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An Expanded Phylogeny of the Entodiniomorphida (Ciliophora: Litostomatea)

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Summary. The Entodiniomorphida are a diverse and morphologically complex group of ciliates which are symbiotic within the digestive tracts of herbivorous mammals. Previous phylogenies of the group have exclusively considered members of one family, the Ophryoscolecidae, which are symbiotic within ruminants. We sought to improve understanding of evolution within the entodiniomorphs by expanding the range of ciliates examined to include the Cycloposthiidae and Macropodiniidae (symbionts of equids and macropodids respectively). The entire SSU-rRNA gene was sequenced for 3 species, *Cycloposthium edentatum*, *Macropodinium ennuensis* and *M. yalanbense*, and aligned against 14 litostome species and 2 postciliodesmatophoran outgroup species. *Cycloposthium* was consistently grouped as the sister-taxon to the Ophryoscolecidae although support for this relationship was low. This suggests that there is more evolutionary distance between the Cycloposthiidae and Ophryoscolecidae than previously inferred from studies of gross morphology, cell ontogeny or ultrastructure. In contrast, *Macropodinium* did not group with any of the entodiniomorphs, instead forming the sister group to the entire Trichostomatia (Entodiniomorphida + Vestibuliferida). This early diverging position for the macropodiniids is concordant with their morphology and ontogeny which failed to group the family with any of the entodiniomorph suborders. The currently accepted classification of the Trichostomatia is thus deficient and in need of review.

Key words: Ciliophora, Cycloposthiidae, Entodiniomorphida, evolution, Macropodiniidae, ribosomal RNA genes, Trichostomatia.

INTRODUCTION

The Entodiniomorphida are endosymbiotic ciliates which inhabit the fermentative digestive organs of most mammalian herbivores (Williams and Coleman 1991). Currently 3 suborders with the Entodiniomorphida are recognised: the Archistomatia, Blepharocorythina and Entodiniomorphina (Lynn and Small 1997). The archistomes include a single family, the Buetschliidae,

which are characterised by the possession of simple conical vestibulum, a holotrichous covering of longitudinal somatic kineties and fully developed concrement vacuoles (Wolska 1964). The blepharocorythines are also monofamilial (Blepharocorythiidae) and possess a complicated oral apparatus consisting of a conical vestibulum, a dorsal overture, an external adoral ciliary band and triangular vestibular ciliary band (Wolska 1971). The Entodiniomorphina are the most diverse group characterised by reduced somatic ciliation, forming tufts or bands, semi-rigid pellicle covering extensive non-ciliated areas and an adoral band of cilia around the cytostome. Nine families are currently assigned to this suborder including the best studied entodiniomorphs, the

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families Ophryoscolecidae and Cycloposthiidae, endo-symbionts of ruminants and equids respectively (Williams and Coleman 1991).

The most recently described family of entodiniomorphs has been the most difficult to classify: the monogeneric Macropodiniidae, endosymbiotes of macropodid marsupials (kangaroos and allies). The family was originally described by Dehority (1996) who did not assign them to any entodiniomorph suborder as their distinctive morphology differed substantially from all previously described families. Cameron *et al.* (2001a) examined the affinities of the macropodiniids by comparing subcellular features to those of other entodiniomorphs but found that different features suggested affinities with different entodiniomorph suborders and no consensus could be found. Our subsequent study of stomatogenesis within *Macropodinium* (Cameron and O'Donoghue 2001) similarly suggested different affinities depending on which feature's ontogenic sequence was considered. It is thus most probable that structural and developmental homologies between the macropodiniids and other entodiniomorphs have been obscured by the long isolation of the former within Australian marsupials (all other entodiniomorphs inhabit eutherian mammals). To determine the relationship of the macropodiniids to the entodiniomorphs it is therefore necessary to study their genetic sequence evolution.

Previous molecular phylogenetic studies on the entodiniomorphs have been confined to members of a single family, the Ophryoscolecidae. Embley *et al.* (1995) was the first study to examine the small subunit (SSU) ribosomal RNA gene of ophryoscolecids (*Entodinium caudatum* misidentified as *Polyplastron multivesiculatum*) which supported the placement of the entodiniomorphs as the sister-group of the vestibuliferans within the Class Litostomatea. Subsequent studies by Wright and Lynn (1997a) and Wright *et al.* (1997) expanded the phylogeny of the ophryoscolecids by the addition of 5 extra genera (*Diplodinium*, *Eudiplodinium*, *Ophryoscolex*, *Epidinium* and *Polyplastron*) and confirmed the hypotheses of Lubinsky (1957a, b) regarding structural evolution within the Ophryoscolecidae (i.e. basal position of *Entodinium* and reversal of orientation of the adoral ciliation in other genera). Despite excellent coverage of the ophryoscolecids, phylogenetic studies of the remaining entodinimorph families are totally lacking. This study therefore sought to examine the phylogenetic relationships of 2 additional families, the Macropodiniidae and Cycloposthiidae, to the Ophyroscolecidae.

## MATERIALS AND METHODS

Source of samples

Samples of stomach content were collected from macropodid hosts shot in the field or euthenased in zoos and preserved in 9 volumes of 100% ethanol. Individual ciliate species were harvested from mixed species communities under a dissecting microscope by micromanipulation using fine drawn soda glass pipettes. Monospecific pellets of approximately 10 µl volume were obtained for *Cycloposthium edentatum*, *Macropodinium yalanbense* and *Macropodinium ennuensis* from the hosts, *Macropus dorsalis*, *Macropus robustus* (captive animal) and *Macropus robustus* (wild animal) respectively. *Cycloposthium edentatum* is ordinarily a symbiote of horses however the samples used here were recovered from the black-striped wallaby, *M. dorsalis* as described previously (Cameron *et al.* 2000).

DNA extraction and nucleotide sequencing

DNA was extracted using the cetyltrimethylammonium bromide (CTAB) extraction technique of Wright *et al.* (1997). The pellet was air-dried, resuspended in 30 µl of Tris-EDTA (TE) buffer and stored frozen at -20°C. The SSU-rRNA gene was amplified by polymerase chain reaction (PCR) using the primers JB1 and R18A (Cameron *et al.* 2001b). Amplification was performed on a Corbett Research thermal sequencer using the following conditions: denaturation at 95°C (1 min); primer annealing at 50°C (30 s); and chain extension at 72°C (1 min) for 31 cycles. On the final cycle, chain extension was extended for 7 min. The size of PCR bands was estimated by comparison to a 100 bp standard ladder and DNA concentration by comparison to a Mass standard ladder. PCR products were purified to remove unincorporated nucleotides using the QIAquick PCR purification kit (Qiagen, Inc). The 18S region was sequenced using dye terminator automated sequencing (ABI Prism and Big Dye automated sequencing kits). Sequencing of the 18S gene was performed using 6 nested sequencing primers (18B, 18C, SB2, R18B, R18C, R18D, Cameron *et al.* 2001b). Sequence results were examined and ambiguous bases resolved in the Sequence Navigator and Sequencher 3.0 programs. Sequence fragments were reconstructed into whole gene sequences using the Sequencher 3.0 program.

Phylogenetic analysis

A phylogeny of the Litostomatea was reconstructed by alignment of the 3 novel SSU-rRNA gene sequences of entodiniomorph ciliates against 14 other trichostome ciliates and 2 postciliodesmatophorean outgroup taxa (*Eufolliculina uhligi* and *Loxodes striatus*). As the monophyly of the Litostomatea is virtually beyond question (Wright *et al.* 1997; Wright and Lynn 1997a, b; Cameron *et al.* 2001b) sequences from other ciliate orders were not included. Sequences were aligned by eye using the Se-Al program ver 1.0a1 (Rambaut 1996) and modified after reference to secondary structure (Wright 1998). The accession numbers of the additional taxa are: *Dasytricha ruminantium* U57769 (Wright and Lynn 1997b); *Didinium nasutum* U57771 (Wright and Lynn 1997b); *Diplodinium dentatum* U57764 (Wright and Lynn 1997a); *Enchelydon* sp. U80313 (Wright unpub. data); *Entodinium caudatum* U57765 (Wright *et al.* 1997); *Epidinium caudatum* U57763 (Wright *et al.* 1997); *Eudiplodinium maggii*
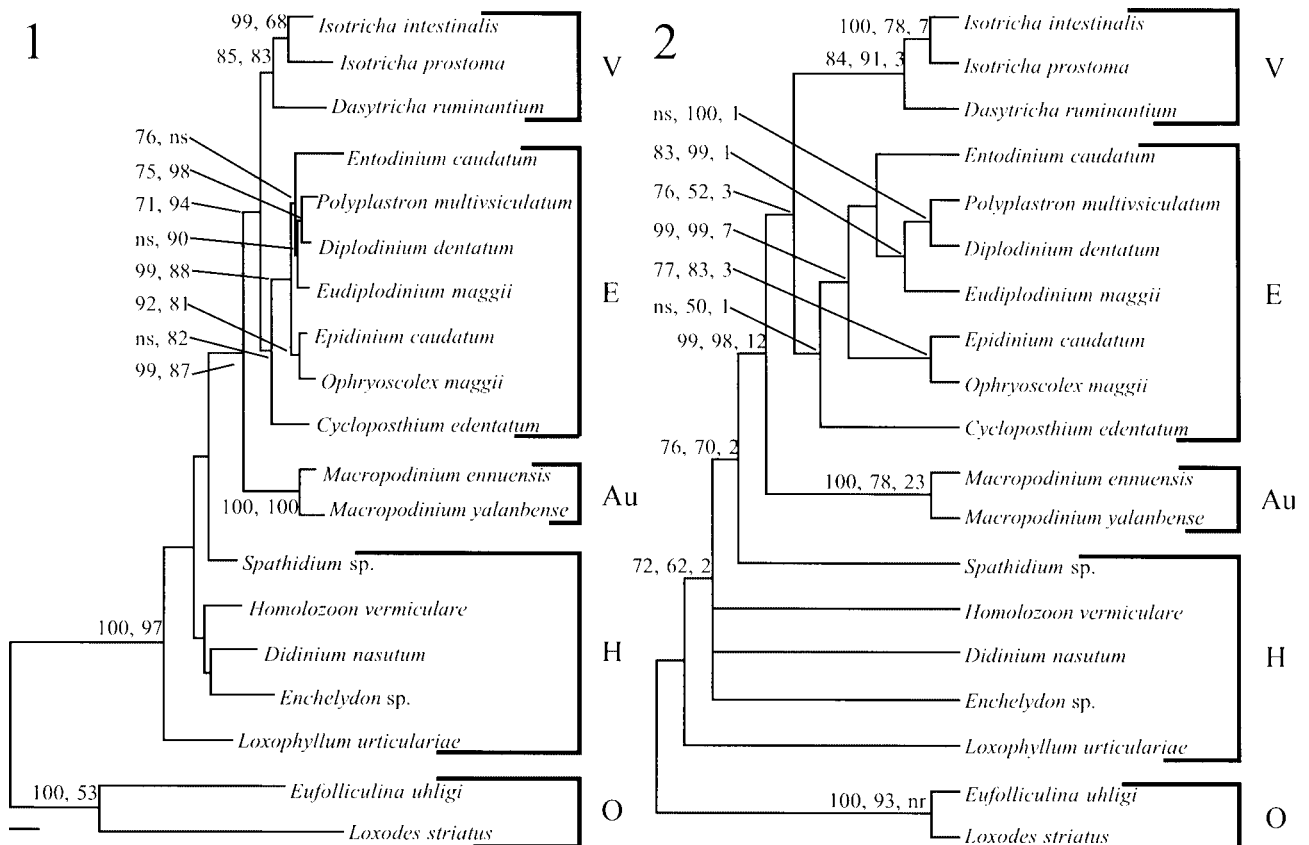
Figs 1-2. Phylogeny of the trichostome ciliates. **1** - maximum likelihood phylogram. Numbers on nodes indicate support: bootstrap and puzzle. **2** - maximum parsimony dendrogram. Numbers on nodes indicate support: bootstrap, puzzle and decay score. Higher taxonomic groups are bracketed: Au - Australian taxa, E - Entodiniomorphida, H - Haptoria, O - Outgroup, V - Vestibulifera. ns - not supported, nr - not reported.

U57766 (Wright and Lynn 1997a); *Eufolliculina uhligi* U47620 (Hammerschmidt *et al.* 1996); *Homolozoon vermiculare* L26447 (Leipe *et al.* 1994); *Isotricha intestinalis* U57770 (Wright and Lynn 1997b); *Isotricha prostoma* AF029762 (Wright and Lynn 1997b); *Loxodes striatus* U24248 (Hammerschmidt *et al.* 1996); *Loxophyllum utriculariae* L26448 (Leipe *et al.* 1994); *Ophryoscolex purkinjei* (Wright and Lynn 1997a); *Polyplastron multivesiculatum* U57767 (Wright *et al.* 1997) and *Spathidium* sp. Z22931 (Hirt *et al.* 1995). Regions with hypervariable sequences which could not be unambiguously aligned to the outgroup taxa were excluded. Eight regions totaling 369 bases were thus omitted; these regions correspond to 9a'→E10-1; loop within E10-1; loop within 11; loop within 17; 23b→23-2a; 29a→29a'; 43b→43b'; and 49g→49g' stem coordinates respectively. These omitted regions have previously been shown to produce misleading phylogenetic results in ciliates due to their arbitrary alignment (Cameron *et al.* 2001b).

Tree construction was performed using PAUP 4.0b8 (Swofford 1998). Two types of tree building algorithms were used: maximum likelihood (ML) and maximum parsimony (MP). Initial trees were constructed using heuristic search parameters. Three confidence indices were calculated: bootstraps (ML and MP); quartet puzzles (ML and MP); and Bremer support indices (MP only). Bootstrapping and quartet puzzling were performed using 1000 replicates in PAUP 4.0b8 (Swofford 1998). Bremer support indices were calculated using TreeRot ver. 2 (Sorenson 1999).

RESULTS

The entire SSU-rRNA gene was sequenced for the three entodiniomorph ciliates *Cycloposthium edentatum*, *Macropodinium ennuensis* and *M. yalanbense*. The SSU-rRNA gene was 1641 bp long in *C. edentatum*, 1639 bp in *M. ennuensisi* and 1639 bp in *M. yalanbense*. These sequences are lodged with Genbank under the accession numbers AF042485 (*C. edentatum*), AF298820 (*M. ennuensis*) and AF042486 (*M. yalanbense*).

Both parsimony and likelihood analyses supported the monophyly of the endosymbiotic litostomes, the

Trichostomatia (Figs 1, 2). In contrast their free-living sister group the Haptoria were paraphyletic in all analyses as *Spathidium* sp. consistently grouped with the trichostomes. Support for *Spathidium* + Trichostomatia was however not strong (unsupported in ML, low support MP). Within the Trichostomatia, support for the monophyly of the Vestibuiferida was consistent and strong. There was limited support for the monophyly of Entodiniomorphida as traditionally conceived. As in all previous, studies the Ophryoscolecidae are monophyletic with high levels of support for this node. Phylogenetic relationships within the Ophryoscolecidae are similar to those reported previously (Wright *et al.* 1997; Wright and Lynn 1997a, b) i.e. division of the family into 3 major clades: *Entodinium* (representing the subfamily Entodiniinae); *Polyplastron* + *Diplodinium* + *Eudiplodinium* (representing the Diplodiniinae); and *Epidinium* + *Ophryoscolex* (representing the remaining subfamilies). It differed with respect to the branching order of these clades; *Entodinium* has been traditionally considered the most basal member of the family (Crawley 1923, Lubinsky 1957a, Wright *et al.* 1997) however here the branching order of the ophryoscolecid clades is unresolved.

While *Cycloposthium* was consistently placed as the sister-taxon to the Ophryoscolecidae in the heuristic searches, there was little support for this relationship, unsupported by all analyses except MP quartet puzzling and then just 50%. The macropodiniids did not fall within the Entodiniomorphida in any of the analyses, rather forming the sister-group to the remainder of the Trichostomatia (Vestibuiferida *sensu* Lynn and Small 1997 + Entodiniomorphida *sensu* Lynn and Small 1997). *Macropodinium* is thus not an entodiniomorph.

DISCUSSION

The phylogeny of the Entodiniomorphida was examined by addition of sequences from the Cycloposthiidae (*C. edentatum*) and Macropodiniidae (*M. ennuensis* and *M. yalanbense*) to sequences of Ophryoscolecidae previously determined (Wright *et al.* 1997; Wright and Lynn 1997a, b). Neither group was found to be particularly closely related to the ophryoscolecids; the cycloposthids were shown to be a weakly supported sister group of the ophryoscolecids while the macropodiniids were much more distantly related forming the earliest diverging branch of the Trichostomatia.

The classification of *Macropodinium* has proven to be troublesome from its first description (Dehority 1996) to the present time. Dehority (1996) assigned the macropodiniids to the Entodiniomorphida primarily on the basis of its reduced somatic ciliation, even though more classical entodiniomorph characters such as retractable vestibula and somatic ciliary tufts were absent (Corliss 1979). Careful examination of the gross morphology of the group using silver staining and scanning electron microscopy (Cameron *et al.* 2001a) failed to resolve the affinities of the group; the vestibular characters were suggestive of blepharocorythiid affinities, the somatic kineties of vestibuliferan affinities and the pellicular folds of cycloposthiid or rhinozetid affinities. Subsequent studies of stomatogenesis in *Macropodinium yalanbense* again failed to unambiguously resolve the affinities of the group (Cameron and O'Donoghue 2001); the replication of the vestibular kineties was similar to that seen in the higher entodiniomorphs, the adoral kineties to that of haptorians and the somatic kineties to that of vestibuliferans. Indeed, rather than suggest which trichostome groups may be closely related to the macropodiniids, these studies highlighted the unique features of the group. The dorsoventral groove, pellicular plates and the ontogeny of the oral kineties *via* two separate processes are features without parallel amongst the ciliates (Cameron *et al.* 2001a, Cameron and O'Donoghue 2001). The current study places these findings into context, the macropodiniids form a distinct monophyletic assemblage which is not closely related to any other group within the Litostomatea. Those features which the macropodiniids share with other groups are therefore either probably convergences (e.g. most oral or pellicular features) or retained pleisomorphies (e.g. somatic ciliary characters).

In contrast, the Cycloposthiidae have been consistently grouped with the Ophryoscolecidae as members of the Entodiniomorphina (so-called higher entodiniomorphs) (Corliss 1979, Grain 1994). The two families share a large number of ultrastructural features in common (Grain 1966; Furness and Butler 1983, 1985a, b). The oral kineties are very similar, both are composed of polybrachykineties which form an adoral loop surrounding the anterior cytostome and a descending vestibular loop (Fernandez-Galiano 1959, Fernandez-Galiano *et al.* 1985). The somatic kineties differ substantially because they are primitively absent in the ophryoscolecids and secondarily derived in the higher genera from the oral kineties whereas they have several forms in the

cycloposthiids (Lubinsky 1957a, b; Corliss 1979). Cytoplasmic features shared include nuclear location, endoplasm/ectoplasm delimination and contractile vacuole structure and location (Ito and Imai 1998, 2000; Cameron *et al*. 2000). Given the number of similarities in cell structure and ultrastructure between the two families, our finding that *Cycloposthium* was only distantly related to the Ophryoscolecidae was surprising. While the heuristic analyses consistently placed *Cycloposthium* as the sister-group to the Ophyroscolecidae, only 1 of the 5 support indices (quartet puzzling of the ML trees) showed significant support for this relationship. It is unknown at this time whether this is due to restricted taxon sampling, lack of phylogenetic signal in the dataset or representative of the real phylogeny of the trichostomes. *Cycloposthium* is traditionally regarded as one of the more derived cycloposthiid genera (Corliss 1979) and the inclusion of more basal representatives of the family may break up the branch length between the two families and strengthen the analysis. Lack of phylogenetic signal from the SSU-rRNA gene has been found previously within the ciliates whereby traditional groups supported by considerable morphological, ultrastructural and developmental data were not recovered e.g. Oligohymenophora (Strüder-Kypke *et al*. 2000, Cameron *et al*. 2001b). Finally, it is possible that the similarities reported above are the result of convergence and are not indicative of phylogenetic relationship. While such convergences have been reported frequently amongst the ciliates (Embley *et al*. 1995, Hammerschmidt *et al*. 1996), it appears unlikely that this is the case in the present study as the relationship between *Cycloposthium* and the ophryoscolecids is simply not significantly supported rather than being contraindicated by any well supported relationships with other taxa.

The present study has significantly increased the taxonomic coverage of the trichostomes for the purposes of phylogenetic analysis. Our understanding of the evolution of the group is, however, still significantly hampered by the large number of taxa for which phylogenetically useful data, gene sequences or complete ultrastructural data, is available. Of the 14 families of trichostomes currently recognised (Corliss 1979, van Hoven *et al*. 1987, Dehority 1996), sequence data is available for only 4 families. More tellingly, some key families have yet to be examined at all; Wolska (1965) has proposed that the Buetschlidae are the most basal representative of the Entodiniomorphida and further that the Blepharocorythidae form a link between the basal buetschlids and the remaining families (Wolska 1971).

No representatives of either family have been sequenced. Examination of such key taxa will be necessary before the major divisions within the Trichostomatia can be recovered. Recovery of these divisions is necessary before a robust classification of the trichostomes can be proposed to adequately classify taxa such as *Macropodinium* which do not fit into any of the presently proposed orders.

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