Phylogenetic consideration of two scuticociliate genera, Philasterides and Boveria (Protozoa, Ciliophora) based on 18 S rRNA gene sequences

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A B S T R A C T
Many scuticociliates are facultative parasites of aquatic organisms and are among the most problematic ciliate taxa regarding their systematic relationships. The main reason is that most species, especially taxa in the order Thigmotrichida have similar morphology and have not been studied yet using molecular methods. In the present work, two scuticociliate genera, represented by two rare parasitic species, Philasterides armatalis (order Philasterida) and Boveria subcylindrica (order Thigmotrichida), were studied, and phylogenetic trees concerning these two genera were constructed based on their 18 S rRNA gene sequences. The results indicate that: 1) Philasterides forms a sister group with Philaster, supporting the classification that these two genera belong to the family Philasteridae; 2) it is confirmed that the nominal species, Philasterides denticranchi Dragesco et al., 1995 should be a junior synonym of Miamiensis avidus as revealed by both previous investigations and the data revealed in the present work; and 3) the poorly known form B. subcylindrica, the only member in the order Thigmotrichida, of which molecular data are available so far, always clusters with Cyclidium glaucoma, a highly specialized scuticociliate, indicating a sister relationship between the orders Thigmotrichida and Pleuronematida.

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1. Introduction
Scuticociliates are found in a variety of habitats with great biodiversity regarding their life styles, structure, behaviour and many other biological characters [1–11]. Although scuticociliates were separated into different groups by different taxonomists [12–15], three scuticociliate orders, namely Philasterida, Pleuronematida and Thigmotrichida, are generally well recognized [14,16].

The genus Philasterides is characterized by a Tetrahymena-like silverline system, the bipartite paroral membrane, and three well-defined oral membranelles, which are arranged in a Paramoophrya-like pattern [17,18]. This genus was originally assigned to the family Philasteridae based on its morphological and morphogenetic features [17–19], but recently transferred to the family Uronematidae according to its stomatogenesis pattern [20]. However, the phylogenetic placement of Philasterides, especially its relationships to other morphologically similar genera, such as Philaster, has never been assessed using molecular data.

The order Thigmotrichida, characterized by a highly differentiated thigmotactic somatic ciliature and a subeuctatorial oral region [16], has received relatively little attention since the monographic works of Chatton and Lwoff [21,22], Fenchel [23], and Raabe [24–28], and no molecular information is available for any species of this order. Phylogenetic analyses based on molecular data (e.g., 18 S rRNA and RFLP) have been successfully used to identify and separate scuticociliate species, however, only few representative pleuronematids, and no thigmotrichid species have been examined by molecular techniques so far [29–35]. Thus, there are still large gaps in our knowledge about these taxa.

As a new contribution, we isolated and sequenced 18 S rRNA gene of Philasterides armatalis, a philasterid species, and of Boveria subcylindrica, a thigmotrichid species. The main goal is to provide molecular evidence for the phylogenetic assignments of the genus Philasterides and the order Thigmotrichida. The present study also provides photomicrographs and a brief redescriptions of the morphology of both species.

2. Materials and methods
2.1. Ciliate collection and identification
B. subcylindrica was isolated from the mantle cavity of the marine mollusk, Pinna pectinata on March 3 rd, 2009 from Qingdao (36° 04’ N; 120° 23’ E), China, by flushing the gills of hosts with seawater. P. armatalis was collected on June 26th, 2008 from the coastal area, the same place as above. Microscopical observations and silver
impregnations are according to Wang et al. [9] and Wilbert [36]. Terminology and systematics are mainly following Lynn [16].

2.2. DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from cells using REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) as described by Yi et al. [37]. The PCR amplifications of 18 S rRNA genes were performed with primers Euk A (5′-AAC CTT GAT CCG CCT ACC AGT-3′) and Euk B (5′-TGA TCC TTC TGC AGC ACC TAC-3′) [38]. PCR amplification, cloning and sequencing were done according to Yi et al. [31].

2.3. Sequence availability and phylogenetic analyses

Besides the newly sequenced 18 S rRNA genes, all other sequences used in the present analysis were obtained from the NCBI GenBank database (Table 1). Three Colpodea species were selected as the outgroup for all analyses. All available 18 S rRNA gene sequences of the subclass Scuticociliatia were included in our phylogenetic analyses. However, Schizocaryum sp. EC13, Cyclidium citrinus and Entorhipidium pilatum were excluded due to their limited sequence length (441 bp, 836 bp and 1376 bp, respectively).

The secondary structure-based 18 S rRNA gene sequence alignment of ciliates downloaded from the European Ribosomal Database [39] was used as the “seed” alignment to build a profile hidden Markov model (HMM) using Hmmer Package version 2.3.2 [40]; the HMM profile obtained was then used to create an alignment of the 56 sequences we assembled using Hmmalign function in Bioedit 7.0.0 [41]. The final alignment of 1704 characters was used to construct phylogenetic trees according to Yi et al. [42]. The program MrModeltest v2 [43] selected the GTR + I + T (0.3430 + G (0.4870) as the best model with AIC criterion, which was then used for both Bayesian inference (BI) and maximum likelihood (ML). The MrBays 3.1.2 program [44] was used for Bayesian analyses, and four simultaneous chains were run for 1,500,000 generations sampling every 100 generations. The first 4000 trees were discarded as burn-in. All trees remaining after the discarding were used to calculate posterior probabilities (PP) using a majority rule consensus. A ML tree was constructed with the PhyML V2.4.4 program [45]. The reliability of internal branches was assessed using a nonparametric bootstrap method with 1000 replicates. A maximum parsimony (MP) tree incorporating 1000 bootstrap replicates was constructed using PAUP* v. 4.0b10 [46] by means of a heuristic search with all characters coded as unordered.

3. Results

3.1. Morphological redescription of P. armatalis Song, 2000 (Fig. 1)

Considering the morphology and infraciliature, the current population corresponds well to the populations described by Song [47] (Fig. 1 I–K) and Hu et al. [19] (Fig. 1 L, M). Therefore, our description concentrates only on those features differing from the previous report.

Cells approximately 60–70 × 20–25 μm in vivo (Fig. 1 A, B), generally thicker than the populations reported previously with asymmetric body shape when viewed from ventral. Cytoplasm mostly full of large globular granules (about 3 μm in diameter), which indicates that the cells might be newly left from the host (Fig. 1 A–G). Always several to many sparsely distributed biconcave crystals detected (Fig. 1 G). Contractile vacuole terminally located at posterior end of cell, formed slowly and then contracted rapidly. Locomotion generally hastily and swimming helically ahead or motionless for a long while while feeding on surface of debris. Infraciliature and buccal apparatus (Fig. 1 H) as described by Song [47] and Hu et al. [19].

3.2. Morphological redescription of B. subcylindrica Stevens, 1901 (Fig. 2)

Our isolation resembles the original redescription very well [24,48], thus only some features are emphasized/documented here. Body usually 40–60 × 20–25 μm in vivo with both ends conspicuously truncated. Cytoplasm colourless and hyaline, often with many to numerous food granules/vacuoles (Fig. 2 A–D). Somatic cilia about 10 μm in length, while cilia in buccal area about 30 μm in length (Fig. 2 A, D, arrow). Contractile vacuole located near the posterior cell end, about 10 μm in diameter (Fig. 2 D, arrowhead). Locomotion by rotating around long axis of body. Isolated cells can be maintained in Petri dish for about 1 h. Infraciliature (Fig. 2 F, K, L) corresponds well with previous reports [24,48]. Number of some kineties (SK) about 27.

3.3. Phylogenetic analyses

The two new 18 S rRNA gene sequences have been deposited in the GenBank database with the length and the accession numbers as follows: P. armatalis, 1758 bp, FJ848877; B. subcylindrica, 1768 bp, FJ848878.

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Table 1

List of the 18 S rRNA nucleotide sequences used in the present work.

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<th>Species</th>
<th>Accession number</th>
<th>Species</th>
<th>Accession number</th>
<th>Species</th>
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* First reported at the name of Philippidens dicentrurchi in Kim et al. [33].
The ML tree (Fig. 3) and the BI/MP trees (Fig. 4) showed a similar topology. Within the class Oligohymenophorea, the subclass Peniculia branched basal, followed by an assemblage including the subclasses Astomatia, Apostomatia, Hymenostomatia, Peritrichia, and the genus Dexiotrichides, and finally the subclass Scuticociliatia (Figs. 3 and 4). The differences between the ML tree and the BI/MP trees are mainly in the phylogenetic positions of some philasterid genera, especially the clade of *Paranophrys magna* + *Uronema elegans* and *Pseudocohnilembus*.

Within the subclass Scuticociliatia, there were four orders: (1) Philasterida was represented by 11 out of 15 families [16] in the present study. All analyses (Figs. 3 and 4) recovered the order Philasterida as a monophyletic group with strong support, whereas the relationships among these families remained unresolved. Of these 11 families, the family Philasteridae appeared to be a well supported monophyletic clade (92% ML, 1.00 BI, 80% MP), in which *P. armatalis* formed a sister group to *Philaster* spp. (2) Pleuronematida included the genera *Schizocalyptra*, *Pleuronema*, *Histiobalantium*, *Eurystomatella* and *Cyclidium*. The genera *Pleuronema* and *Cyclidium* were not monophyletic. Especially species of the genus *Cyclidium* showed great genetic divergences, which placed *C. glaucoma* outside the order Pleuronematida and as a sister taxon to *B. subcylindrica*, instead close to its congeners. However, three *Schizocalyptra* species grouped together with strong support (99% ML, 1.00 BI, 97% MP). (3) Thigmotrichida, represented by *B. subcylindrica*, always grouped with *Cyclidium glaucoma* (100% ML, 1.00 BI, 100% MP). (4) Loxocephalida, whose validity is still under discussion, was polyphyletic with *Paratetrahymena* and *Cardiostomatella* consistently clustering together, and *Dexiotrichides* forming a clade with Apostomatia + Hymenostomatia + Peritrichia.

### 4. Discussion

#### 4.1. Species in Philasterides

Currently, three morphospecies have been reported in the genus *Philasterides*: the type species *Philasterides armatalis*, *P. dicentrarchi* [49] and the recently described *P. armatalis* (Song, 2000). *P. dicentrarchi* is a histophagous parasite of sea bass (*Dicentrarchus labrax*) found by Dragesco et al. [49] in the Mediterranean Sea. Since *Miamiensis avidus* exhibits macro- and microstome stages [50], Song and Wilbert [8] proposed *P. dicentrarchi* as a junior synonym of *M. avidus*, based on their extremely similar morphological character (e.g. body size, buccal apparatus, number of somatic kineties,
position of contractile vacuole pore and life style, etc.). Jung et al. [51] also concluded that *M. avidus* and *P. dicentrarchi* are synonymous, based not only on morphological but also on 18 S rRNA gene sequence data. In the present work, we have sequenced the 18 S rRNA gene sequence of *P. armatalis*, and detected 95 site differences between *P. armatalis* and *P. dicentrarchi* (sequence identity, 94.5%), while only 3 site differences were detected between *P. dicentrarchi* and *M. avidus* (AY550080) (sequence identity, 99.8%). Our phylogenetic trees inferred from the 18 S rRNA gene sequences also indicate that *P. dicentrarchi* (AY642280) and *M. avidus* (AY550080) always cluster in a fully supported (100% ML, 1.00 BI, 100% MP) clade divergent from the newly sequenced *P. armatalis* (Figs. 3 and 4). Therefore, we confirm that *P. dicentrarchi* is a junior synonym of *M. avidus* and that there are only two species in this genus, *P. armata* and *P. armatalis*. Meanwhile, the name of *P. dicentrarchi* has been corrected as *M. avidus* in GenBank by its submitter (personal communication with Sung Mi Kim).

### 4.2. Systematic position of Philasterides and related genera

The assignment of different genera to the families within the Scuticociliatia has been generally controversial, because no widely acceptable criteria have been presented for them [12,14–16,20]. Based on patterns of the stomatogenetic process, the data of apomorphic–plesiomorphic characters, as well as the morphological features, Ma et al. [20] assigned *Philaster* and *Philasterides* to two different families, the Philasteridae and the Uronematidae, respectively. However, based on their similar morphology, especially the “similar” structure of the buccal apparatus (e.g. the dominant membranelles 1 and 2), these two genera were placed in the same family Philasteridae by some other investigators [12,14–16]. Notably, misled by the wrong species name of AY642280 (first named as *Philasterides dicentrarchi*, and recently corrected to *M. avidus*) in GenBank, Miao et al. [35] concluded that *Philaster* and *Philasterides* were conspicuously separated in the topology, and suggested removing *Philasterides* from the family Philasteridae. By adding the sequence of *P. armatalis*, a typical species of this genus, our phylogenetic trees indicate that *Philasterides* and *Philaster* group together in a well supported clade with strong support (1.00 BI, 93% ML, 80% MP) (Figs. 3 and 4), which suggests that they have a reliable close relationship. Therefore, we suggest retaining both *Philasterides* and *Philaster* in the family Philasteridae.

### 4.3. Systematic position of Boveria and the order Thigmotrichida

According to Lynn [16], the order Thigmotrichida includes six families: Ancistridae, Hemispeiridae, Hysterocinetidae, Protanoplophryidae,
Paraptychostomidae, and Nucleocorbulidae. B. subcylindrica, the only species with a 18 S rRNA gene sequence being sequenced (in the present work), belongs to the family Hemispeiridae. Based on the morphology, especially the presence of a 'scutica-field' [48,52], Thigmotrichida should undoubtedly be assigned to the Scuticociliatia. However, their systematic position remains uncertain because neither ontogenetic nor molecular data are available so far.

In our phylogenetic trees, B. subcylindrica clusters with C. glaucoma, a well-known form in the order Pleurostomatida, which is acceptable considering the morphological features: both have the extremely long paroral membrane (PM) which surrounds the whole buccal field and simple membranelles (Fig. 2K–M). The major difference is that the buccal field in B. subcylindrica moves to the posterior end of the cell with clearly differentiated three membranelles (Fig. 2K, L) ([vs. the buccal field in Cyclidium/C. glaucoma is on the ventral cell side with membranelles more or less reduced or incompletely differentiated, Fig. 2M]. We suppose that the morphological divergence might be explained by their different ways of living (parasitic vs. free living). Therefore, the order Thigmotrichida, represented by Boveria, might have a sister relationship with Cyclidium-related pleuronematids, which is consistent with the morphological data.

Unexpectedly, the three Cyclidium species do not cluster together, and C. porcatum even branches outside the order Pleuronematida, which is inconsistent with their morphological similarity (the site changes detected among these three species are 222–275 bp, and the sequence identities among them are 84.0%–87.2%). Moreover, the present phylogenetic trees (Figs. 3 and 4) depicted C. glaucoma and C. porcatum as very divergent from other pleuronematids. There are possibly two reasons: 1) considering that misidentification of Cyclidium species is a potential problem [47,53], their 18 S rRNA gene sequences are very possibly in question; and 2) the genus Cyclidium itself is not a monophyletic assemblage, possibly based on a fast evolutionary divergence rate of these Cyclidium-like species, and the topology shown in the present work exhibits only the gene trees based on few species (under sampling). Thus further studies are needed.

Since only a few representative pleuronematid and one thigmotrichid sequences are available so far, it is too early to draw a final conclusion about the exact relationship among them. However, we are confident that, based on both morphological and molecular data, the order Thigmotrichida falls into the subclass Scuticociliatia, and has a close genetic relationship with the order Pleuronematida.

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Fig. 4. Bayesian tree inferred from 18 S rRNA gene sequences. Numbers at nodes represent posterior probability of Bayesian analysis and the bootstrap percentages for maximum parsimony (1000 replicates). Hyphen (-) indicates disagreement between Bayesian and MP *.

References


