (MP) using PAUP* 4.0b10 (Swofford, 2002), and Neighbour-Joining (NJ) using PHYLIP ver. 3.67 (Felsenstein, 2004). A GTR+G (=0.4918) +I (=0.3329) model of nucleotide substitution generated by likelihood ratio tests and AIC criteria was estimated in MrModeltest v.2 (Nylander, 2004). For Bayesian analyses, four simultaneous chains were run for 1 000 000 generations, with sampling at intervals of 100 generations (10 000 trees), and a burn-in of 25% (2500 trees discarded), and posterior probabilities were estimated for all nodes. Bootstrapping with 1000 replicates was performed on both distance and parsimony datasets (Gao *et al.*, 2008). *Loxophyllum rostratum* was used as outgroup in all analyses. TreeView v1.6.6 (Page, 1996) and MEGA 4.0 (Tamura *et al.*, 2007) were used to visualise tree topologies. The hypothesis that species of *Philaster* and *Philasterides* group into a common clade was tested with the approximately unbiased (AU) test, Kishino–Hasegawa (KH) test, and Shimodaira–Hasegawa (SH) test using the CONSEL package (Shimodaira & Hasegawa, 2001) to compare a constraint tree with the consensus BI tree.

Character	Min	Max	Mean	SD	CV	n
Body length	67	137	101.6	19.4	19.1	24
Body width	22	64	42.6	9.0	21.2	24
Length of BF	33	53	42.5	5.6	13.1	23
Number of SK	34	47	39.8	3.5	8.7	24
Number of Ma	1	1	1	0	0	25
Length of Ma	13	29	20.0	4.6	23.2	25

Table 2 Measurements of *Philaster apodigitiformis* sp. nov. performed on specimens after protargol impregnation. Measurements in μm. BF, buccal field; CV, coefficient of variation; Mean, arithmetic mean; Ma, macronucleus; Max, maximum; Min, minimum; n, sample size; SD, standard deviation; SK, somatic kineties.

Results

Morphology of *Philaster apodigitiformis* sp. nov. (Fig. 2, Table 2)

Diagnosis. Parasitic *Philaster*, measuring 50–140 × 30–60 μm *in vivo*, body oval in shape and flattened slightly to create bilateral symmetry; cilia of paroral membrane

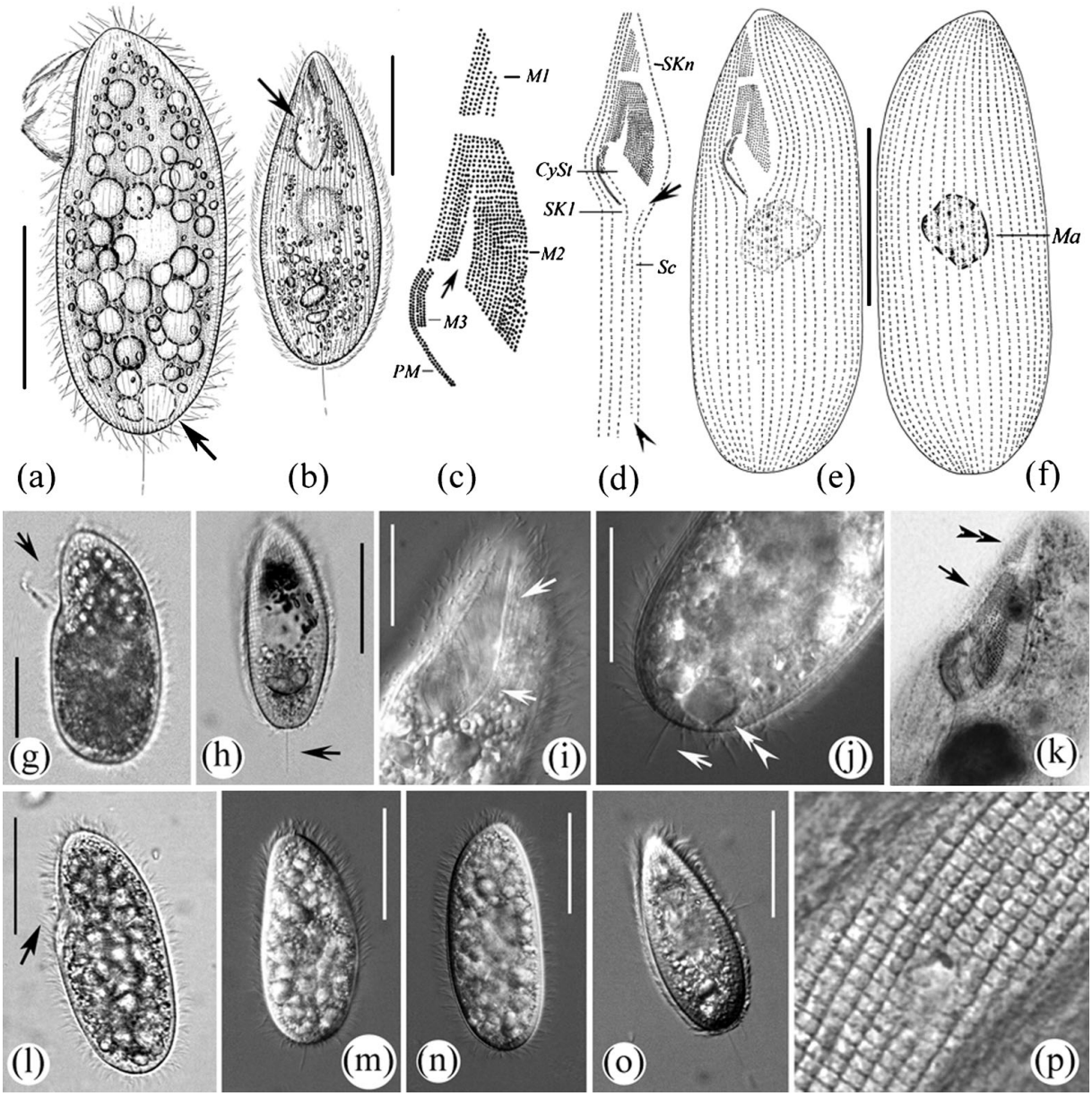

Figure 2 Morphology and infraciliature of *Philaster apodigitiformis* sp. nov. *in vivo* (a, b, g–j, l–o), after protargol (c–f, k) and silver nitrate (p) impregnations. (a) Left lateral view of a representative individual, arrow marks the contractile vacuole; (b) Ventral view, note the slender body shape and the pointed anterior end; (c) Details of buccal apparatus, arrow marks the splitting of the membranelle 2; (d) Ventral view of the buccal field, to show both the buccal apparatus and the ciliature in the postoral area, arrow and arrowhead mark the single-rowed scuticus; (e, f) Infraciliature of ventral and dorsal views; (g, h) Lateral and ventral views of two specimens, arrow in g indicates the membrane/membranelles of the oral apparatus, whereas in h marks the caudal cilium; (i) Ventral view of the buccal area, arrows point to the left margin of buccal field; (j) Caudal view, arrow marks the caudal cilium, double-arrowheads indicate the contractile vacuole; (k) Buccal field, arrow marks the M2, while double-arrowheads indicate the M1; (l–o) To show different body shapes, arrow in l marks the buccal field where no sail-like ciliary structure is recognisable; CySt, cytostome; $M_{1–3}$, membranelle 1–3; Ma, macronucleus; PM, paroral membrane; Sc, scutica; $SK_{1, n}$, the first, and the last somatic kinety. Scale bars: in a, b, e–h, l–o = 30 μm; in i, j = 20 μm.

conspicuously long and forming a sail-shaped structure; both membranelle 1 and 2 dominant consisting of many rows; one long caudal cilium and one oval macronucleus; contractile vacuole caudally positioned; approximately 40 somatic kineties; marine habitat.

Type location and host. Parasitic within 1-day-old eggs of the puffer *Takifugu rubripes*, Qingdao, China.

Slide deposition. One holotype slide (protargol preparation; registry no. 2009:3:10:1) has been deposited in The Natural History Museum, London; one paratype slide (protargol preparation; registry no. WYG-07053101) has been deposited in the collection of the Laboratory of Protozoology, OUC.

Etymology. The specific epithet *apodigitiformis* (*apo*-; Greek, away from), refers to the fact that the new species differs

from the well-known *Philaster digitiformis* despite some similarities in infraciliature.

Description of morphology. Size of living cell highly variable, mostly 60–90 μm long but some up to 140 μm in length, with length to width ratio of approximately 2:1. Body shape elongate, cylindrical, and tapering slightly toward anterior when viewed laterally, shape uniform among individuals, usually bilaterally flattened with thickness to width ratio of 3:4 (Fig. 2a, g, h, l–o). Viewed ventrally, cells oval with rounded posterior end and anterior part conspicuously narrowed, no apical plate (Fig. 2a, b, g, h, o). Buccal field large, extending to approximately 1/3 of body length, with shallow depression; no conspicuous cavity in posterior end when viewed laterally (Fig. 2e, g, h, k, l). Pellicle smooth, with bar-shaped, very fine, short (2 μm long) extrusomes only visible under high magnification in living cell. Cytoplasm colourless to greyish, often darkened by inclusion of many large granules (Fig. 2a, b, e, g). Macronucleus spherical to ovoid, located centrally, and often appearing as a large hyaline area in living cells (Fig. 2e, f). One contractile vacuole (CV) positioned caudally and slightly toward dorsal side, measuring 6–10 μm across, and pulsating at long intervals (1–2 minutes) (Fig. 2a, arrow; j, double-arrowheads); no CV pore observed after staining with protargol.

Somatic cilia approximately 6 μm long, densely arranged; one caudal cilium measuring approximately 12 μm in length (Fig. 2a). Cilia of buccal apparatus approximately twice as long as somatic cilia, forming sail-shaped profile extending beyond margin of buccal cavity (Fig. 2a, g). Sail-shaped profile sometimes not visible (Fig. 2l, arrow).

Locomotion usually rapid, rotating around longitudinal axis of body. Possibly a true parasite because all cells die within 1–2 hours after being released from eggs of host.

Somatic kineties numbering approximately 40, extending entire length of body, composed of dikinetids (Fig. 2e, f). Somatic kinety 1 extending over oral cavity (Fig. 2d, e). One long kinety forming scutica between first and last kineties, consisting of more than 20 pairs of basal bodies and terminating at ¾ of distance from anterior to posterior of cell (Fig. 2d).

Buccal apparatus typical of genus (Fig. 2c): Membranelle 1 triangular, consisting of 7 rows of basal bodies; membranelle 2 largest, branching deeply in posterior part (Fig. 2c, arrow), containing approximately 20 rows of basal bodies; membranelle 3 short and reduced in comparison to other membranelles, consisting of 3 rows of basal bodies. Paroral membrane terminating at anterior edge of membranelle 3. Silverline system typical of genus (Fig. 2p).

Gene sequence and phylogenetic analyses

The SSU rRNA sequence (GenBank Accession No. FJ648350) of *Philaster apodigitiformis* had a length of 1675 nucleotides and a GC content of 44%. A within-genus sequence divergence value for *Philaster* was 98.7%. The primary structure of the sequence in *Philaster apodigitiformis* showed a similarity of 92.0% and 91.8% to the species in the most closely related clade, *Paranophrys magna* and *Uronema elegans*, respectively.

Phylogenetic trees produced with three methods were congruent (Fig. 1) and showed three main clades within the subclass Scuticociliatia: clade I consisting of the order Loxocephalida (1.00 BI, 53% MP, 97% NJ) and including the genera *Cardiostomatella* and *Dexiotrichides*; clade II consisting of: the order Pleuronematida (1.00 BI, 97% MP, 99% NJ) and including the genera *Schizocalyptra*, *Pleuronema*, *Histiobalantium*, *Eurystomatella* and *Cyclidium*; and clade III consisting of the order Philasterida (1.00 BI) represented by nine families Cohnilembidae, Entorhipidiidae, Orchitorphryidae, Parauronematidae, Philasteridae, Pseudocohnilembidae, Schizocaryidae, Thyrophylacidae and Uronematidae.

All trees showed the order Philasterida (according to Lynn, 2008) as a well-defined, monophyletic group, but relationships between most families of philasterids were still unresolved. *Homalogastra setosa* branched most basally in the philasterids, followed by *Miamiensis avidus* and *Philasterides dicentrarchi*. The positions of the three genera *Anophryoides*, *Mesanophrys* and *Metanophrys* were ambiguous in all trees. An unexpected result was the association of two species of *Parauronema* into different clades (Fig. 1). *Philaster* always clustered with *Paranophrys magna* and *Uronema elegans*, supported strongly by the BI posterior probability (0.92) but poorly by MP and NJ bootstrap values (50%, 50%). Results of AU, KH and SH tests supported the hypothesis represented by the BI tree (Fig. 1) that there is a lack of association between *Philaster* and *Philasterides*. All tests gave significant differences ($P < 0.0001$) when the constraint of the two species of *Philaster* being in the same clade with *Philasterides* was applied, indicating that monophyly of the family Philasteridae was rejected.

Discussion

Systematic position of *Philaster* and related genera

Until now, the phylogenetic position of the family Philasteridae has been uncertain because neither ontogenetic nor molecular data were available for species of *Philaster*. Corliss (1979) placed *Metanophrys*, *Paranophrys*, *Parauronema*, *Philaster* and *Philasterides* into the family Philasteridae on the basis of similar morphology. This concept of the family has been questioned repeatedly with respect to assignment of different genera (Jankowski, 1980, 2007; Puytorac, 1994; Lynn & Small, 2002), with the result that only *Philaster* and *Philasterides* remained assigned to the Philasteridae in Lynn (2008). For the latter, *Philasterides dicentrarchi* is the only species, of which the SSU RNA gene was sequenced (AY642280). The reason for this is that both genera have a similar buccal morphology, i.e. the large size of membranelles 1 and 2 in relation to membranelle 3 (Song, 2000). However, the phylogenetic trees obtained in the present study show that the clade comprising *Philasterides* and *Miamiensis* is basal to and distant from the clade containing *Philaster* (Fig. 1), which suggests strongly that they do not belong to the same family. This

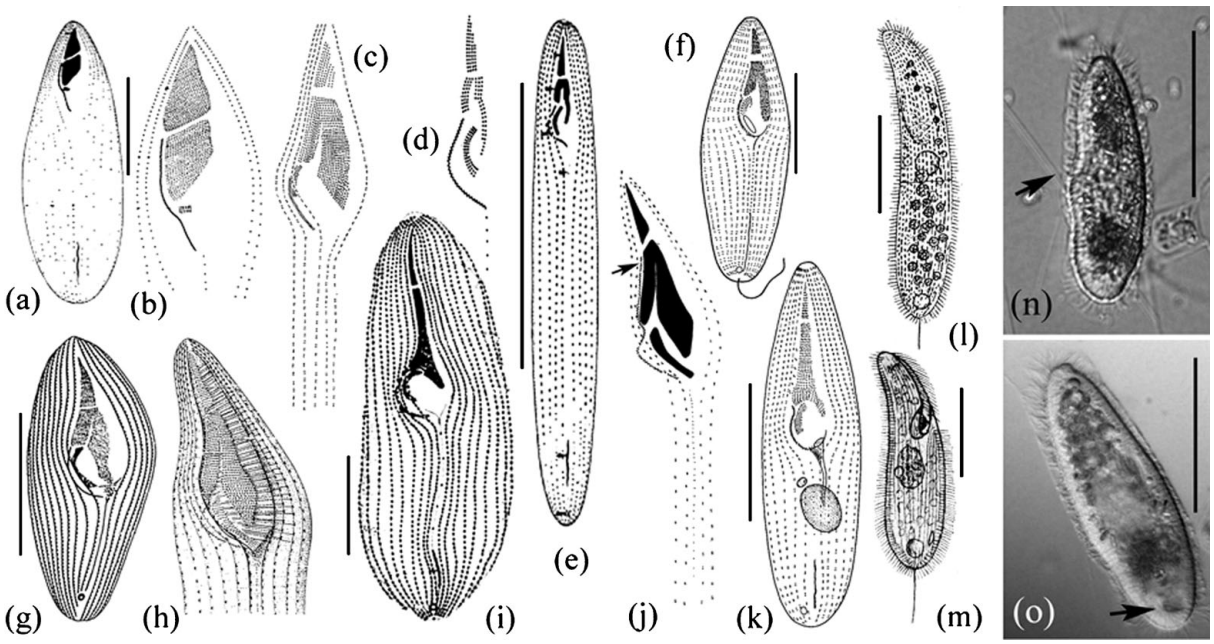

Figure 3 Morphology and infraciliature of *Paraphilaster echini* (a, b from Grolière *et al.*, 1980) and four *Philaster* species: *P. apodigitiformis* sp. nov. (c, from present work); *P. bergeri* (d, e from Grolière *et al.*, 1980); *P. digitiformis* (f, from Small & Lynn, 1985; g, from Mugard, 1948; h, from Puytorac *et al.*, 1966; j, from Grolière, 1974, the PM is possibly misinterpreted, see text; l, from Fabre-Domergue, 1885; m, from Kahl, 1931), *P. hiatti* (i, from Thompson, 1969; k, from Small & Lynn, 1985) (b, c, d, h, j, showing the buccal apparatus), and *Philaster* sp-SZ-07041501 (n, o, from present work; arrow in (n) marks the posterior end of the inconspicuous paroral membrane, while in (o) indicates the contractile vacuole; Ma, macronucleus). Scale bars = 40 μm.

indicates that the similarities in the oral ciliature of *Philaster* and *Philasterides*, traditionally used as the most critical criteria to outline the families and genera (Corliss, 1979; Small & Lynn, 1985; Song, 2000; Lynn & Small, 2002; Jankowski, 2007), are probably convergent features. Another reasonable case is that the identification of the samples was incorrect, that is, the so-called *P. dicentrarchi* might be a species of *Miamiensis* since such organisms are small and difficult to identify even for experienced researchers (Song, 2000). In addition, other families in the order Philasterida, such as the Uronematidae (*Uronema*, *Uronemella*, *Homalogastra*) and Parauronematidae (*Parauronema*, *Miamiensis*), also were shown to be paraphyletic or polyphyletic in our phylogenetic trees (Fig. 1). For the time being, we suggest removing *Philasterides* from it as an *insertae sedis*.

Comparison of *Philaster apodigitiformis* sp. nov. with its congeners

Three species have been reported so far in the genus *Philaster*, *P. digitiformis* Fabre-Domergue, 1885, *P. bergeri* Grolière *et al.*, 1980, and *P. hiatti* Thompson, 1969. *Philaster bergeri* is smaller than *P. apodigitiforms* with a conspicuously slender body shape (Fig. 3e), a much smaller buccal field (only 1/4 length of cell), a 'simplified' buccal apparatus with only several longitudinal rows in M1 and M2 (Fig. 3d), and far fewer somatic kineties (22–23 vs. 34–47 in *P. apodigitiformis*) (Grolière *et al.*, 1980). *Philaster hiatti* is approximately the same size as *P. apodigitiformis* but has an extremely elongate, narrow buccal field with highly developed but slender membranelles 1 and 2 (Thompson, 1969; Small & Lynn, 1985). Other differences between the two species are a lower number of 33 somatic kineties in *P. hiatti* (vs. mean of 40 in *P. apodigitiformis*), free-living lifestyle (vs. parasitic), slender body shape (vs. ovoid shape), and a much less densely ciliated scutica (vs. densely ciliated) (Table 3). In addition, the posterior part of M2 is almost unbranched in *P. hiatti* (vs. conspicuously branched and thus Y-shaped in *P. apodigitiformis*) (Fig. 3i, k).

The well-known form *Philaster digitiformis* has been described repeatedly either from observations of living cells (Fabre-Domergue, 1885; Kahl, 1931) or after silver impregnation (Mugard, 1948; Puytorac *et al.*, 1966; Grolière, 1974; Small & Lynn, 1985). According to both the original description by Fabre-Domergue (1885) and Kahl's (1931) redescription, *P. digitiformis* is characterised by its slender, finger-shaped body, the anterior part of which bends backwards (Fig. 3l, m). As Kahl (1931) emphasised, its buccal groove (buccal field) is narrow and long, with the posterior part conspicuously invaginated. Both authors depicted the buccal cilia as being approximately the same length as somatic cilia, i.e. short and uniform, never forming a sail-like outline (see Fig. 3l, m vs. Fig. 2a, g). All descriptions of *P. digitiformis* depict it with 25–30 somatic kineties and an MI that is narrower and smaller than that of *P. apodigitiformis* (Fig. 3f–h). The paroral membrane of *P. apodigitiformis* appears to have been depicted incorrectly by Grolière (1974) (Fig. 3j, arrow) because the anterior end of the PM always terminates at or near the anterior

Characters	Eurystomatellidae	Cyclidiidae	Pleuronematidae
Paroral membrane	**circular, almost completely closed**	hook-like, on right margin of buccal field	as in Cyclidiidae
Oral membranelles	**all together and close-set,** well-developed; **anteriorly positioned**	non-differentiated, not clearly defined; longitudinally arranged	highly differentiated, all parts clearly separated; longitudinally arranged
Cytostome position	**top of buccal field**	near bottom of buccal field	as in Cyclidiidae
Post-oral cilia-free field	present	absent	present
Pre-oral kineties	absent	absent	present
Anterior/ posterior sutures	absent	absent	present
Scutica	absent	present	absent

Table 3 Morphological comparison of the families Eurystomatellidae, Cyclidiidae and Pleuronematidae (data from the present authors). Unique features of the new family in boldface type.

end of M3 (Grolière *et al.*, 1980; Small & Lynn, 1985). Thus, living cells of *P. apodigitiformis* can be clearly separated from those of *P. digitiformis* by a body that is ovoid and bilaterally flattened vs. slender and finger-shaped with laterally curved anterior, long buccal cilia and a sail-like paroral membrane vs. short and uniform buccal cilia, more somatic kineties (approximately 40 vs. < 30), and parasitic lifestyle vs. free-living.

Another philasterid species, *Paraphilaster echini* (Fig. 3 a, b), also lives as an endocommensal, but can be distinguished from *P. digitiformis* by having a completely different structure of the M2 and M3. Additionally, its paroral membrane terminates approximately at the anterior end of M2 (vs. posterior end of M2 in *P. apodigitiformis*) (Grolière *et al.*, 1980).

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