J. Euk. Microbiol., 44(1), 1997 pp. 61–67

Phylogeny of the Rumen Ciliates *Entodinium, Epidinium* and *Polyplastron* (Litostomatea: Entodiniomorphida) Inferred from Small Subunit Ribosomal RNA Sequences

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ABSTRACT. Three complete 18S ribosomal RNA gene sequences from the rumen ciliates, *Entodinium caudatum* (1,639 bp), *Epidinium caudatum* (1,638 bp), and *Polyplastron multivesiculatum* (1,640 bp) were determined and confirmed in the opposite direction. Trees produced using maximum parsimony and distance-matrix methods (least squares and neighbour-joining), with strong bootstrap support, depict the rumen ciliates as a monophyletic group. *Entodinium caudatum* is the earliest branching rumen ciliate. However, *Entodinium simplex* does not pair with *En. caudatum,* but rather with *Polyplastron multivesiculatum.* Signature sequences for these rumen ciliates reveal that the published SSrRNA gene sequence from *En. simplex* is in fact a *Polyplastron* species. The free-living haptorian ciliates, *Loxophyllum, Homalozoon* and *Spathidium* (Subclass Haptoria), are monophyletic and are the sister group to the rumen ciliates. The litostomes (Class Litostomatea), consisting of the haptorians and the rumen ciliates, are also a monophyletic group.

Supplementary key words. Alveolata, Ciliophora, parsimony, Trichostomatia.

THE rumen ciliates, discovered 153 years ago [25], are the most abundant protozoans in the rumen and are involved in host metabolism and digestion of plant material. Over the last 40 years, relatively little phylogenetic research has been attempted on the rumen ciliates, as most studies have centred around new species' descriptions, redescriptions or species distributions. Therefore, little is known about the phylogenetic relationships within and among the rumen ciliates. Dogiel [12–14] and Lubinsky [34–36] were the first to suggest evolutionary relationships of the entodiniomorphid rumen ciliates based upon morphological characters and structural complexity within the largest family, the Ophryoscolecidae. Dogiel [12–14] and Lubinsky [34–36] believed that *Entodinium* species were the most primitive of the entodiniomorphids because they lack skeletal plates, had no more than one spine, and had only one ciliary band. They concluded that *Entodinium*-like species were probably the first to colonize the rumen.

Furness and Butler [21] included a suite of ultrastructural data on the rumen ciliates and further evaluated these data in functional and evolutionary terms: they correlated the complexity of the cell's cytoalimentary organization with typical size of fibre ingested by the ciliate. For example, *Epidinium,* thought to be evolutionarily advanced by Lubinsky [36], has a tube-like cytopharynx and shows the greatest development of cytoalimentary organization over *Polyplastron* (an evolutionary intermediate form) and *Entodinium* (ancestral representative). This allows *Epidinium* to ingest large plant fragments in the ruminal fluid not available to the intermediate or smaller forms, which ingest smaller plant fibres and bacteria respectively. The phylogenetic analysis of Furness and Butler [21], based on ultrastructural characters, corroborated the earlier work of Dogiel [14] and Lubinsky [34–36].

The rumen ciliates are characterized by generally having: (1) unspecialized oral ciliature, (2) a somatic kinetid that is typically made up of one kinetosome with usually two transverse microtubular ribbons evident only during kinetosomal replication, and (3) microtubular bundles (nematodesmata) that extend into the cytoplasm from the bases of kinetids that surround the cytostome [38]. At present, there are two major classification schemes for the Phylum Ciliophora, based primarily upon ultrastructural features of the ciliate cortex. For de Puytorac and his collaborators [8–10], the rumen ciliates entirely comprise the Class Vestibuliferea, with three orders (Trichostomatida, Entodiniomorphida and Blepharocorythida), and are a sister group to the Class Litostomatea within the Subphylum Filicorticata. Lynn and his collaborators [38, 48, 49] recognize two orders of rumen ciliates, Vestibuliferida and Entodiniomorphida, within the Subclass Trichostomatia, a sister group to the Subclass Haptoria within the Class Litostomatea. Despite the differences in rank for the rumen ciliates, both schemes are basically quite similar. Molecular data will be useful in testing these proposed relationships.

Therefore, we sequenced in both directions the complete small subunit ribosomal RNA (SSrRNA) genes from *Entodinium caudatum, Epidinium caudatum,* and *Polyplastron multivesiculatum* and compared these to SSrRNA gene sequences from other ciliates and eukaryotes. Phylogenetic analyses were used to determine (1) if the rumen ciliates form a monophyletic group, (2) if *En. caudatum* and *En. simplex* form a clade basal to the other two entodiniomorphids, (3) if the rumen ciliates are the sister group to the free-living haptorian ciliates represented by *Homalozoon, Loxophyllum,* and *Spathidium,* and (4) if the Class Litostomatea, to which the rumen ciliates belong, is monophyletic.

MATERIALS AND METHODS

Source of samples and culture conditions. Cells of *En. caudatum* were collected from rumen-fistulated monofaunated sheep and were a kind gift from Dr. Mike Ivan and Linda Neill (Centre for Food and Animal Research, Central Experimental Farm, Ottawa, Ontario, Canada K1A 0C6). Rumen fluid containing *Ep. caudatum* and *P. multivesiculatum* was collected from a rumen-fistulated sheep (BAD). *Epidinium caudatum* was cultured from single cells isolated from the rumen fluid, while *P. multivesiculatum* was collected from rumen fluid.

For culturing *Ep. caudatum,* rumen fluid was filtered through two layers of cheese cloth to remove plant and feed material, and put into a separatory funnel for 1 h at 39° C to sediment protozoa. The sedimented ciliates formed a noticeable white layer and approximately 20 ml of this layer were removed and cells of *Ep. caudatum* were picked using a hand-drawn Pasteur pipette. Individual cells of *Ep. caudatum* were used to inoculate Dehority's rumen fluid-M (DRFM) broth. DRFM broth contains per litre: (1) 500 ml mineral mix M, (2) 100 ml rumen fluid supernatant, (3) 50 ml 1.5% (w/v) CH_3COONa, (4) 83.3 ml 6% (w/v) $NaHCO_3$, (5) 6.7 ml 3% (w/v) cysteine-HCL, and (6) 260 ml dH_2O. Mineral mix M is made as follows: dissolve in 1 L of dH_2O, 6.0 g NaCl, 0.2 g $MgSO_4$, 0.26 g $CaCl_2 \cdot 2H_2O$, and 2.0 g KH_2PO_4. Rumen fluid supernatant is prepared as follows: strain 200 ml of rumen fluid through cheese cloth, centrifuge filtered fluid at 1,000 *g* for 10 min and retain supernatant.

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DRFM broth was prepared anaerobically by gassing with 100% CO_2 until reduced. If required, the pH was adjusted to 6.6. Ten millilitres of DRFM were anaerobically transferred into 16 × 150-ml test tubes and autoclaved. Test tubes of inoculated DRFM broth were slanted at a 10° angle and incubated at 39° C. Each day, under anaerobic conditions, ciliates were fed 0.1 volumes of an orchard grass/wheat substrate. The food substrate is prepared as follows: air-dried orchard grass and whole kernels of wheat are ground to pass a 40-mesh screen. Then, 1.5 g ground wheat and 1.0 g orchard grass are suspended in dH_2O, reduced with CO_2, and stored in 2.0-ml aliquots at −20° C. Five millilitres of each culture were anaerobically transferred twice a week into new test tubes containing an equal volume of DRFM broth and 0.1 volumes of the food substrate. After approximately 4 wk, cells were filtered through cheese cloth, collected by centrifugation, and fixed in 70% ethanol.

DNA extraction and sequencing. A DNA extraction procedure using the non-ionic detergent, cetyltrimethylammonium bromide (CTAB) [41, 44, 56], commonly used for bacterial and plant DNA extraction and purification, was slightly modified for use with the rumen ciliates, which are full of polysaccharides. The CTAB protocol for bacteria was modified as follows: after picking individual cells (i.e. *P. multivesiculatum*) or harvesting rumen culture or fluid, (i.e. *En. caudatum, Ep. caudatum,* respectively), ciliates were pelleted in a 1.5-ml microcentrifuge tube by centrifugation at 6,000 *g* for 2 min. Cells were resuspended in 500 µl 1× TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and 30 µl of 20 mg/ml proteinase K and incubated at 37° C for 1 h. Following incubation, 140 µl of 5 M NaCl were added and thoroughly mixed before the addition of CTAB/NaCl [2% (w/v) CTAB, 100 mM Tris-Cl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl]. The lysate was completely mixed by inverting the microcentrifuge tube several times and incubated at 65° C for 10 min. An equal volume of chloroform, containing isoamyl alcohol (24:1), was added to the lysate and mixed to extract CTAB from the solution, and then centrifuged at 7,000 *g* for 5 min. The aqueous phase was transferred to a new microcentrifuge tube with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), mixed, and centrifuged at 7,000 *g* for 5 min. The aqueous phase was transferred to a new tube with an equal volume of chloroform/isoamyl alcohol, mixed, and centrifuged at 7,000 *g* for 2 min. Again the aqueous phase was removed and the DNA was precipitated with 0.6 volumes of isopropanol at room temperature for 5 min. DNA was collected by centrifugation at 14,000 *g* for 10 min and the nucleic acid pellet was washed with 70% ice-cold ethanol. The pellet was air dried and resuspended in 50 µl of dH_2O. Universal SSrRNA primers [40] were used in a polymerase chain reaction (PCR) amplification using a Perkin-Elmer 9600 thermal cycler. The following parameters constituted one PCR cycle: 1 min denaturation at 94° C, 1:05 min primer annealing at 37° C, and 3:00 min primer extension at 72° C. On the 35th and last cycle, the primer extension was extended for 6 min. Cloning was performed by blunt-end ligation of the SSrRNA insert into a specially prepared plasmid using the Sure Clone Ligation Kit (Pharmacia, Baie D'Urfé, Québec) and *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, CA) competent cells [5]. The Flexi-Prep 100 Kit (Qiagen Inc., Chatsworth, CA) was used to harvest and purify plasmid DNA from confirmed clones for sequencing.

The SSrRNA gene was sequenced in both directions using an ABI Prism 377 Automated DNA Sequencer (Applied Biosystems Inc, Foster City, CA) using diterminator and Taq FS with three forward and three reverse internal universal 18S primers [15] and the M13 vector forward and reverse primers.

Sequence availability and phylogenetic analysis. The nucleotide sequences in this paper are available from the GenBank/EMBL databases under the following accession numbers: *Climacostomum virens* X65152 [26], *Colpidium campylum* X56532 [23], *Colpoda inflata* M97908 [24], *Cyclidium glaucoma* Z22879 [17], *Discophrya collini* L26446 [32], *Entodinium simplex* U27815 [17], *Eufolliculina uhligi* U47620 [26], *Euplotes aediculatus* X03949, M14590 [52], *Furgasonia blochmanni* X65150 [4], *Glaucoma chattoni* X56533 [23], *Homalozoon vermiculare* L26447 [32], *Ichthyophthirius multifiliis* U17354 [57], *Labyrinthuloides minuta* L27634 [33], *Loxodes striatus* U24248 [26], *Loxophyllum utricullariae* L26448 [32], *Metopus contortus* Z29516 (Embley et al., unpubl. data), *Metopus palaeformis* M86385 [16], *Ophryoglena catenula* U17355 [57], *Opisthonecta henneguyi* X56531 [23], *Oxytricha granulifera,* X53486 [47], *Paramecium tetraurelia* X03772 [51], *Plagiopyla nasuta* Z29442, Z29543 [17], *Prorocentrum micans* M14649 [27], *Protocruzia* sp. X65153 [26], *Pseudomicrothorax dubius* X65151 [4], *Sarcocystis muris* M64244, M34846 [22], *Spathidium* sp. Z22931 (Dyal and Embley, unpubl. data), *Stylonychia pustulata* M14600, X03947 [15], *Symbiodinium pilosum* X62650, S44661 [45], *Tetrahymena corlissi* U17356 [57], *Tetrahymena empidokyrea* U36222 [29], *Theileria buffeli* Z15106 [1], *Tracheloraphis* sp. L31520 [28], *Trimyema compressa* Z29438, Z29556 [17], *Trithigmostoma steini* X71134 [32], and *Uronema marinum* Z22881 (Dyal et al., unpubl. data).

Sequences were globally aligned using the Dedicated Comparative Sequence Editor (DCSE) program [11]. The sequence alignment was further refined by considering secondary structural features of the SSrRNA gene. Hypervariable regions that could not be unambiguously aligned were omitted from the matrix since the alignment for these regions remains arbitrary. The elimination of these hypervariable regions from the data is recommended by most researchers [7, 54]. *Labyrinthuloides,* the dinoflagellates (*Prorocentrum, Symbiodinium*), and the apicomplexans (*Theilera, Sarcocystis*) were used as the "outgroups" for the ciliates [39, 50, 53, 55].

For distance-matrix analyses, the alignment was reduced to 1,732 positions. PHYLIP (ver. 3.51C) [19] was used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using both the Jukes and Cantor [30] model and the Kimura [31] two-parameter model. A distance-matrix tree was then constructed using the Fitch and Margoliash [20] least squares (LS) method and the neighbour-joining (NJ) method [46].

Sequence data for the maximum parsimony (MP) analysis were further reduced to 778 phylogenetically informative sites and trees were constructed using PHYLIP's DNAPARS program to find the most parsimonious tree. Prior to tree construction, all sequences were randomly entered and jumbled 15 times. Both parsimony and distance data were bootstrap resampled [18] 500 times.

RESULTS

The lengths of the complete SSrRNA gene were as follows: *Entodinium caudatum* 1,639 nucleotides; *Epidinium caudatum* 1,638 nucleotides; and *Polyplastron multivesiculatum* 1,640 nucleotides. These three new SSrRNA gene sequences have been deposited in the GenBank/EMBL databases under the accession numbers: U57765 (*Entodinium caudatum*), U57763 (*Epidinium caudatum*), and U57767 (*Polyplastron multivesiculatum*).

Molecular phylogenies. The complete SSrRNA gene sequences of the three new rumen ciliates were examined and compared against other SSrRNA gene sequences from ciliates, representing all the major classes of the phylum, and some alveolates [6]. Bootstrap values of both distance matrix (Fig. 1) and parsimony (Fig. 2) trees strongly support the ciliates

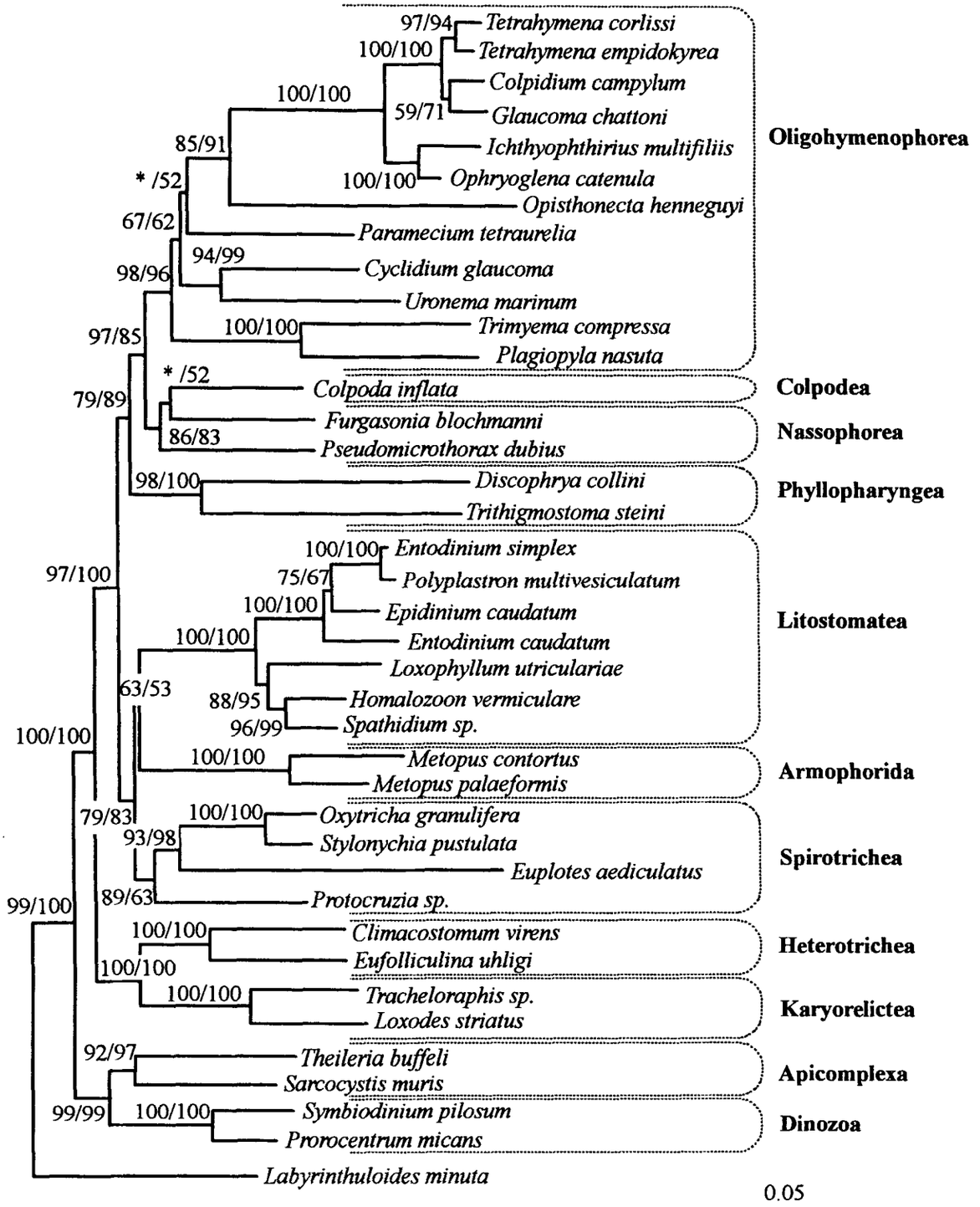

Fig. 1. An SSrRNA distance tree of the ciliates derived from evolutionary distances produced by the Jukes and Cantor [30] correction model and constructed using the Fitch and Margoliash [20] least squares (LS) method and the neighbour-joining (NJ) method [46]. The consensus trees for 500 bootstrap resamplings of the data set were computed independently and the bootstrap resamplings of SSrRNA gene sequences are included as a percentage at the forks. The LS bootstrap value is followed by the NJ bootstrap value. Bootstrap values less than 50% are indicated by an asterisk (*). Evolutionary distance is represented by the horizontal component separating species in the figure. The scale bar corresponds to 5 changes per 100 positions. Names for suprageneric taxa appear in boldface.

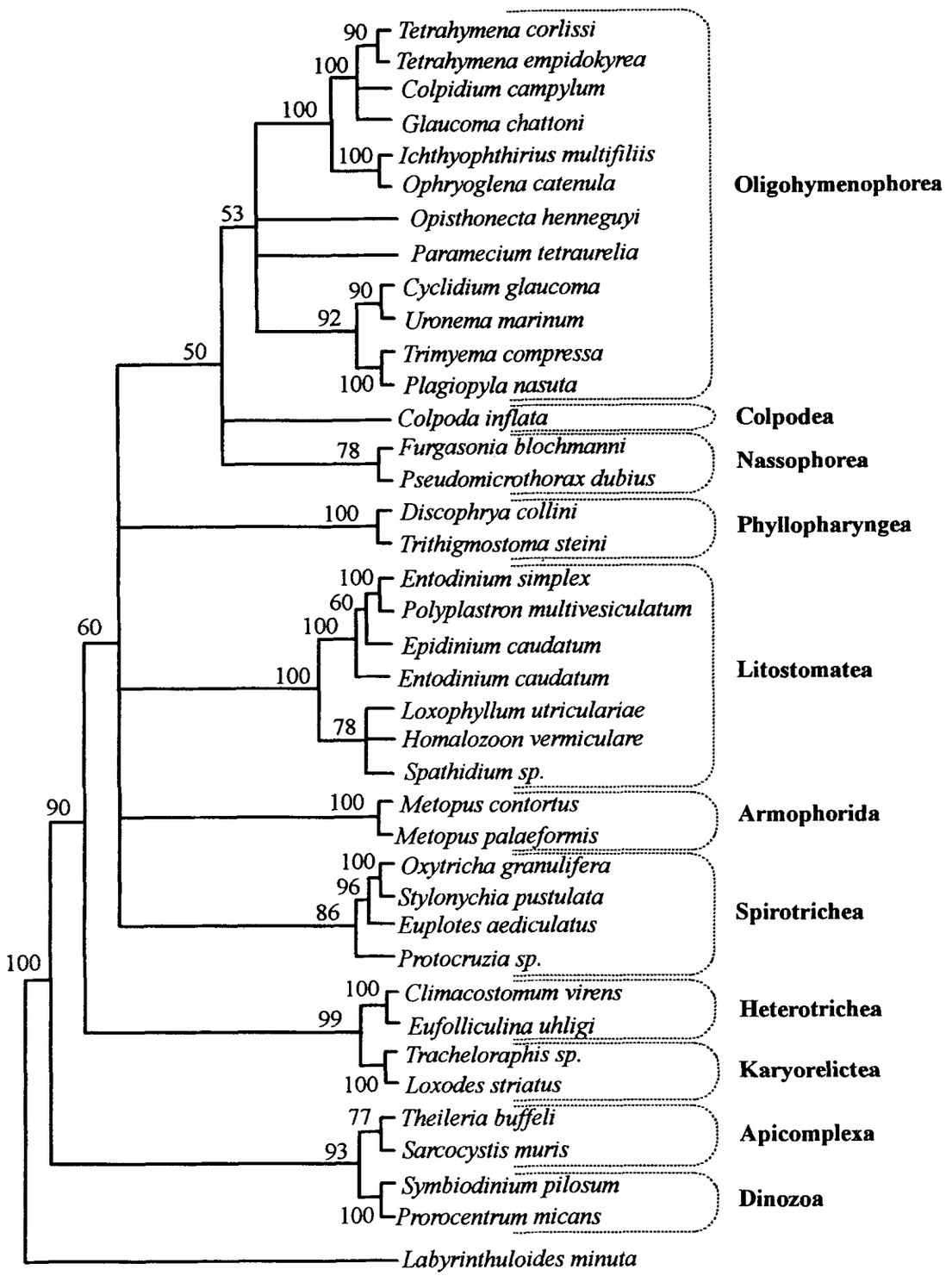

Fig. 2. A maximum parsimony tree of the ciliates inferred from complete SSrRNA gene sequences using a bootstrap resampling of the data set. The numbers at the forks represent the percentage of times the group occurred out of 500 trees. Bootstrap values less than 50% are not indicated on the tree and those branches were collapsed. No significance is placed on the lengths of the branches connecting the species. Names for suprageneric taxa appear in boldface.

Table 1. Differences between British and North American *Polyplastron multivesiculatum.*

Sequence position 5′–3′	400	601	1047	1048	1050	1051	1375	1601
P. multivesiculatum (N. American)	G	A	A	G	C	T	C	T
P. multivesiculatum (British)	A	G	—	—	A	N	T	G

(>90%) as a monophyletic group and as the sister group to the apicomplexans and the dinoflagellates, consistent with their placement in the parvkingdom Alveolata [6].

In all trees (Fig. 1, 2), the rumen ciliates form a monophyletic group. However, *Entodinium* appears to be paraphyletic as *En. simplex* is closer to *P. multivesiculatum* than to *Entodinium caudatum.*

The free-living haptorian ciliates, *Homalozoon, Loxophyllum,* and *Spathidium* (Subclass Haptoria), form a monophyletic group 88% (LS), 95% (NJ) (Fig. 1) and 78% (MP) (Fig. 2) of the time, with distance analyses suggesting a closer relationship between *Homalozoon* and *Spathidium* (96% LS, 99% NJ) than with *Loxophyllum* (Fig. 1). Parsimony analysis could not resolve phylogenetic relationships among these haptorians (Fig. 2). The haptorian ciliates are a sister group, 100% of the time, to the rumen ciliates. These two major lineages comprise the litostome ciliates (Fig. 1, 2).

Bootstrap values for the distance analyses weakly support (63% LS and 53% NJ) the metopids, *Metopus contortus* and *Metopus palaeformis,* as the sister group to the litostomes (Fig. 1). Parsimony analysis could not resolve the sister group to the litostomes (Fig. 2). In all trees, bootstrap analyses strongly support the karyorelicteans-heterotrichs as the sister group to all other ciliates (Fig. 1, 2).

DISCUSSION

Bootstrap data from maximum parsimony, neighbour-joining, and the least squares method strongly support the rumen ciliates as a monophyletic assemblage and as the sister group to the haptorid ciliates within the Class Litostomatea (Fig. 1, 2). *Entodinium caudatum* is the earliest branching entodiniomorphid before *Epidinium* and a dichotomy containing *Polyplastron* and *En. simplex.* Thus, at first glace, it appears that the genus *Entodinium* is paraphyletic. However, the published *En. simplex* sequence [see 17] was misidentified and is actually that of *P. multivesiculatum* (Hirt, R. P., pers. commun.).

We found eight nucleotide differences between the British *P. multivesiculatum* and our *P. multivesiculatum* (Table 1). Two possible explanations for the differences between the two *P. multivesiculatum* sequences are intraspecific variation or sequencing error. Embley et al. [17] do not indicate if both strands of their *P. multivesiculatum* SSrRNA gene were sequenced for confirmation. If these nucleotide differences are confirmed not to be sequencing errors by subsequent research, there would be a significant amount of genetic divergence between British and North American populations of *P. multivesiculatum,* suggesting that molecular evolution has proceeded quite rapidly in the genus.

Four signature sequences (13–26 bp) for *Entodinium caudatum,* two for *Epidinium caudatum,* and three for *P. multivesiculatum* (Table 2) distinguish these sequences from those of all other ciliates. These signature sequences are currently being investigated for use as oligonucleotide probes to study rumen protozoal ecology (Forster et al., unpubl. data).

Our study and the study by Leipe et al. [32] could not unambiguously resolve the sister taxon to the litostomes. Our distance trees depict the metopids as the closest relatives to the litostomes, similar to the least squares tree by Leipe et al. [32]. Although they did not show bootstrap values for that tree, the bootstrap data in our analyses, 63% (LS) and 53% (NJ) (Fig. 1), weakly support this arrangement. Our parsimony analysis could not resolve the sister group to the litostomes (Fig. 2).

All trees indicated, with very high bootstrap support (Fig. 1, 2), that the ciliates are a monophyletic group. The "first" branch in the ciliate tree of descent is a dichotomy shared by the karyorelicteans (Class Karyorelictea), *Loxodes* and *Trachelloraphis,* and their sister group, the heterotrichs (Class Heterotrichea), *Climacostomum* and *Eufolliculina,* consistent with recent phylogenies inferred from large and small subunit rRNA data [2, 3, 26, 28]. The Classes Karyorelictea and Heterotrichea constitute the Subphylum Postciliodesmatophora Gerassimova and Seravin, 1976 sensu Small and Lynn [49], which is characterized by strongly overlapping postciliary microtubular ribbons [38]. Within the Postciliodesmatophora, the heterotrichs divide their macronucleus with extramacronuclear microtubules, while the karyorelicteans have non-dividing macronuclei [26, 43]. Lynn [37] has proposed that the remaining ciliates, united by using intramacronuclear microtubules to divide their macronucleus, should be assigned to the Subphylum Intramacronucleata Lynn, 1996.

Hammerschmidt et al. [26] suggested transferring *Protocruzia* from the Class Karyorelictea to the Class Spirotrichea because their analysis of SSrRNA sequences placed it closer to the spirotrichs than to the karyorelicteans. However, no bootstrap data were shown on their least squares tree and they were unable to resolve *Protocruzia*'s phylogenetic position using maximum parsimony. In our analyses, all trees depicted *Protocruzia* in close association with the spirotrichs, with bootstrap

Table 2. Signature sequences of *Entodinium, Epidinium* and *Polyplastron.*

Species	Signature sequences	Sequence position 5′–3′	Helix #
Entodinium caudatum	5′-GAGACCTTAAATTTC-3′	442–456	17
	5′-GATTCTTCTATACTATAGATGATATC-3′	1224–1249	43
	5′-TGTTATACAAATA-3′	1265–1277	44
	5′-CTCCTTTGGGAAAGATA-3′	1552–1568	49
Epidinium caudatum	5′-GTTCTCAATACTCTGTATTCTGCAAC-3′	1221–1246	43
	5′-CTCCGTACGGGGAAGATA-3′	1262–1274	49
Polyplastron multivesiculatum	5′-GCGGTTATTATCGC-3′	442–455	17
	5′-GATTCTATCATCTTATGATTGATATC-3′	1225–1250	43
	5′-CCTGTAAGGGGAAGATA-3′	1553–1569	49

data (89% LS and 86% MP) strongly supporting the placement of *Protocruzia* within the Class Spirotrichea.

The ciliates, *Plagiopyla* and *Trimyema,* were included within the order Vestibuliferida as a relative to the rumen ciliates [10]. Only recently were these two ciliates placed in different classes by de Puytorac [8]. de Puytorac [8] placed *Plagiopyla* (Class Plagiopylea) and *Colpoda* (Class Colpodea) within the Superclass Transversala and *Trimyema* within the Class Nassophorea with *Pseudomicrothorax* and *Furgasonia.* In our analyses, *Plagiopyla* does not pair with *Colpoda* and *Trimyema* does not form a monophyletic group with *Pseudomicrothorax* and *Furgasonia.* In fact, all trees depict *Plagiopyla* and *Trimyema* as sister taxa. Our trees also indicate, with strong bootstrap support (Fig. 1, 2), that the branch including *Plagiopyla* and *Trimyema* belongs within the Class Oligohymenophorea. Thus, our results support the placement by Small and Lynn [49] of *Plagiopyla* to the Subclass Plagiopylia within the Class Oligohymenophorea and the proposal by Nerad et al. [42], based upon ultrastructure, that *Trimyema* may also belong within the Class Oligohymenophorea. Our data further suggest that *Trimyema* should be placed in the Subclass Plagiopylia with *Plagiopyla.*

Finally, Leipe et al. [32] noted that the SSrRNA gene sequences of the haptorian ciliates, *Homalozoon* and *Loxophyllum,* were approximately 100 nucleotides shorter than the average length of the ciliate 18S rRNA gene. The three new rumen ciliate sequences are the same length as the haptorian SSrRNA genes. From our secondary structure analyses, we have concluded that the litostomes have "lost" helix E23-5 and have a "reduction" in the length of other helices within Variable Region 4. Clearly, this is a diagnostic feature of the litostomes.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Paul Hebert for the use of essential equipment; Dr. Michael Ivan for allowing A-DGW to work in his laboratory in Ottawa; Linda Neill for collecting the rumen fluid from monofaunated sheep and for assisting A-DGW while he was in Dr. Ivan's laboratory; Angela Holliss for generating sequences using the ABI Automated DNA Sequencer; and Dr. Donald S. Martin and Darlene Hack for critical comments on an earlier version of this manuscript. This work was completed as part of a University of Guelph Doctor of Philosophy dissertation by A-DGW. The work was funded by a Natural Sciences and Engineering Research Council of Canada research grant awarded to DHL.

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Received 6-12-96, 8-12-96, 10-4-96; accepted 10-15-96