Phylogenetic analysis and taxonomic distinction of six genera of pathogenic scuticociliates (Protozoa, Ciliophora) inferred from small-subunit rRNA gene sequences

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Pathogenic scuticociliates, which are common in the haemolymph or tissues of maricultured animals and often cause serious diseases, are a species-rich assemblage with mostly unresolved systematic relationships, especially in some less-studied groups. In the present study, we sequenced the small-subunit rRNA gene of six species of scuticociliates, Uronemella parafilificum, Metanophrys sinensis, Parauronema longum, Cohnilembus verminus, Porpostoma notata and Ancistrum crassum, the last two of which have not been studied previously using molecular analyses. Phylogenetic trees were constructed using Bayesian inference, maximum-likelihood and maximum-parsimony methods to assess the inter- and intra-generic relationships of scuticociliates. Results revealed the following: 1) Porpostoma did not cluster with the Philasteridae, Cohnilembidae or any other family of the order Philasterida; 2) sequences of Uronemella parafilificum and Uronemella filificum showed a difference of 1.02 % (15 nt sites), revealing a close relationship between them; 3) the approximately unbiased test rejected monophyly of both Metanophrys and Parauronema, indicating that the terminal position of the anterior end of the paroral membrane and the structure of membranelle 1 are unreliable characters for distinction of genera in this group of scuticociliates; 4) Ancistrum crassum grouped with Boveria subcylindrica, showing a close phylogenetic relationship between the orders Thigmotrichida and Pleuronematida; and 5) Parauronema longum, Cyclidium plouneouri and Cyclidium porcatum should be removed from their currently assigned genera.

INTRODUCTION

Scuticociliates are ciliated protists that are either free living, found in all aquatic habitats, or parasitic to aquatic animals. They are generally small in size and show similar features in vivo as well as in the basic pattern of silverline system and infraciliature (Fan et al., 2010; Small, 1967; Song et al., 2003). Hundreds of scuticociliate species have been reported since the 1960s, using silver impregnation methods, and many new taxa have been described (Evans & Thompson, 1964; Fan et al., 2009; Foissner et al., 2009; Pan et al., 2010; Small, 1967; Song, 2000; Wang et al., 2009). The scuticociliates were first established as an order by Small (1967) based on stomatogenetic data and were subsequently elevated to subclass rank (Lynn, 2008; Lynn & Small, 1997, 2002; de Puytorac, 1994). In his newly revised classification system, Lynn (2008) recognized three scuticociliate orders: Philasterida, Pleuronematida and Thigmotrichida. Considering the high number of scuticociliate morpho-species that have been described to date, the number of available small-subunit (SSU) rRNA gene sequences is low. Currently, the SSU rRNA gene sequences of only two dozen philasterid and one thigmotrichid species are available. Consequently, the relationships among most families of the Philasterida remain unresolved (Foissner et al., 2009; Gao et al., 2010; Lynn & Strüder-Kypke, 2002; Miao et al., 2008, 2009; Yi et al., 2009). Therefore, more taxa are needed in order to achieve a better understanding.
and interpretation of the phylogenetic relationships of these organisms.

In the present investigation, we isolated and sequenced the SSU rRNA genes of five philasterid species (Porpostoma notata, Uronemella parafilificum, Metanophrys sinensis, Parauronema longum and Cohnilenbus verminus) and one thigmotrichid species (Ancistrum crassum). We also performed silver impregnation techniques and obtained data for analyses of morphological features. We combined and analysed molecular as well as morphological data and were able to gain a better understanding of the relationships among scuticociliates within the class Oligohymenophorea.

METHODS

Ciliate collection and identification. Porpostoma notata was isolated from the dead bodies of crab larvae in a mariculture pond in Weifang (37° 07’ N 119° 49’ E), north China. Sediments containing water and dead bodies were taken directly after draining the pond. Uronemella parafilificum was collected from a small shallow water puddle in a dry aqueduct near mariculture ponds. Metanophrys sinensis was isolated from mariculture water from Weifang (37° 07’ N 119° 49’ E) and cultured in sample water with a dead flounder larva (Paralichthys sp.) to enhance bacterial growth. Cohnilenbus verminus was detected in the water sample of the mariculture pond about a week after rice grains were added. Parauronema longum was isolated from coastal waters in a harbour of Qingdao (36° 08’ N 120° 31’ E), China. Glass slides were fixed to a frame to serve as artificial substrates immersed in water and left for about 10 days to allow for colonization. Ancistrum crassum was isolated from the mantle cavity of the marine bivalve Rudinapes philippinarum, bought from a market in Qingdao in March, 2009, by flushing the gills with seawater. Microscopic observations and silver impregnations were performed according to Wilbert (1975). Terminology and systematics mainly follow Lynn (2008) except for the order Loxocephalid (vs Loxocephalidae).

DNA extraction, PCR amplification and sequencing. Total genomic DNA was extracted from cells using the REDExtract-N-Amp Tissue PCR kit (Sigma) as described by Zhang et al. (2010). PCR amplifications of SSU rRNA genes were performed with the universal primers Euk A (5’T-AACCTGTTGATCCGTCCGAGT-3’) and Euk B (5’T-GATCTTTCTGCCGTTCCACTAC-3’) (Medlin et al., 1988). Cycling parameters for PCR amplification were as follows: 1 cycle (94 °C, 5 min); 30 cycles (94 °C, 1 min; 62–50 °C touch down, 1 min; 72 °C, 1 min 30 s); 1 cycle (72 °C, 10 min). Purified PCR products of the appropriate size were inserted into the pUCm-T vector (Shanghai Sangon Biological Engineering & Technical Service Company) and sequenced at the Invitrogen sequencing facility in Shanghai, China.

Sequence availability and phylogenetic analyses. With the exception of the newly sequenced SSU rRNA gene, the rest of the sequences used in the study were obtained from the NCBI/GenBank. All available SSU rRNA gene sequences of the subclass Scuticociliata were included in our phylogenetic analyses. However, Schizocaryum sp. EC13, Cyclidium citrinum and Eintorhipidium pilatum were excluded due to their short sequence length (441, 836 and 1376 bp, respectively). Three Colpodea species were selected as the outgroup for all analyses.

The secondary structure-based SSU rRNA gene sequence alignment of ciliates downloaded from the European Ribosomal Database (Wyts et al., 2002) was used as the ‘seed’ alignment to build a profile hidden Markov model (HMM) using HMMER package version 2.3.2 (Eddy, 2001). The resulting HMM profile was then used to create an alignment of the 65 sequences using HMMLIGN within the package. Regions that could not be aligned unambiguously were masked using BioEdit 7.0.0 (Hall, 1999). The final alignment of 1650 characters was used to construct phylogenetic trees according to Yi et al. (2009). The program MrModeltest v.2 (Nylander, 2004) selected the GTR+I+G model as the most suitable model according to the AIC criterion for Bayesian inference (BI). The software MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) was used for Bayesian analyses and four simultaneous chains were run for 4600000 generations, sampling every 100th generation. The first 12000 trees were discarded as burn-in. All remaining trees were used to calculate posterior probabilities using a majority rule consensus. A maximum-likelihood (ML) tree was constructed with the program PhyML V2.4.4 (Guindon & Gascuel, 2003) using the best model according to the AIC criterion selected by the program Modeltest v.3.4 (Posada & Crandall, 1998). The reliability of internal branches was assessed using non-parametric bootstraping with 1000 replicates. A maximum-parsimony (MP) tree incorporating 1000 bootstrap replicates was constructed using PAUP* v.4.0b10 (Swofford, 2002) using a heuristic search with all characters coded as unordered. Phylogenetic trees were visualized with TreeView v.1.6.6 (Page, 1996) and MEGA4 (Tamura et al., 2007).

To test the monophyly of the focal group against competing phylogenetic hypotheses, the approximately unbiased (AU) test was used (Shimodaira, 2002). ML trees were generated with a constraint block, enforcing the monophyletic constraint of the respective focus group in PAUP (Swofford, 2002), under a GTR+I+G model, which was identical to the one used when estimating the global ML tree. The three best-scoring trees (i.e. with lowest – ln likelihood score) that met the constraint of each alternative hypothesis were used for comparison (Table 1). The site-wise likelihoods were calculated for each tree topology under the same model (GTR+I+G) with other parameters estimated by the PAUPUP (Calendini & Martin, 2005) interface relying on PAUP* DOS version and were then subjected to the AU test (Shimodaira, 2002) as implemented in Consel (Shimodaira & Hasegawa, 2001).

RESULTS

Sequence information

Lengths and G + C contents of the six SSU rRNA gene sequences determined in this study were: Porpostoma notata, 1755 bp, 44.27 mol%; Metanophrys sinensis, 1754 bp, 44.13 mol%; Uriennema parafilificum, 1756 bp, 42.65 mol%; Parauronema longum, 1759 bp, 44.80 mol%; Cohnilenbus verminus, 1759 bp, 44.74 mol%; and Ancistrum crassum, 1753 bp, 44.10 mol%.

Phylogenetic analyses

Topologies of the BI and ML trees were similar so the two trees were combined (Fig. 1). With the exception of Cyclidium poratum and Cyclidium glaucoma, the two orders Thigmotrichida and Pleuronematida were monophyletic and had a strongly supported sister relationship (1.00 BI, 99% ML). The order Philasterida was a well-defined monophyletic group (1.00 BI, 59% ML); however, the relationships among its families remained unresolved. Topology of the MP tree (Fig. 2) differed from that of the BI/ML tree (Fig. 1). Porpostoma notata and the clade
Table 1. Results of AU tests comparing trees that are representative of alternative hypotheses about the phylogenetic associations of groups of interest in this study

<table>
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<th>AU</th>
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Fig. 1. Bayesian tree inferred from SSU rRNA gene sequences. Numbers at nodes represent the posterior probability of Bayesian analysis and the bootstrap values of maximum-likelihood out of 1000 replicates. Asterisks (*) indicate disagreement between Bayesian and ML. Bar, 5 substitutions per 100 nt positions. Newly sequenced species are shown in bold.
formed by *Metanophrys sinensis* and *Paranophrys magna* branched differently. *Porpostoma notata* formed a polytomy with four other clades of philasterids in the BI/ML analyses, but the topology was poorly supported (0.60 BI, 12 % ML); similarly, its grouping with *Schizocaryum dogieli* in the MP analysis was supported only weakly (29 % MP). *Uronemella parafillicum* and *Uronemella filificum* formed a fully supported clade (1.00 BI, 100 % ML, 100 % MP) that was basal to the clade formed by *Parauronema virginianum, Entodiscus* and *Uronema*. *Metanophrys sinensis* was distant from *Metanophrys similis*, but grouped with *Paranophrys magna* with full support (1.00 BI, 100 % ML, 100 % MP). Both then clustered with *Pseudocohnilembus* species (0.62 BI) or Philasterides and *Philaster* in MP analysis (50 % MP). The newly sequenced population of *Parauronema longum* formed a fully supported clade (1.00 BI, 100 % ML, 100 % MP) with a previously determined sequence of *Parauronema longum* (accession no. AY212807), being basal to the clade of *Entorhipidium, Thyrophylax* and *Plagiopyliella*. The two *Cohnilembus vermis* individuals grouped together (1.00 BI, 100 % ML, 100 % MP), forming a distinct clade within the Philasterida. Only four nucleotide differences were detected (sequence divergence 0.23 %) between these two sequences despite the large geographical distance between collection sites (China vs UK) and different sampling times (2009 vs 1993). The two thigmotrichid species *Ancistrum crassum* and *Boveria subcylindrica* formed a branch with strong support (1.00 BI, 100 % ML, 99 % MP), which then grouped with *Cyclidium glaucoma*. The semi-conserved, parsimony-informative regions of the SSU rRNA gene sequence alignment of the focal group were also created by BioEdit 7.0.0 and MEGA4. The signature nucleotides in a focal group are shaded in Figs 3 and 4.

**DISCUSSION**

The subclass Scuticociliatia comprises over 100 genera. It is a very problematic group because morphological and morphogenetic characteristics do not necessarily correspond to the actual genetic diversity that we observe (Lynn, 2008; Lynn & Strüder-Kypke, 2005; Ma et al., 2005). Similarly to previous investigations, we were unable to clearly assign genera, especially within the philasterids, into single families (Figs 1 and 2) (Gao et al., 2010; Miao et al., 2008, 2009; Yi et al., 2009). In our discussion, we will
therefore focus on relationships at the intra- and inter-generic level rather than on classification at the family level.

**Phylogeny of Porpostoma (Fig. 5f, g, t, u, v)**

To date, only two species of the genus Porpostoma have been described, *Porpostoma grassei* and the type species *Porpostoma notata* (Petz et al., 1995; Song & Wilbert, 2000a; Song, 2000). However, due to the unique character of multiple (up to 18) polykinetal segments in the M1 at some stages in the life cycle (Corliss, 1979), the histophagous *Porpostoma* has always been considered as a taxonomically curious organism. Corliss (1979) considered *Porpostoma* as incertae sedis in the family Philasteridae, Lynn & Small (2002) placed it in the family Cohnilembidae and Lynn (2008) transferred it to the family Philasteridae. All of the above-mentioned taxonomic placements were based solely on morphological features. However, the phylogenetic position of *Porpostoma* has not been studied previously.

In our SSU rRNA gene tree (Fig. 1), *Porpostoma* clustered neither with the Philasteridae nor with the Cohnilembidae. Generally, its placement among the scuticociliates was ambiguous and not well supported. Furthermore, analysis of the semi-conserved, parsimony-informative regions of the alignment of SSU rRNA gene sequences also revealed *Porpostoma* to be distinct from both the Philasteridae and Cohnilembidae (Fig. 3b). Considering the morphological characters, there are distinct differences between the groups:

1) ciliates of the family Philasteridae have three typical adoral membranelles (M1–M3), with M1 being triangular and equal in size or smaller than M2 (Lynn, 2008);
2) *Cohnilembus* species usually have slender fusiform or lancet-like body shape and M1 is quite long and single-rowed, forming a conspicuous, false 'double-membrane' with the

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**Fig. 3.** Regions of alignment of SSU rRNA gene sequences of *Parauronema* (a) and *Metanophrys* (b) with related species. Regions are separated by vertical lines and the reference sequence, and first and last nucleotide positions of each region are given at the top. Insertions and deletions were compensated by introducing alignment gaps (–). Matched sites are represented by dots (·). Distinct sequence signatures of each clade are shaded.
anterior dense dikinetids of the somatic kinety n (Fig. 5a, h–j) (Lynn, 2008; Song et al., 2009); 3) Porpostoma has many somatic kineties (up to 50) with densely arranged cilia and a prominent M1, which is transversely subdivided into ten or more segments (Song, 2000; Song et al., 2009). Based on molecular and morphological data, it is probably not justifiable to place Porpostoma in either the Philasteridae or the Cohnilembidae.

In our phylogenetic trees (Figs 1 and 2), Porpostoma associated with Schizocaryum in all analysis but with only poor to moderate support (0.60 BI, 12 % ML, 29 % MP). There are 15 unique nucleotide identities between Porpostoma and Schizocaryum in the alignment of SSU rRNA gene sequences (e.g. Fig. 3b); however, Schizocaryum has some morphological features (e.g. a ciliated vestibulum) unlike those of any other known scuticociliates, including Porpostoma (Berger, 1961; Lynn & Strüder-Kypke, 2002), prompting Lynn & Strüder-Kypke (2002) to assign the genus and its family to the order Philasterida based solely on the information from SSU rRNA gene sequences. Considering the poor support for the association between Porpostoma and Schizocaryum, placing the former either in the Schizocaryidae or in a new family of its own would certainly be premature, especially in light of the polyphyly of some other scuticociliate families (e.g. Orchitophryidae). More data, especially sequences of additional related taxa, are needed to provide an adequate resolution of the taxonomic placement of the two genera.

### Phylogeny of Uronemella (Fig. 5b, r, s)

**The genus Uronemella was proposed by Song & Wilbert (2002) for Uronemella filificum on the basis of the following genus-specific characteristics: thigmotactic uronematid with pear-shaped body and subequatorially positioned cytostome; dominant apical plate; and a Uronema-like oral apparatus. Based on these morphological features, Uronemella was assigned to the family Uronematidae (Lynn, 2008; Song & Wilbert, 2002). The validity of Uronemella as a genus has been supported by the SSU rRNA gene trees, as well as by the predicted secondary structures of the V4 region of SSU rRNA gene sequences (Yi et al., 2009).**
Uronemella parafilificum was found by Gong et al. (2007) in the top layer of sediment of a muddy site in the Ganghwa tidal flat in Incheon, South Korea. Gong et al. (2007) split it from the Uronemella filificum-complex as it possesses fewer somatic kineties (16–19 vs 21–24) and four extremely long posterior cilia. Our individuals corresponded well with the description of Gong et al. (2007). In particular, some individuals had only one posterior cilium. As explained by Gong et al. (2007), there is only one caudal cilium complex in protargol-impregnated specimens, indicating only one true caudal cilium and the other three ‘extra’ long cilia may either be artefacts or arise from normal somatic basal bodies. It is probable that the individuals with only one posterior cilium lose their three ‘extra’ long cilia but the reason for this remains unknown.

Uronemella parafilificum and Uronemella filificum grouped together in the phylogenetic analyses, with a genetic divergence of 1.02% (15 nt sites). The primary structure of the aligned SSU rRNA gene sequences revealed several distinct sequence signatures for both species, but also some differences (Figs 1, 2 and 3a). Based on molecular data, our study shows the close relationship between Uronemella parafilificum and Uronemella filificum. However, more molecular work should be done on the Uronemella complex to identify sequence similarities and also the evolutionary rate of the SSU rRNA gene at the species and/or population level.

Moreover, phylogenetic trees revealed that the genus Uronema is polyphyletic: Uronema marinum clustered with the clade formed by Parauronema virginianum and Entodiscus, whereas the other two species of Uronema formed a separate clade with considerable genetic divergence (6.07%). This genetic divergence was also recognizable in the primary structure of the aligned SSU rRNA gene sequences (Fig. 3a). The genus Uronemella was basal to the clade formed by Parauronema virginianum, Entodiscus and Uronema. Thus, we have shown that the family Uronematidae is not monophyletic based on SSU rRNA gene sequences. More broad and detailed multi-gene phylogenetic analyses of Uronematidae will be discussed in later work.

**Phylogeny of Metanophrys (Fig. 5e, k, l)**

The genus Metanophrys was established about three decades ago (de Puytorac et al., 1974) and the generic definition was revised recently (Song & Wilbert, 2000b). Morphologically, Metanophrys is similar to Paranophrys and Mesanophrys. The main character used to distinguish these three genera is the terminal position of the anterior end of the paroral membrane, extending anteriorly to the middle of M2 (Metanophrys), the anterior end of M2 (Paranophrys) or the posterior end of M2 (Mesanophrys) (Song et al., 2009).

Up to now, only one SSU rRNA gene sequence has been available for the genus Metanophrys, namely that of...
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Metanophrys similis (Shang et al., 2006). Phylogenetic analyses indicated that *Metanophrys similis* was closely related to *Mesanophrys carcini* and *Anophryoides haemophilia*. In the present study, we isolated *Metanophrys sinensis* (Fig. 5e) and sequenced its SSU rRNA gene. Interestingly, the SSU rRNA gene sequence of *Metanophrys sinensis* had 150 nt site changes compared to that of *Metanophrys similis* (sequence divergence of 8.50%), whereas it differed in only 53 positions from the sequence of *Paranophrys magna* (sequence divergence of 3.01%). Furthermore, *Metanophrys sinensis* and *Paranophrys magna* shared more than 50 semi-conserved, parsimony-informative sites between their SSU rRNA gene sequences. In comparison, only 17 sites were identical between *Metanophrys similis* and *Metanophrys sinensis* (e.g. Fig. 3b). Based on these data, the monophyly of *Metanophrys* was evaluated using AU tests. Results showed that the monophyly of *Metanophrys* was rejected with this dataset (AU<0.001). Hence, the terminal position of the anterior end of the paroral membrane should not be used as a key character for defining genera within this clade.

According to Lynn (2008), *Metanophrys* was placed in the family Orchitophryidae along with *Mesanophrys, Anophryoides* and *Paranophrys*. However, our phylogenetic trees revealed that the four genera did not cluster together, but were distributed over three clades: 1) *Metanophrys sinensis* and *Paranophrys magna*; 2) *Mesanophrys carcini* and *Metanophrys similis*; and 3) *Anophryoides haemophilia* with *Miambiensis avidus*. In addition, the primary structure of the SSU rRNA gene sequence alignment did not show any family-specific sequence signatures that would support the monophyly of Orchitophryidae. However, considering that only a couple of *Metanophrys* species were sequenced, it would not be appropriate to revise the genus at present. Sequences from more taxa, especially type species of genera, should be added to draw clear conclusions.

**Phylogeny of Parauronema (Fig. 5c, p, q)**

The genus *Parauronema* was first reported by Thompson (1967) and comprises three species: *Parauronema virginianum* Thompson 1967, *Parauronema acutum* Small and Lynn 1985, and *Parauronema longum* Song 1995. *Parauronema longum* differs from *Parauronema virginianum* and *Parauronema acutum* mainly in body shape, the large apical plate and the higher number of somatic kineties (Song & Wilbert, 2000b). *Parauronema virginianum* has a very similar morphology to *Uronema marinus*, the only exception being a two-rowed M1, which means that *Parauronema virginianum* is morphologically closer to *Uronema marinus* than to its congener, *Parauronema longum*. Lynn (2008) placed *Parauronema* in the family Parauronematidae with *Glaucophora, Miambiensis* and *Potomacus*, whereas Corliss (1979) placed it in the family Philasteridae. However, based on our analysis (Figs 2 and 3), *Parauronema* is phylogenetically distant from the family Philasteridae and thus from *Miambiensis.

*Parauronema longum* from the current study (Fig. 5c) was very similar morphologically to the original description. In addition, only four nucleotide substitutions (sequence divergence of 0.23%) were detected between the SSU rRNA gene sequences of the two *Parauronema longum* isolates (accession nos HM236338 and AY212807). Finally, each of the two *Parauronema* species shared more semi-conserved, parsimony-informative sites with members of the other scuticociliate families than with each other (e.g. Fig. 3a). These relationships were also reflected in the phylogenetic analyses. Specifically, *Parauronema longum* had more sites in common and thus grouped with *Entorhizidium, Thyrophylax* and *Plagiopyliella*, whereas *Parauronema virginianum* clustered with the *Uronema* clade. The hypothesis that all *Parauronema* species clustered together was rejected by the AU test (AU<0.001), thus supporting the polyphyly of the genus. The results were congruent with the information obtained from the morphological studies mentioned above. Therefore, the structure of M1 should not be used as a diagnostic character for *Parauronema* or *Uronema*.

Since *Parauronema virginianum* is the type species of the genus *Parauronema*, we propose that *P. longum* be removed from the genus *Parauronema*, based on both morphological and molecular characters. We recommend that *Parauronema longum* be assigned as *incertae sedis* within the clade containing the families *Entorhizidiiidae* and *Thyrophylacidae*. Relationships within this clade are difficult to resolve and, until more taxa are added, we feel that it would be premature to create a new monotypic genus for *Parauronema longum* based on molecular data alone.

**Phylogeny of Ancistrum (Fig. 5d, m, n, o) and related genera**

The genus *Ancistrum* was created by Maupas (1883) to include a species that was described inaccurately by Quennerstedt (1867) and then transferred improperly to the astome genus *Anophrya* by Kent (1882). *Ancistrum* was assigned to the family Ancistridae Issel 1903 of the order Thigmotrichida. The order includes ciliates inhabiting the mantle cavity of marine bivalve molluscs (Corliss, 1979; Lynn, 2008). As the least-specialized genus of the order Thigmotrichida, *Ancistrum* has the main morphological characters of scuticociliates, such as membranelles M1–M3, paroral membrane, scutic and caudal cilium. The silverline system is very similar to that of *Cyclidium*, and the infraciliature (especially the oral apparatus) is similar to that of *Pleuronema* (Song, 2000; Xu & Song, 2000).

In this study, we isolated *Ancistrum crassum* (Fig. 5d), which corresponded well to previous descriptions (Song, 2000; Song et al., 2003; Xu et al., 1997). The SSU rRNA gene was sequenced for the first time in this species and sequence divergence from *Boveria subcylindrica*, the only other thigmotrich ciliate that has been sequenced, was
4.24%. As the phylogenetic trees showed, Ancistrum crassum clustered with Boveria subcylindrica with strong support (1.00 BI, 100% ML, 99% MP), and both grouped with the pleuronematids. In addition, numerous thigmotrich-specific nucleotide signatures were observed in the SSU rRNA gene sequence alignment (e.g. Fig. 4).

The positions of the three Cyclidium species in our phylogenetic trees were markedly dispersed (Figs 1 and 2); Cyclidium porcatum branched with the Loxocephalida in the BI/ML analyses, Cyclidium glaucoma clustered with Ancistrum and Boveria with full support, and Cyclidium plouneouri grouped with the core pleuronematids (Figs 1 and 2). In the primary structure of the SSU rRNA sequence alignment, Cyclidium plouneouri, Eurytomatella and Wilbertia shared 60 signature nucleotides, Cyclidium glaucoma, Ancistrum and Boveria shared 30 signature nucleotides, and Cyclidium porcatum and the Loxocephalida shared 28 signature nucleotides. Thus, based on the information of the existing SSU rRNA gene sequences, we propose that: 1) the genus Cyclidium, represented by Cyclidium glaucoma, should be transferred to the Thigmotrichida; 2) Cyclidium plouneouri should be removed from the genus Cyclidium and instead be assigned to the Pleuronematida as an incertae sedis; and 3) Cyclidium porcatum should be taken out of the genus Cyclidium and be placed as an incertae sedis in the Loxocephalida.

**Conclusion**

Scuticociliates are an extremely divergent group and our study shows that there are major discrepancies between their morphological and molecular diversity. Assignments to genera and families based solely on morphological features do not correspond to those based on the phylogeny inferred from SSU rRNA gene sequence data. The first reason is that the current taxonomic system is based mainly on morphology, especially the structure of buccal ciliature. However, there are far too few distinguishable morphological features among scuticociliates and almost no intermediate forms among them regarding the buccal structures (Fan et al., 2009, 2010; Foissner et al., 2009; Pan et al., 2010; Wang et al., 2009). The second reason is that most species descriptions lack ontogenetic data and the existing ones show great resemblance (Hu et al., 2008; Ma et al., 2001, 2002, 2005, 2006; Morado & Small, 1994). Therefore, stomatogenesis does not seem to be a useful criterion for scuticociliate classification at the present time. The third reason is that SSU rRNA gene sequences are available for only a few species and, thus, undersampling as well as long branch attraction artefacts may be masking true phylogenetic relationships. Due to the small amount of molecular data, it is premature to draw final conclusions about the relationships among scuticociliate taxa or to evaluate the evolutionary relationships among the group. More taxa sampling and more gene information needs to be added before a thorough revision of the subclass is possible.

**ACKNOWLEDGEMENTS**

This work was supported by the Natural Science Foundation of China (project no. 41006098) and King Saud University. We are grateful for the thoughtful comments and advice of Dr John Clamp, Dr Eleni Gentekaki, the anonymous reviewer and the Associate Editor. Many thanks are also due to Mr Xinpeng Fan and Ms Jie Huang for their kind help with species identification and experiment operation.

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