Parallel evolution of histophagy in ciliates of the genus *Tetrahymena*

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**Abstract**

**Background:** Species of *Tetrahymena* were grouped into three complexes based on morphological and life history traits: the *pyriformis* complex of microstomatous forms; the *patula* complex of microstome-macrostome transformers; and the *rostrata* complex of facultative and obligate histophages. We tested whether these three complexes are paraphyletic using the complete sequence of the small subunit rDNA (SSrDNA).

**Results:** In addition to the 16 species of *Tetrahymena* whose SSrDNA sequences are known, we sequenced the complete SSrDNA from the following histophagous *Tetrahymena* species; *Tetrahymena bergeri*, *Tetrahymena mobilis*, *Tetrahymena rostrata*, and *Tetrahymena setosa* as well as the macrostome species *Tetrahymena vorax*. We also included a ciliate tentatively identified as *Lambornella* sp., a parasite of the mosquito *Aedes* sp. We confirmed earlier results using SSrDNA, which showed two distinct clusters of *Tetrahymena* species: the *australis* group and *borealis* group. The genetic distances among *Tetrahymena* are in general very small. However, all nodes were supported by high bootstrap values. With the exception of *T. bergeri* and *T. corlissi*, which are both histophagous and group as sister species, all other histophagous *Tetrahymena* species are most closely related to a bacterivorous species. Furthermore, *Lambornella* sp. and *T. empidokyrea*, both mosquito parasites, are sister species, although there is a considerable genetic distance between them.

**Conclusions:** There has been parallel evolution of histophagy in the genus *Tetrahymena* and the three classical species complexes are paraphyletic. As the genus *Lambornella* arises within the *Tetrahymena* clade, it is not likely a defensible one.

**Background**

All species of the genus *Tetrahymena* are morphologically very similar. As such, ecological, morphological, biochemical, and molecular features have been used over the years in attempts to classify them. The earliest classifications were based on morphological and ecological data. Czapik [1] regarded the presence or absence of a caudal cilium as an important character. Later, Corliss [2] distinguished three morphological species complexes: the *pyriformis* complex with smaller, bacterivorous...
species and fewer somatic kinetics; the rostrata complex with larger parasitic or histophagous species, more somatic kinetics, and the ability to form resting cysts; and the patula complex with species that undergo microsome-macrostome transformation. Within the complexes, particularly the pyriformis complex, species are distinguishable by mating capacity and/or isozyme patterns [3–5]. Finally, Corliss [6] suggested another approach based on the degree of parasitism. Since, the Tetrahymena species are free-living, as well as facultative and obligate parasites, Corliss [6] suggested an evolutionary lineage from free-living species, considering T. pyriformis to be the basal species, to facultative parasites, and then to obligate parasites.

More recently, gene sequences of ribosomal DNA (rDNA) and histones have been used to determine relationships among Tetrahymena species. Phylogenies based on these sequences revealed that there is little divergence between the Tetrahymena species [7–12]. The 5.8S rDNA sequence is too short and consists of relatively conserved regions, which make it difficult to resolve the phylogenetic relationships among the species of a complex [13,14]. Partial regions of the large subunit ribosomal DNA (LSrDNA) that have been sequenced are identical for some species [9,10]. The small subunit ribosomal DNA (SSrDNA) is longer and there are sufficient differences between the sequences to infer a stable topology for most Tetrahymena species, with the exception of several species of the australis group that share identical or almost identical sequences [12]. Despite the high degree of relatedness, the tree topologies inferred from these analyses are consistent and well supported, separating the species into two branches – the australis group and the borealis group. According to the data inferred from 5S, 5.8S, and 23S rRNA, the species within the genus Tetrahymena were clustered into six ribosets (i.e., sets of species with similar sequences in the regions studied [11]) and later molecular analyses by Nanney et al. [10] generally confirmed these groupings. Riboset C corresponds to the australis group while ribosets A1, A2, and B include members of the borealis group.

In their LSrDNA analysis of several Tetrahymena species, Nanney et al. [10] demonstrated that histophagous and macrostome species grouped within clades of bacterivorous species, and they concluded therefore that macrostomy and histophagy arose by parallel evolution. By sequencing and analyzing the SSrDNA of more histophagous species, we further tested whether histophagy evolved several times independently within the genus Tetrahymena.

Results

**Sequences and primary structure**

The length of the SSrDNA sequences and EMBL/GenBank accession numbers are as follows: Lambornella sp., 1749 nucleotides (AF364043); Tetrahymena bergeri, 1748 nucleotides (AF364039); Tetrahymena mobilis, 1749 nucleotides (AF364040); Tetrahymena rostrata (strain ID-3), 1750 nucleotides (AF364042); Tetrahymena setosa (strain HZ-1), 1749 nucleotides (AF364041); Tetrahymena vorax (strain V2S), 1672 nucleotides (AF364038).

The SSrDNA sequences of all investigated Tetrahymena species differ only in 69 positions, which are located in the variable regions V1-V9 of the SSrRNA molecule Additional file 1 Fig. 1). Over half the variable positions are found in variable regions V2 and V4 (Fig. 1). The sequence of the histophagous T. mobilis is identical to those of the two microstome species, T. tropicalis and T. vorax.

**Figure 1**

Secondary structure model of the small subunit ribosomal RNA molecule of Tetrahymena bergeri. The model was constructed with the RNA Viz program [42] and shows 65 variable sites (in red). The additional 4 variable sites are missing in the Tetrahymena bergeri sequence (cf. Additional file 1: Table 1.xls).
While the histophagous *T. setosa* shares an identical SSrDNA sequence with the bacterivorous *T. pyriformis* Additional file 2. *Tetrahymena rostrata* shows only one mismatch in its sequence to *T. canadensis* and *T. borealis*. The SSrRNA sequences of the latter two species are identical as are those of *T. hyperangularis* and *T. pigmentosa* Additional file 1 Additional file 2. *Tetrahymena bergeri*, which was described by Roque et al. [15] and which has been regarded as doubtful species, has a unique SSrDNA sequence that differs in 9 nucleotide positions from its sister species *T. corlissi*.

**Phylogenetic analyses**

The two ophryoglenid species *Ophryoglena cantenula* and *Ichthyophthirius multifiliis* and the tetrahymenid species *Glaucoma chattoni* and *Colpidium campylum* were chosen as outgroup species to test relationships within the genus *Tetrahymena*. Since several species of *Tetrahymena* show identical SSrDNA sequences, not all sequenced *Tetrahymena* species were included in the phylogenetic analysis.

The general topologies of the trees inferred from the four different methods were quite similar (least-squares [LS], neighbor-joining [NJ] – Fig. 2; maximum parsimony [MP] – tree not shown; maximum likelihood [ML] – Fig. 3). The evolutionary distances within the *australis* group and *borealis* group are very small Additional file 2. Between the species of these two main groups, however, the distances are larger. Although the two main groups and some of the branches therein were very well supported by bootstrap values (Fig. 2) and ML support values (Fig. 3), other relationships within the genus *Tetrahymena* remain unresolved. This is demonstrated by the MP analysis, which computed 21 equally parsimonious trees that all supported the two major groups, but differed in the placement of the species within the clusters. The consensus tree of the MP analysis could only resolve three clusters: the *australis* group, the *borealis* group, and the *T. empidokyrea/Lambornella* sp. pair. All other branches were collapsed (tree not shown). Both distance analyses (LS and NJ) computed stable and comparable trees with high bootstrap support for the *australis* group and
the *borealis* group and sufficient bootstrap support to place *T. empidokyrea* and *Lambornella* sp. within the *australis* group (Fig. 2). The ML analysis also showed three clusters, with *T. empidokyrea* and *Lambornella* branching basal to all other *Tetrahymena* species (Fig. 3).

In all analyses, *T. bergeri* and *T. corlissi* branched basal within the *borealis* group (Fig. 2, 3). The other relationships within the *borealis* group, however, have to be regarded as unresolved. The bootstrap support for a closer relationship of the *T. pyriformis* and *T. rostrata* ribosets is very low (39% [LS], 40% [NJ]), as well as the bootstrap support for the cluster of the *T. thermophila* and *T. tropicalis* ribosets (42% [LS], 50% [NJ]). Within the *T. pyriformis* branch, the relationship to *T. vorax* is only supported by 50% [LS, NJ] bootstrap (Fig. 2).

*Tetrahymena bergeri* is confirmed as a valid species, closely related to *T. corlissi* but only distantly related to *T. rostrata*, which it resembles morphologically (Fig. 2, 3). The three newly-sequenced species, *T. rostrata*, *T. setosa*, and *T. mobilis*, each group together with a bacterivorous species, relationships supported by high bootstrap values (Fig. 2, 3).

The two *Tetrahymena* species isolated from mosquitoes, *Lambornella* sp. and *T. empidokyrea*, grouped together within the *Tetrahymena* clade. The evolutionary distance that separates *Lambornella* sp. and *T. empidokyrea* (i.e., \(d = 0.0042\)) is within the range of those separating other valid species Additional file 2.

If the different modes of nutrition are traced on the phylogenetic tree, it is evident that the two macrostome species *T. vorax* and *T. patula* are interspersed among the microstome species and that most of the histophagous species have a microstome species as sister group (Fig. 4, 5). To address the question which nutritional strategy can be regarded as ancestral, we performed character tracing with two different assumptions. In the first tree (Fig. 4), bacterivory is considered to be ancestral, and it shows under these circumstances that histophagy would have evolved five times independently within the genus *Tetrahymena* and that macrostomy would have evolved twice from bacterivory. If histophagy is considered to be the ancestral nutritional strategy (Fig. 5), bacterivory would have evolved five times within the genus and macrostomy would have evolved once from a bacterivorous and once from a histophagous ancestor. In both models five parallel steps are necessary to construct the topology.

**Figure 4**
Character state distribution of nutritional life history strategy of *Tetrahymena* species. The tree topology was derived by maximum parsimony analysis [38] and characters were traced using MacClade [41]. Bacterivory was assumed to be ancestral.

**Figure 5**
Character state distribution of nutritional life history strategy of *Tetrahymena* species. The tree topology was derived by maximum parsimony analysis [38] and characters were traced using MacClade [41]. Histophagy was assumed to be ancestral.
Discussion

Several phylogenies of the genus *Tetrahymena* have been constructed, based on various molecules like 5S and 5.8S rRNA [13,14], SSrRNA [12], LSrRNA [9–11], and histone H3II/H4II [7]. They are consistent in their topologies and separate two main clusters within the genus *Tetrahymena*: the *australis* and the *borealis* groups. The first group is homogenous and consists of species of the riboset C as defined by Preparata et al. [11] (= *T. australis* group). The second group is more heterogeneous and comprises both ribosets A (A1: *T. thermophila* group and A2: *T. tropicalis/borealis* group) as well as riboset B (T. *pyriformis* group). In the analyses of Preparata et al. [11], the two macrostome species *T. paravorax* and *T. caudata* did not group with any of the ribosets but branched basal to all other *Tetrahymena* species. As in the analyses of Nanney et al. [9,10], our trees depict the *australis* group as a separate branch, coinciding with riboset C. The parasitic species *T. empidokyrea* and *Lambornella* sp. grouped basal within this clade. The topologies within the *borealis* group are in agreement with the SSrRNA tree of Soin et al. [12]. However, the branching pattern is not highly supported by the bootstrap values, since the genetic distances for the SSrRNA within those main clusters are small, even for *Tetrahymena* species. In comparison with the results of Brunk et al. [7] who sequenced the histone H3II/H4II regions, our tree topologies show only minor differences. In their analysis, *T. borealis* and *T. rostrata* were closely related to *T. tropicalis* whereas in our analyses, *T. borealis* and *T. rostrata* were always grouped with the *pyriformis* group (=riboset B).

The three newly sequenced species *T. mobilis*, *T. setosa*, and *T. rostrata* each grouped with a bacterivorous species as the closest relative. Moreover, the sequences of *T. mobilis* and *T. setosa* are identical with their sister species and *T. rostrata* shows only one mismatch to *T. borealis*, a pattern that appears in other sequenced molecules as well. Nanney et al. [10] found the partial LSrRNA sequences of *T. pyriformis* identical to that of *T. setosa* while the partial LSrRNA sequence of *T. canadensis* (identical to *T. borealis* in their SSrDNA) was identical to that of *T. rostrata*. Soin et al. [12] identified identical SSrRNA sequences for several *Tetrahymena* species. Our results increase the number of *Tetrahymena* species that share identical SSrDNA sequences. However, those species that are identical in their rDNA sequence can be distinguished morphologically or isozymically. Since *Tetrahymena* species are polymorphic for many isozymic traits, some of the species were defined on the basis of their specific isozyme characteristics [3,5]. However, the data derived from isozyme studies cannot be reliably used for the construction of phylogenetic trees within the genus *Tetrahymena*. If the rDNA trees are compared to the tree inferred from isozymeic characters, a general accordance is achieved, but the species of ribosets A1 and A2 are scattered throughout the isozyme dendrogram [9].

*Tetrahymena bergeri* is confirmed as a valid species with a unique SSrDNA sequence and life cycle. *Tetrahymena bergeri* is closely related to *T. corlissi*, but several morphological and biological characteristics distinguish them from each other [15,16]. The main differences are the rostrum of *T. bergeri*, the infraciliature on the apical part of the cell, the oral infraciliature, the location of the pores of the contractile vacuole, and the resting cyst, which has not been observed for *T. bergeri*. *Tetrahymena rostrata* resembles *T. bergeri* morphologically [15], but based on life cycle features and such morphological characters as the polar basal body complex and minor differences in the buccal structures, Lynn [16] recognized them as two different species. Our results reveal that there is a large genetic distance between them, supported by high bootstrap values of the branching pattern. Thus, the *rostrata* complex as defined by Corliss [2] is shown to be a paraphyletic assemblage of species with a convergent life cycle but not a close genetic relationship.

The *Lambornella* species, presumably derived from the tree-hole *Aedes* mosquito, grouped with *T. empidokyrea* within the clade of *Tetrahymena* species. There is enough evolutionary distance between *Lambornella* sp. and *T. empidokyrea* to separate them as two different species. Another species of *Lambornella, L. clarki*, grouped within the genus *Tetrahymena* in an analysis of the D2 domain of the LSrDNA, much closer to other *Tetrahymena* species than *T. paravorax* [10]. In fact, Nanney et al. [10] showed that *Lambornella clarki* had a close relationship to *T. corlissi*. However, the *Lambornella* species in our analysis grouped with a different major cluster from *T. corlissi*, and this had high bootstrap support. Since we were unable to culture and stain our *Lambornella* species, it might have been a contaminant *Tetrahymena* from the tree-hole habitat. In our analysis *T. corlissi* is the sister species to *T. bergeri* and these two species grouped basal to the ribosets A and B (i.e., the *thermophila/borealis/tropicalis* and *pyriformis* groups). Since *Lambornella* sp. and *T. empidokyrea* also branched basally within the riboset C (i.e., *T. australis* group), this might explain the affinity of *L. clarki* and *T. corlissi* observed in the analysis of Nanney et al. [10], which included a different set of *Tetrahymena* species. Additionally, the method used by Nanney et al. [10] is most reliable for closely related species (i.e., within a species group), but shows limitations for more distantly related taxa. Taxonomically, another mosquito-parasitizing hymenostome species, *Lambornella stegomyiae* Keilin, 1921, had been assigned to the genus *Tetrahymena*.
na as T. stegomyiae [17]. Corliss & Coats [18] transferred it back to the genus Lambornella when they described a second species, L. clarki. The main generic character separating Lambornella from Tetrahymena is the cuticular cyst of Lambornella from which it invades the haemocoel of its larval host. Our data show that the generic distances between Lambornella sp. and the Tetrahymena species are in most cases smaller than the ones between the mosquito parasite T. empidokyrea and other species of Tetrahymena (cf. Fig. 2). Further analyses must be performed to test these placements of Lambornella within the genus Tetrahymena. If they prove to be correct, the genus Lambornella has to be regarded as invalid.

Our results support the claim that histophagy has evolved within the genus Tetrahymena several times independently. If the mode of food uptake is traced on the phylogenetic distance tree, it is evident that the two macrostome species are interspersed among the microstome species. This confirms the LSU rRNA trees of Nanney et al. [9,10] in which the macrostome species are interspersed among the bacteria-feeding species while the other two macrostome species – T. paravorax and T. caudata – grouped basal to all Tetrahymena species. Most of the histophagous species we studied have a microstome species as sister taxon. Therefore, the three complexes – pyriformis, patula, and rostrata – must be regarded paraphyletic.

Based on our genetic distance tree we traced the character of food uptake under two different assumptions: the first assumption was that bacterivory was ancestral; and the second assumption was that histophagy was ancestral. Under both assumptions, there would have been five parallel steps necessary to construct the topology. The two macrostome species that we studied both grouped among the more derived Tetrahymena species; therefore, we did not assume macrostomy to be ancestral. Macrostome Tetrahymena species can be bacterivorous and morphologically similar to other bacterivorous Tetrahymena under certain conditions, but they are able to rearrange and enlarge their buccal ciliature and subsequently live as carnivores (see [19]).

Histophagous species have a rather complex life cycle, often with cyst formation and some morphological transformation. The histophagous species are either mostly free-living (i.e., T. bergeri, T. mobilis, T. setosa), facultatively parasitic (i.e., T. corlissi in invertebrates and lower vertebrates, T. rostrata in invertebrates), or apparently obligate parasites of mosquitoes (i.e., T. empidokyrea, Lambornella sp.). Hill [20] made the observation that Tetrahymena species must be highly derived based on their loss of biosynthetic abilities: they require 10 amino acids, 6 vitamins, guanine, and uracil; they have no urea cycle enzymes; and they probably make neither sterols, glutathione, nor carbamylphosphate. Could the genus have evolved from a Tetrahymena-like ancestor that was histophagous and that reverted to bacterivory or did histophagy emerge numerous times within the genus whose ancestor was bacterivorous? Since character state distributions for these two scenarios require an equal number of steps, we can make no certain conclusion at this time. However, we prefer the first model, which suggests the parallel evolution of histophagy from a bacterivorous ancestor. As different potential invertebrate and vertebrate host species evolved, it is possible that parallel evolution of histophagy within the genus Tetrahymena occurred to exploit these new habitats. An intriguing nutritional correlation is recorded by Hill [20]. He noted that, compared to bacterivorous pyriformis species, sterol is required for growth of T. corlissi, T. setifera (=T. setosa), and T. paravorax while phospholipid additions aid the growth of these fastidious species, T. corlissi, T. limacis, T. patula, and T. vorax. Could it be that sterol and phospholipid dependence evolved as these species exploited histophagy and macrostomy as nutritional life history strategies?

Our study demonstrates that the tetrahymenine ciliates have diversified as genetically isolated gene pools that are adapted to a variety of distinctive ecological niches. This rapid diversification may have occurred within the last 158 million years, since the late Jurassic [21]. Based on the genetic diversity within the genus Tetrahymena, the hypothesis that protist diversity is limited [22,23] must be questioned. It is obvious that morphological similarity does not reflect genetic identity nor does it necessarily reflect the ecological niche: morphologically similar Tetrahymena species may be genetically very different and may be either bacterivorous or histophagous. Further research on the genetic diversity within and between species of ciliates is needed to determine how widespread is this disconnect between morphology and genetics.

Conclusions

Within the genus Tetrahymena, two main clusters can be separated by molecular phylogenetic analyses: the australis and the borealis group. Generally, genetic distances for the SSrDNA among the species within those two clusters are very small. Our results distinguish Tetrahymena bergeri from any other Tetrahymena species. The other three newly sequenced, histophagous species T. mobilis, T. setosa, and T. rostrata each group with a bacterivorous species as the closest relative and show identical or almost identical sequences to their sister species. Thus, the rostrata complex of histophagous Tetrahymena is shown to be a paraphyletic assemblage of
species with a convergent life cycle but not a close genetic relationship. This supports the model of parallel evolution of histophagy from a bacterivorous ancestor within the genus *Tetrahymena*, triggered by the evolution of different potential invertebrate and vertebrate host species.

**Materials and methods**

**Source of the species strains and culturing**

The histophages, *Tetrahymena rostrata* (strain ID-3, ATCC #30770) and *Tetrahymena setosa* (strain HZ-1, ATCC #30782), and the macrostome *Tetrahymena vorax* (strain V2S, ATCC #30421) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The histophage *Tetrahymena bergeri* was obtained by D. Lynn from the culture collection at the Université de Clermont-Ferrand in 1976, and has been maintained in our laboratory since then. A culture of the histophage *Tetrahymena mobilis* was a gift from W. Foissner, Salzburg, Austria. The species was described as *Saprophilus mobilis* (Kahl, 1926). In an investigation, Foissner W & Schiftner U (in prep.) found that it belongs to the genus *Tetrahymena* (W. Foissner, pers. comm.). All species except *T. mobilis* were cultured in proteose peptone-yeast extract medium (1.25 g/l dextrose anhydrous, 5 g/l proteose peptone, 5 g/l yeast extract) with a biweekly transfer. *Tetrahymena mobilis* was cultured in spring water with fragmented mealworms (*Tenebrio molitor*) as food source. The species, tentatively identified as a *Lambornella* species, was isolated from a sample derived from a tree-hole *Aedes* mosquito and provided to us by the laboratory of J. O. Washburn and J. R. Anderson (University of California, Berkeley).

**DNA extraction and sequencing**

*Lambornella* sp., *T. bergeri*, *T. mobilis*, *T. rostrata*, and *T. setosa* were harvested by centrifugation, and washed in TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). DNA extraction followed the standard protocol of Sambrook, Fritsch and Maniatis [24]. The DNA extraction of *T. vorax* followed the protocol of Walsh, Metzger and Higuchi [25]. One ml of the culture was centrifuged and the supernatant was discarded. Then, 200 µl of 5% Chelex® 100 (Sigma, Oakville, ON, Canada) were added to the pellet. The mixture was vortexed, and incubated for 30 min in a water bath at 56°C. Then, the mix was boiled for 8 min at 100°C and vortexed again. Finally, the sample was centrifuged at 16,000 g for 3 min in an Eppendorf Microcentrifuge 5415C, and 15 µl of this template were used for the subsequent PCR reaction. The PCR amplification of the SSrRNA genes was performed in a PTC-100™ thermal cycler (MJ Research Inc., Watertown, MA) or in a Perkin-Elmer GeneAmp 2400 thermal cycler (PE Applied Biosystems, Mississauga, ON, Canada). The SSrDNA of *T. vorax* was amplified using the internal forward primer 82F (5’-GAAACTGCGAATGGCCT-3’ [26]) and the Medlin B reverse primer (5’-TGATCCCTCTGCAGTTCCATAC-3’ [27]). For all other species, the universal eukaryotic Medlin A forward primer (5’-AACCTGTTGATCCTGCCAGT-3’ [27]) and the reverse primer 5’-TTGGTCCTGTTTCAAGACG-3’ [8] were used in the PCR reactions. The SSrDNA of *Lambornella* sp. was subsequently cloned and sequenced following previously described methods [28]. PCR products were purified using the GeneClean kit (BIO/CAN, Mississauga, ON, Canada). They were sequenced in both directions using an ABI Prism 377 Automated DNA Sequencer (Applied Biosystems Inc., Foster City, CA), using dye terminator and Taq FS with three to four forward and four reverse internal universal SSrRNA primers [26].

**Sequence availability and phylogenetic analysis**

The nucleotide sequences used in this paper are available from the GenBank/EMBL databases under the following accession numbers: *Colpidium campylum* X56532 [29]; *Glaucoma chattoni* X56533 [29]; *Ichthyophthirius multifilis* U 17354 [30]; *Ophryoglena catenula* U 17355 [30]; *Tetrahymena australis* X56167 [12]; *Tetrahymena borealis* M98020 [12]; *Tetrahymena capricornis* X56172 [12]; *Tetrahymena corlissi* U 17356 [31]; *Tetrahymena empidokyrea* U 36222 [31]; *Tetrahymena hegewischi* X56166 [12]; *Tetrahymena hyperangularis* X56173 [12]; *Tetrahymena malaccensis* M26360 [12]; *Tetrahymena patula* X56174 [12]; *Tetrahymena pigmentosa* M26358 [12]; *Tetrahymena pyriformis is* X56171 [12]; *Tetrahymena thermophila* M 10932 [32]; and *Tetrahymena tropicalis* X56168 [12].

The alignment of the sequences was performed with the Dedicated Comparative Sequence Editor (DCSE) program [33] and further refined by considering secondary structural features of the SSrRNA molecule. Genetic distances were calculated with the DNADIST program of the PHYLIP package, ver. 3.51e [34] based on the Kimura 2-parameter model [35]. The programs FITCH (Fitch-Margoliash least squares method [36]) and NEIGHBOR (neighbor-joining method [37]) of this package were used to construct distance trees. A maximum parsimony analysis was performed with PAUP*, ver. 4.0 [38]. Both parsimony and distance data were bootstrap resampled 640 times (FITCH) and 1,000 times (NEIGHBOR, PAUP) [39] respectively. PUZZLE, ver. 4.0.2 (maximum likelihood method [40]) was used to construct a maximum likelihood tree with support values for the internal branches and maximum likelihood branch lengths.

Out of the most parsimonious trees we chose the one that showed basically the same topology as the distance tree and imported it into MacClade ver. 3.0 [41] in order to
perform the character mapping for histophagy, macrostomy, and bacterivory.

Abbreviations

**LS** - least-squares

**LSrDNA** - large subunit ribosomal DNA

**ML** - maximum likelihood

**MP** - maximum parsimony

**NJ** - neighbor-joining

**PCR** - polymerase chain reaction

**SSrDNA** - small subunit ribosomal DNA

Additional material

**Additional file 1**

Table 1: Base pair differences among small subunit ribosomal DNA sequences of the Tetrahymena species. Synapomorphies of the australis group are highlighted in boldface. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2148-1-5-S1.xls]

**Additional file 2**

Table 2: Kimura-2-parameter evolutionary distances (lower left triangle) and absolute base pair differences (upper right triangle) among small subunit ribosomal DNA sequences of Tetrahymena species. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2148-1-5-S2.xls]

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