MOLECULAR PHYLOGENY OF THE ENDOSYMBIOTIC CILIATES (LITOSTOMATEA: TRICHOSTOMATIA) OF VERTEBRATE ANIMALS INFERRED FROM 18S rRNA GENE SEQUENCES

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ABSTRACT

MOLECULAR PHYLOGENY OF THE ENDOSYMBIOTIC CILIATES (LITOSTOMATEA: TRICHOSTOMATIA) OF VERTEBRATE ANIMALS INFERRED FROM 18S rRNA GENE SEQUENCES

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Complete 18S rRNA sequences were elucidated from **(1)** six entodiniomorphid rumen ciliates, *Diplodinium*, *Entodinium*, *Epidinium*, *Eudiplodinium*, *Ophryoscolex*, and *Polyplastron*, **(2)** three vestibuliferid rumen ciliates, *Balantidium*, *Dasytricha*, *Isotricha intestinalis*, and *I. prostoma*, **(3)** two marsupial ciliates, *Cycloposthium* and *Macropodinium*, and **(4)** three free-living ciliates, *Didinium*, *Dileptus*, and *Enchelyodon*, likely the closest relatives to these endosymbionts.

Phylogenetic analysis of these 15 new sequences revealed that ophryoscolecids are a monophyletic group that is divided into three lineages corresponding to the subfamilial divisions of the Ophryoscolecidae, with *Entodinium* branching first. The rumen ciliates are the sister group to *Cycloposthium* consistent with their placement into the order Entodiniomorphida. *Macropodinium* does not group with the other entodiniomorphids, but basal to the vestibuliferid-entodiniomorphid clade. Together, the endosymbionts are the sister group to the free-living haptorians, together constituting the class Litostomatea.

The rate of nucleotide substitution for ciliates was calibrated to be 1% divergence per 72 to 80 million years (My). The origin of ciliates (i.e. crown eukaryotes) is calculated to be much older than previously speculated, dating back to the Paleoproterozoic, 1,980 to 2,200 million years ago. It was also determined that the rate of nucleotide substitution

for rumen ciliates is almost a magnitude faster (1% per 8-11 My) than that for free-living ciliates. This faster clock might be explained by intense selection on survivability as they invaded the rumen, or by the relatively high ambient temperature (39° C) of the rumen environment, as such high temperatures are known to decrease the efficiency of DNA repair mechanisms leading to higher mutation rates.

Intraspecific sequence variation among different hosts and geographical locations was examined using the ITS-1/5.8S/ITS-2 region. Analysis of this region from *I. prostoma* from Canadian, American, and Australian cattle and sheep, showed that there is no sequence variation within this region. This suggests that populations of *I. prostoma* on two continents are very recently diverged, consistent with human colonisation and migration of domestic animals in the 18^{th} and 19^{th} centuries. Finally, secondary structure of these sequences reveal that members of the class Litostomatea have "lost" helix E23-5, indicating a new molecular diagnostic feature for this class of ciliates.

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Finally, to my mother and father (Jean and Kenneth McG. Dick) --- **I DID IT!!** Thank you for all the sacrifices you made, all the prayers you said, and for the love, encouragement, and support you gave me to make this degree possible. I could never repay you for all that you have done and given me. **I LOVE YOU BOTH.**

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"Genius is one percent inspiration and ninety-nine percent perspiration."

Thomas Alva Edison 1847-1931

CHAPTER ONE

General Introduction And Literature Review

"There is no substitute for hard work."

Thomas Alva Edison, 1847-1931

INTRODUCTION

Historical Background

Unicellular microorganisms have been arbitrarily assigned, depending upon their locomotion, to either the animal kingdom or the plant kingdom ever since 1674 when Antony Van Leeuwenhoek first discovered them under his simple microscope. This whimsical assortment of single-celled organisms into either of the two kingdoms continued for almost 200 years until Haeckel, in 1866, saw a need to propose a third kingdom, the Protista, to include those microorganisms that Leeuwenhoek and other microscopists discovered. Haeckel's classification scheme (Figure 1.1) was devised such that lines of descent represented evolutionary histories or phylogenies which could be traced back to a common ancestor (Mayr, 1969). His scheme recognized eight different phyla within his kingdom Protista: Moneres (prokaryotes), Diatomeae, Protoplasta, Myxocystoda, Flagellata, Rhizopoda, Myxomycetes, and Spongiae. Haeckel's classification, as well as other schemes that arose over the next 120 years, were primarily based upon examinations of gross morphological characteristics and fossil records. However, there were two problems associated with these classification schemes: (1) there are few comparable morphological traits shared among the protists; and (2) fossilization does not preserve single-celled organisms well (Corliss, 1979; Sogin *et al.* 1986c). With the widespread use of the electron microscope in the 1960's, ultrastructural data were used to resolve some of the problems/questions left behind from light microscopy. It was also around this time that Zuckerkandl and Pauling (1965) were amongst the first to suggest the use of macromolecules to infer phylogenies amongst all extant life. Not long after their vision, phylogenetic analyses were based upon amino acid sequence changes of

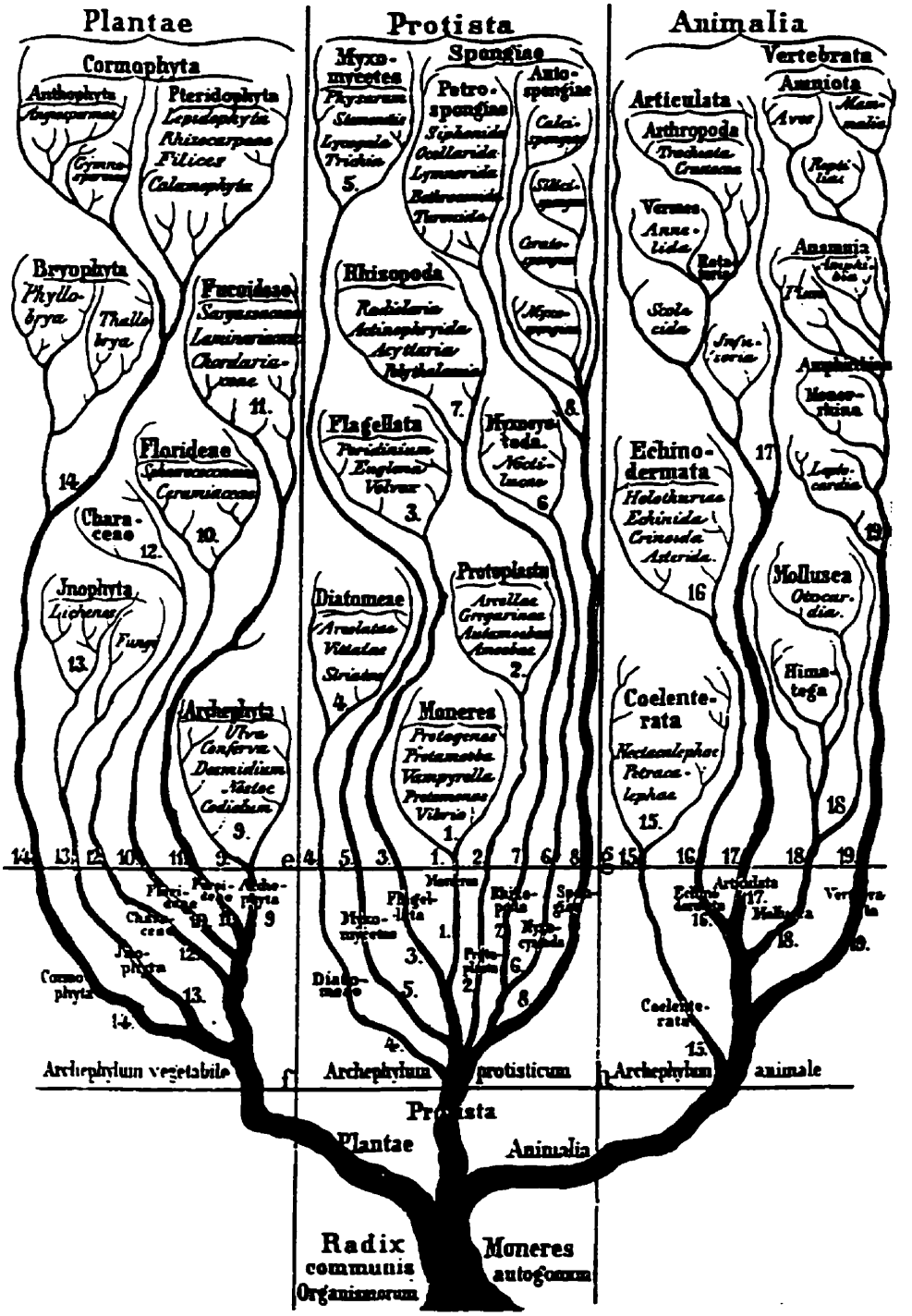


Fig. 1.1 A copy of Haeckel's original tree from 1866 depicting the new kingdom Protista to include those organisms that did not belong to either the plant or animal kingdoms. Copied from Schlegel (1991).

ferrodoxins (Rao and Cammack, 1981), superoxide dismutases (Lumsden and Hall, 1975; Asada *et al.*, 1980), haemoglobin (Ingram, 1963; Fitch and Margoliash, 1967), and cytochrome *c* (McLaughlin and Dayoff, 1973). However, these molecules were of limited phylogenetic use because they were confined to a small number of taxa (Sogin *et al.*, 1986c).

Within the past 20 years, one of the most significant contributions to phylogenetic reconstruction has been the analysis of DNA gene sequences (Hillis and Dixon, 1991; Avise, 1994). However, this new approach to phylogenetic analyses would not have been possible if it were not for at least two major breakthroughs in molecular technology. These were the development of RNA and DNA sequencing protocols (Sanger and Coulson, 1975; Sanger *et al.*, 1977; Maxam and Gilbert, 1977) and the polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Mullis *et al.*, 1986; Mullis and Faloona, 1987; White *et al.*, 1989, Mullis, 1990), in which a very minute amount of DNA, from a crime scene (e.g. dried blood) or an extinct species (e.g. the quagga or woolly mammoth), could be amplified (i.e. copied) and subsequently cloned or sequenced (see Pääbo *et al.*, 1989; Pääbo, 1990; Persing *et al.*, 1990). Moreover, PCR would not have been made possible if it were not for the discovery of a heat stable enzyme, *Taq* polymerase, which is purified from the hot spring bacterium *Thermus aquaticus*. Together, these new technologies permitted the efficient use of DNA gene sequences for inferring phylogenetic relationships.

Characteristics Of The Ribosomal DNA Genes

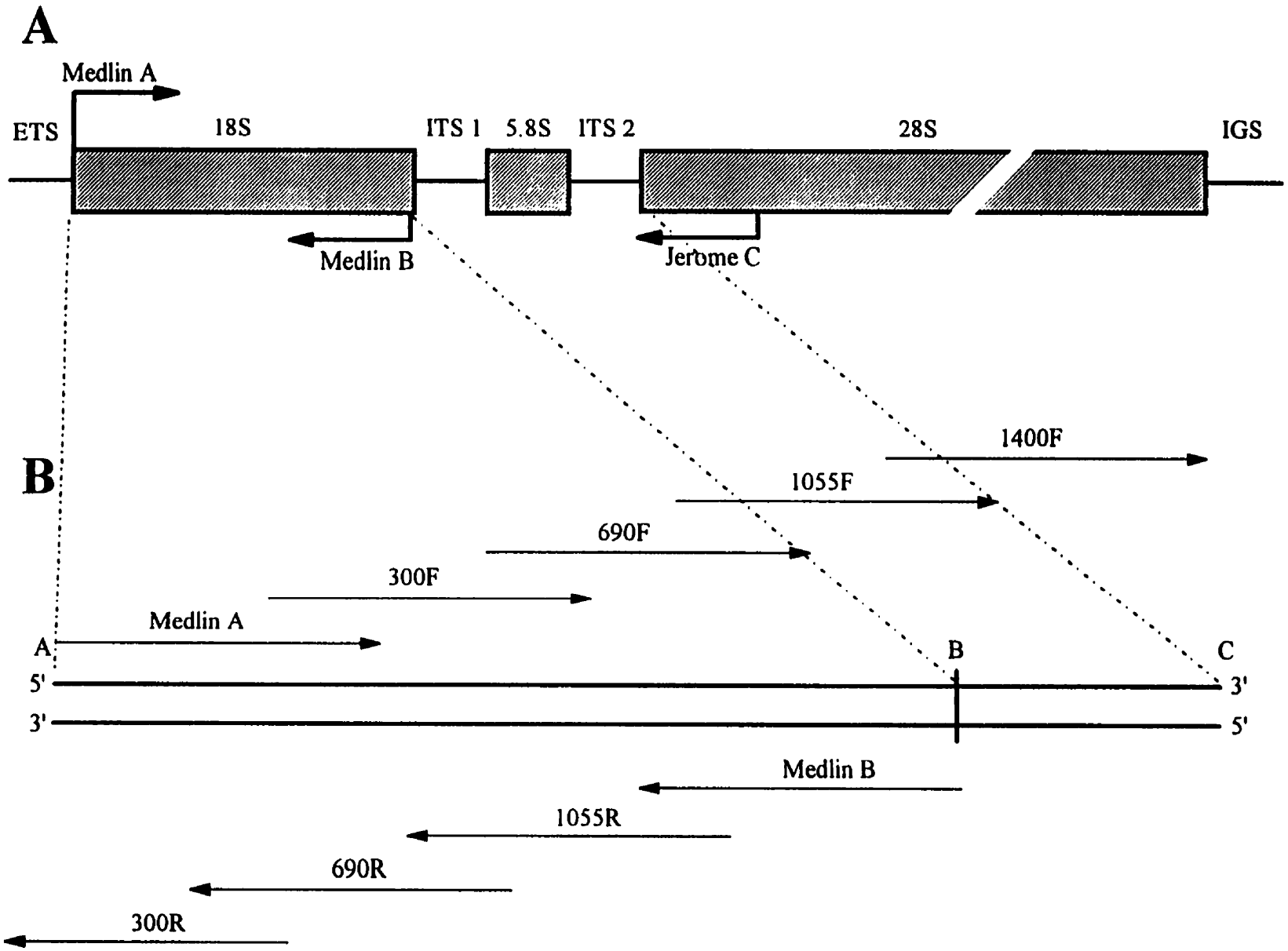
Since there are few morphological traits and macromolecules shared by multicellular and unicellular organisms (Baverstock *et al.*, 1991; Hillis and Dixon, 1991), it was important

for molecular phylogeneticists to select a gene that would be present in all organisms, be easy to isolate, and change sufficiently slowly to accommodate comparisons of distantly related taxa (Olsen, 1988). In addition, the gene must contain an abundance of phylogenetically informative sites and not undergo lateral gene transfer (Olsen, 1988). Since protein synthesis is a prerequisite for all life, ribosomes, and therefore ribosomal RNAs (rRNA), are universally distributed and are functionally equivalent in all cells (Lane *et al.*, 1985; Qu *et al.*, 1988; Raué *et al.*, 1988; Baverstock *et al.*, 1991; Schlegel, 1991).

Typically, there are several hundred tandemly repeated copies of ribosomal DNA (rDNA) in the nuclear genome (Hillis and Dixon, 1991). The number can vary from one or two copies in the ciliate, *Tetrahymena*, to as many as several thousand copies in cereals (Appels *et al.*, 1980). The rDNA transcription unit is made up of the following regions, in order: the external transcribed spacer (ETS), the small subunit rRNA (SSrRNA) gene, an internal transcribed spacer (ITS-1), the 5.8S large subunit rRNA (LSrRNA) gene, a second internal transcribed spacer (ITS-2), and the 28S LSrRNA gene (see Figure 1.2A) (Allard and Honeycutt, 1991; Schlötter *et al.*, 1994). An intergenic spacer region (IGS) separates the rDNA tandem repeat units.

Multiple copies of these rDNA molecules do not normally evolve independently of one another within an individual, or within a species (Dover, 1982; Arnheim, 1983, Hillis and Dixon, 1991). However, since different regions of the rDNA tandem repeat unit evolve at different rates, certain regions, like the ETS and IGS regions, may be too hypervariable for phylogenetic reconstruction. The 5.8S LSrRNA gene [≈160 basepairs (bp)] is not suitable for

Fig. 1.2. (A). A schematic drawing of the ribosomal DNA transcription unit showing the arrangement of the tandemly repeated copies. Each tandem repeat is comprised of an external transcribed spacer (ETS), the 18S SSrRNA gene, an internal transcribed spacer (ITS-1), the 5.8S LSrRNA gene, a second internal transcribed spacer (ITS-2), the 28S LSrRNA gene, and then the intergenic spacer region (IGS) which separates the next tandem repeat. The 28S gene is 2-3 times larger than the 18S gene as suggested by the "break". Medlin A, Medlin B, and Jerome C are PCR primer sites to amplify various regions of the rDNA transcription unit. The arrows determine the direction of the primer extension. **(B).** An enlarged area of the two PCR products [i.e. A - B ($\approx$ 1,800 bp), or A - C ($\approx$ 2,800 bp)] showing the internal primers and sequencing strategy. Sequences were obtained in both directions for confirmation. Arrows indicate the direction of the newly synthesized strand.


A
Medlin A
ETS
18S
ITS 1
5.8S
ITS 2
28S
IGS
Medlin B
Jerome C
B
1400F
1055F
690F
300F
Medlin A
A
B
C
5'
3'
3'
5'
Medlin B
1055R
690R
300R

phylogenetic analysis because of its low number of phylogenetically informative sites. So, the 5.8S sequence is often used in conjunction with the ITS sequences which flank it.

The ITS-1 and ITS-2 regions (see Figure 1.2A) are less conserved than those of the SSrRNA and LSrRNA genes and are potentially of greater use for identification and discrimination of interspecific and intraspecific genetic variation (White *et al.*, 1990; Allard and Honeycut, 1991). The 28S LSrRNA gene (2,900 - 4,800 bp) is approximately double the size of the SSrRNA gene and contains more functional domains and greater variation. However, its larger size has limited the number of complete gene sequences in the database. Instead, partial sequences from various domains within the 28S gene are used for phylogenetic analyses (Baroin *et al.*, 1988; Qu *et al.*, 1988; Preparata *et al.*, 1989; Baroin-Tourancheau *et al.*, 1992, 1995).

The SSrDNA gene has been the molecular marker of choice by molecular phylogeneticists (Hillis and Dixon, 1991). Because this gene is highly conserved, it has been used to study a wide range of relationships from the origin of life to relatively recent evolutionary events (Hillis and Dixon, 1991). Ranging in size from 1,246 bp in the microsporidian *Vairimorpha necatrix* (Vossbrinck *et al.*, 1987) to as long as 2,741 bp in the anaerobic amoeba *Phreatamoeba balamuthi* (Hinkle *et al.*, 1994), the eukaryotic SSrRNA sequences are composed of multiple functional domains with varying degrees of sequence conservation. There are semi-conserved regions that change sufficiently slowly to allow for comparisons of distantly related taxa and there are highly variable regions that contain enough information to resolve phylogenetic relationships among closely related taxa (Elwood *et al.*, 1985). Since molecular divergence, in some instances, is roughly linearly correlated with

divergence time (molecular clock), genetic distance data from rRNA sequences have also been used to predict evolutionary events for major groups of organisms. In the absence of a thorough fossil record, which is especially true for many protist groups, a molecular clock can provide useful insights into a group's evolution (Hillis and Moritz, 1990; Saunders and Druehl, 1992; Wright and Lynn, 1997c -- see chapter 7).

The Impact Of Molecular Data On Phylogenetic Analysis

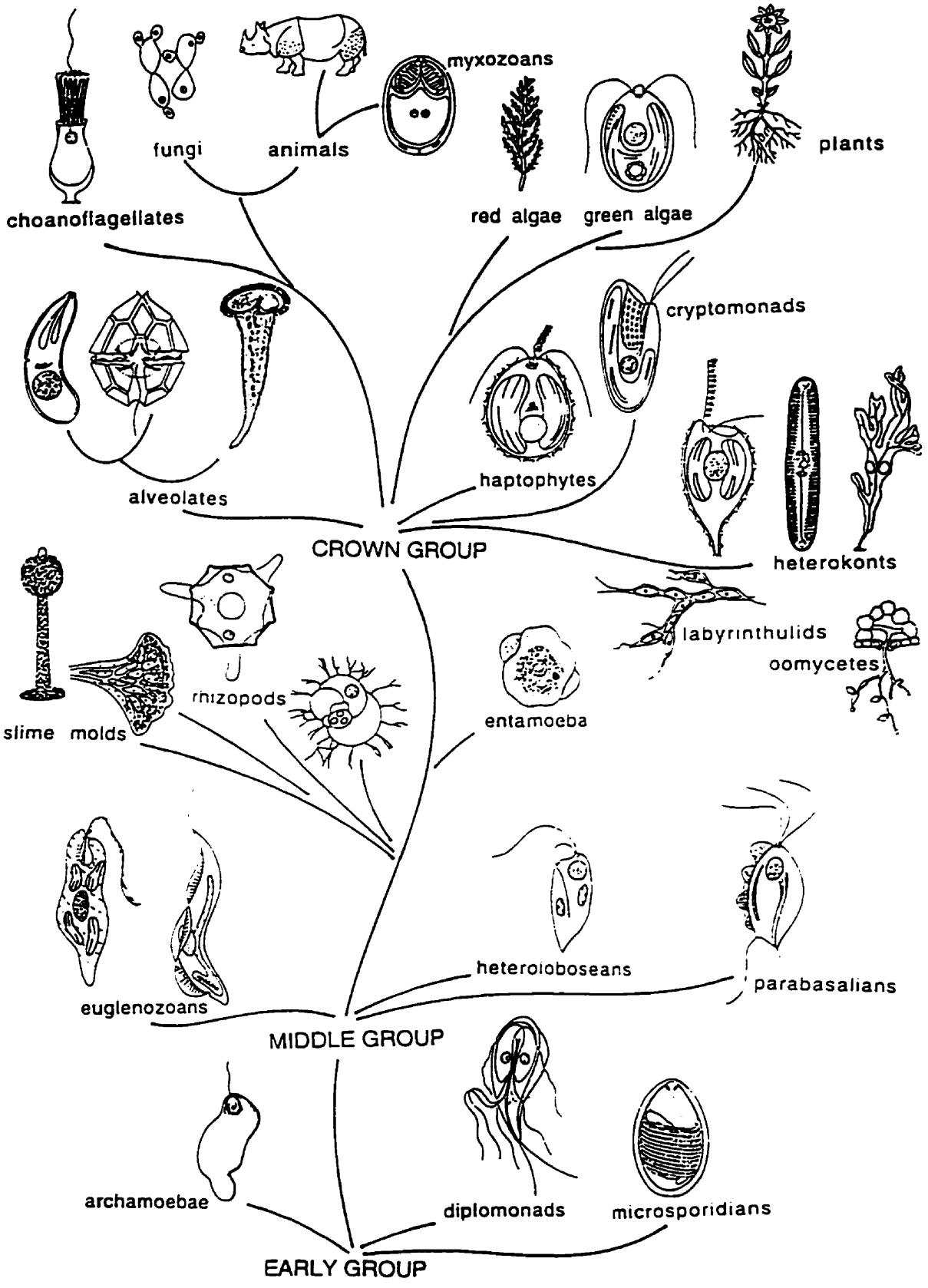
Without a doubt, the effect of molecular data on our understanding of prokaryote and eukaryote phylogeny has been invaluable. In the past, prokaryote phylogenies were primarily based upon cellular and physiological features, which did not provide characteristics that produced reliable phylogenies (Olsen *et al.*, 1994). *Escherichia coli* was the first organism to be characterised by SSrRNA sequencing in 1978. Soon after, rRNA based phylogenies began to emerge and by 1994, over 1,500 complete SSrRNA gene sequences were determined for the prokaryotes (Olsen *et al.*, 1994), and have been very useful in determining evolutionary relationships within and among the archaebacteria (Archaea) and the eubacteria (Bacteria) (Woese, 1987; Woese *et al.*, 1990; Olsen *et al.*, 1994). For example, SSrRNA phylogenetic trees show that the Bacteria divide into 11 major groups, whereas the Archaea divide into two main groups, a methane group – the Euryarchaeota (e.g. *Methanococcus* spp.) and a sulphur group -- the Crenarchaeota (e.g. *Sulfolobus* spp.) (Olsen *et al.*, 1994).

Within the eukaryotes, there are probably more SSrDNA gene sequences available for representatives of the kingdom Protista than from any of the other three classical eukaryotic kingdoms (i.e. Animalia, Fungi, and Plantae). Information from these sequences has been

especially valuable for determining evolutionary relationships within the protists. For example, global phylogenetic trees (Figure 1.3) based mainly on rRNA gene sequences depict the three "amitochondriate" lineages, diplomonads (phylum Metamonada), trichomonads (phylum Parabasala), and microsporidians (phylum Microspora) as the earliest branching eukaryotes before groups having mitochondria (Vossbrinck *et al.*, 1987, 1993; Sogin *et al.*, 1989; Leipe *et al.*, 1993; van Keulen *et al.*, 1993). This suggest that these groups could have diverged before mitochondrial endosymbiosis took place and that their phylogenetic placement is consistent with traditional views based on morphology: that the diplomonads (i.e. *Giardia* and *Hexamita*), trichomonads (ie. *Trichomonas*), and microsporidia (i.e. *Vairimorpha* , *Septata*, and *Encephalitozoon*) are primitive eukaryotes because they have 70S ribosomes like bacteria and they lack a Golgi apparatus (except for the trichomonads), mitochondria, and endoplasmic reticulum as in most eukaryotes. However, in light of very recent findings of nuclear genes encoding proteins that in other eukaryotes are localized in the mitochondrion (i.e. pyridine nucleotide transhydrogenase, chaperonin cpn60, and heat shock protein HSP70), molecular data now strongly suggest that mitochondrial endosymbiosis could have occurred earlier than previously assumed, and because all these organisms live in anaerobic environments, the absence of mitochondria might be due to secondary loss (Clark and Roger, 1995; Bui *et al.*, 1996; Germot *et al.*, 1996, 1997; Horner *et al.*, 1996; Roger *et al.*, 1996, 1998; Hirt *et al.*, 1997; van der Giezen *et al.*, 1997).

Molecular data have also determined that the middle branches of the global eukaryotic tree (Figure 1.3) represent organisms that have mitochondria, but lack chloroplasts, such as, the entamoebas, cellular slime molds, and the kinetoplastids. Further, the crown of the

Fig. 1.3. A global eukaryotic tree inferred from SSrRNA gene sequences (copied from Bardele, 1997). It depicts three main regions of the tree, the "early group" comprised of the "amitochondriates", the "middle group," which consists of the slime molds, amoebae, and some flagellates, and the "crown group", which includes the ciliates, apicomplexans, plants, algae, fungi, and animals.


myxozoans
fungi
animals
plants
choanoflagellates
red algae
green algae
cryptomonads
alveolates
haptophytes
heterokonts
CROWN GROUP
labyrinthulids
oomycetes
rhizopods
slime molds
entamoeba
heteroloboseans
parabasalians
euglenozoans
MIDDLE GROUP
archamoebae
diplomonads
microsporidians
EARLY GROUP

eukaryotic tree (Figure 1.3) illustrates great single-celled diversity and eukaryotic radiation with the appearance of the animals, fungi, multi-cellular plants, algae, ciliates, dinoflagellates, and apicomplexans (Sogin *et al.*, 1986a, 1989; Cavalier-Smith, 1993; Hinkle *et al.*, 1994).

Molecular data have demonstrated the need to separate groups of protists, at various taxonomic levels (e.g. phylum, class, order), to reflect evolutionary lines of descent. For example, controversy has surrounded the members of the protist phylum Myxozoa for almost 100 years (Stolc, 1899). Primarily parasites of fishes, these organisms were thought to be protists, but have an infective spore stage that is multicellular (Siddall *et al.*, 1995). Siddall *et al.* (1995) used SSrRNA gene sequences in a phylogenetic analysis to determine myxozoan origins. Their results indicated that the myxozoa are a clade of highly derived parasitic cnidarians and are the sister group to the narcomedusan *Polypodium hydriforme*. This result, combined with morphological similarities, such as collagen production, cellular junctions, and a redescription of the polar capsules as a typical nematocyst, argued for the demise of the protist phylum Myxozoa and the inclusion of the myxozoa within the phylum Cnidaria.

Molecular data have also illustrated that the ciliated protozoa fall into two main lineages based on their mode of macronuclear division. This led to the erection of a new subphylum within the Ciliophora, the Intramacronucleata, to describe those organisms that divide their macronucleus using intramacronuclear division (Lynn, 1996). The remaining ciliates (i.e. some that use extramacronuclear division) were already assigned to the subphylum Postciliodestmatophora, based on derived features of the somatic kinetid (Small and Lynn, 1981).

Also within the phylum Ciliophora, Small and Lynn (1985) recognized the heterotrichs (e.g. *Blepharisma*) as a subclass within the class Spirotrichea based on a unique ultrastructural fibre. De Puytorac *et al.* (1987) recognized the heterotrichs at the class rank because of the distinctiveness of their adoral zone membranelles. Based on the deep branching of *Blepharisma* (Greenwood *et al.*, 1991; Schlegel *et al.*, 1991; Baroin-Tourancheau *et al.*, 1992; Wright and Lynn, 1995), coupled with the fact that the mean genetic distance that separated *Blepharisma* from the stichotrichs ($d \approx 0.154$) (Wright , 1993) was greater than the distance that separated rat (*Rattus*) from brine shrimp (*Artemia*) ($d = 0.139$) (Greenwood *et al.*, 1991), molecular data suggested that Small and Lynn (1981, 1985) had inaccurately placed the heterotrichs within the class Spirotrichea. In addition, molecular based trees also suggested that the plagiopylids (subclass Plagiopylia) be elevated to class rank (class Plagiopylea) and include the genus *Trimyema* (see Chapter 2) (Wright *et al.*, 1997), thereby increasing the number of classes recognized within the phylum Ciliophora from eight to 10 (Table 1.1).

In recent studies involving analysis of SSrRNA sequences, (Wright and Lynn, 1995, 1997a, 1997b; Wright *et al.*, 1997), a closer relationship was found between *Paramecium* and the oligohymenophoreans (e.g. *Opisthonecta*) than between *Paramecium* and any other ciliates. De Puytorac *et al.* (1987) placed *Paramecium* in the subclass Peniculia within the class Oligohymenophorea. In contrast, Small and Lynn (1985) placed *Paramecium* in the order Peniculida within the class Nassophorea. The SSrRNA results, coupled with phylogenies inferred from partial sequences of the LSrRNA gene (Baroin *et al.*, 1988; Baroin-Tourancheau *et al.*, 1992) and evolutionary distance data [i.e. the distance separating *Opisthonecta* from its oligohymenophorean relatives ($d \approx 0.198$) (Wright and Lynn, 1995), was greater than the

Table 1.1. Classification scheme of the phylum Ciliophora as proposed by Lynn and Small (1998).

PHYLUM CILIOPHORA	
SUBPHYLUM POSTCILIODESMATOPHORA	
Class I. Heterotrichea	
Subclass Heterotrichia	8 orders
Class II. Karyorelictea	4 orders
SUBPHYLUM INTRAMACRONUCLEATA	
Class III. Colpodea	4 orders
Class IV. Prostomatea	2 orders
Class V. Spirotrichea	
Subclass Choreotrichia	2 orders
Subclass Stichotrichia	1 order
Class VI. Litostomatea	
Subclass Haptoria	**3 orders**
Subclass Trichostomatia	**2 orders**
Class VII. Phyllopharyngea	
Subclass Phyllopharyngia	2 orders
Subclass Chonotrichia	2 orders
Subclass Suctoria	3 orders
Class VIII. Nassophorea	5 orders
Subclass Nassophoria	5 orders
Subclass Hypotrichia	1 order
Class IX. Plagiopylea	
Subclass Plagiopylia	1 order
Class X. Oligohymenophorea	
Subclass Hymenostomatia	2 orders
Subclass Peritrichia	2 orders
Subclass Astomatia	1 order
Subclass Apostomatia	3 orders

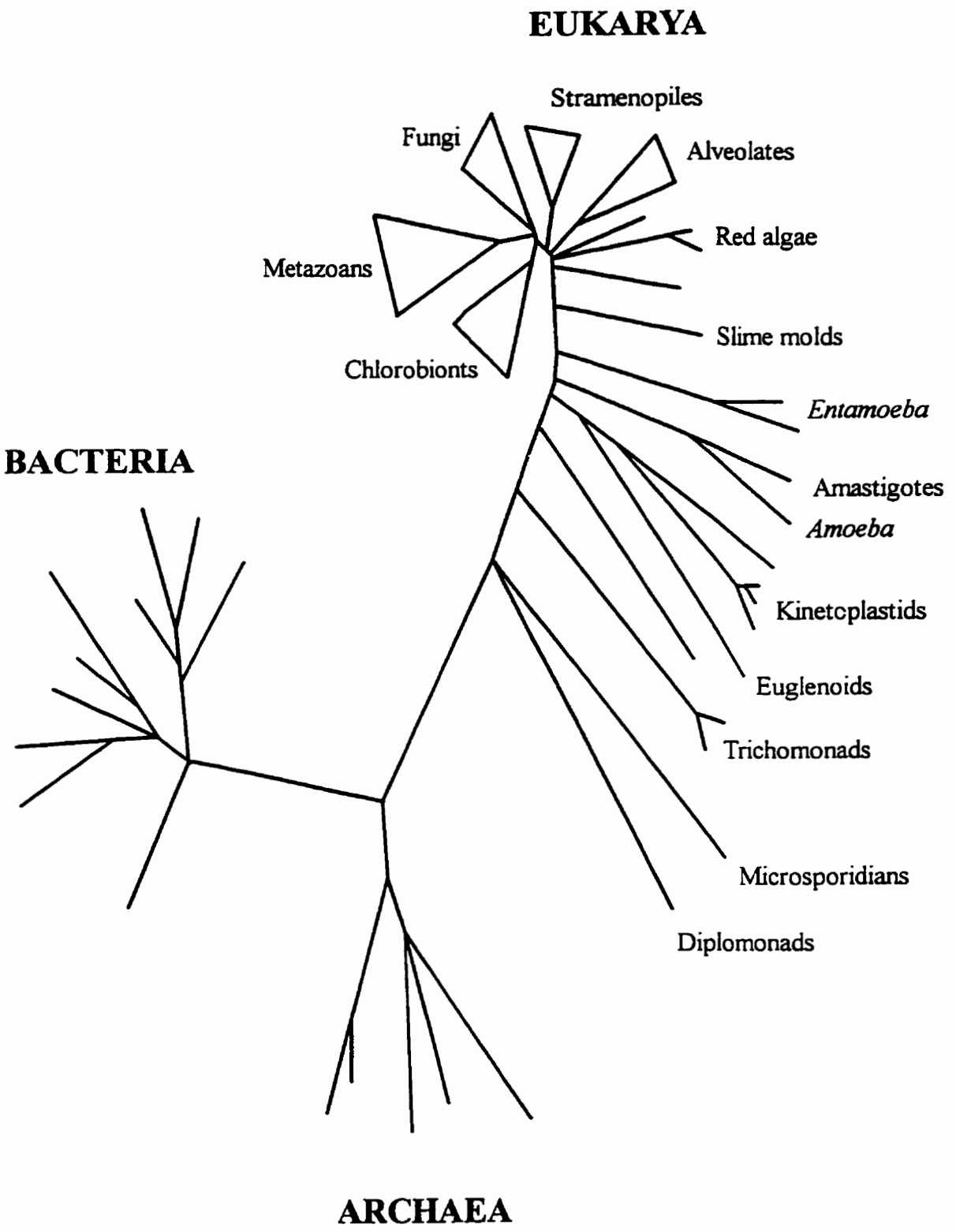
distance separating *Paramecium* from the oligohymenophoreans (d ≈ 0.166) (Wright and Lynn, 1995)], suggested that Small and Lynn's (1981, 1985) placement of *Paramecium* in the class Nassophorea was incorrect. Moreover, even the transferring of the ciliate *Protocruzia* from the class Karyorelictea to the class Spirotrichea was based upon the results of rRNA phylogenies (for more details see Chapter 2).

Molecular analyses have also been used to elucidate the phylogenetic position of organisms whose taxonomic affinity was uncertain. For example, the human parasite *Blastocystis hominis* has been a taxonomic enigma since its description over 100 years ago and it has been misclassified as a sporozoan, a yeast, an amoeba, and the cyst form of a flagellate. Analysis of SSrRNA gene sequences indicated that *B. hominis* belonged within the stramenopile lineage and as the sister taxon to another gut endosymbiont, *Proteromonas* (Silberman *et al.*, 1996)

The Paraphyletic Protists

Global SSrRNA phylogenetic trees of life (Figure 1.4) indicate that extant organisms fall into three major groups or empires, the eukaryotes, bacteria, and archaea (Olsen, 1987; Sogin *et al.* 1989; Hinkle *et al.*, 1994; reviewed by Schlegel, 1994). Sogin *et al.* (1989) and Woese *et al.* (1990) both suggested a reduction from the classical five kingdom system to a three domain system by grouping together all the eukaryotes into the domain Eukarya and by calling the other two major domains Bacteria and Archaea. Also called empires or kingdoms, there is increasing support for the new three "empire" concept.

Fig. 1.4. An unrooted global phylogeny (redrawn from Schlegel, 1994) of Archaea, Bacteria, and Eukarya based on least-squares, distance-matrix analysis of SSrRNA gene sequences. Lengths of the branches corresponds to genetic distances. The genetic distance amongst the "protists" is far greater than that between multicellular taxa and either the Archaea or Bacteria.


EUKARYA
Stramenopiles
Fungi
Alveolates
Red algae
Metazoans
Slime molds
Chlorobionts
Entamoeba
BACTERIA
Amastigotes
Amoeba
Kinetoplastids
Euglenoids
Trichomonads
Microsporidians
Diplomonads
ARCHAEA

Because the eukaryotes are monophyletic and that animals, higher plants, and fungi all evolved from these diverse group of protists, the current classification scheme of the Protista, and others before it, depict the various protist groups as being paraphyletic. For example, Corliss (1984) recognized 45 phyla of protists and assigned them to 18 supraphyletic assemblages within the Protista. However, his scheme did not illustrate the lines of evolutionary descent within the Protista. Because the kingdom Protista is not a natural group, coupled with the fact that genetic distances between groups of protists (i.e. classes or orders) far exceed those genetic distances once observed amongst the classical kingdoms (Greenwood *et al.*, 1991; Wright, 1993; Schlegel, 1994), there has been an increasing tendency to divide the Protista into several "super-groups" to represent monophyletic assemblages (Corliss, 1984, 1994; Cavalier-Smith, 1987, 1989, 1993).

Cavalier-Smith (1993) was one of the first to propose radical changes by dividing his empire Protista into two superkingdoms (Archezoa, Metakaryota) containing three kingdoms (Archezoa, Protozoa, Chromista) with four subkingdoms, two branches, four infrakingdoms, seven parvkingdoms, four superphyla, and 25 phyla (Table 1.2). According to Cavalier-Smith (1993), the addition of these new taxonomic ranks (i.e. superkingdoms, branches, infrakingdoms, parvkingdoms, etc.) was necessary to group together protists with shared derived characters and to depict the evolutionary lines of descent of these diverse unicellular eukaryotes. One year later, Corliss (1994) proposed a less complicated scheme than his previous 1984 classification (Corliss, 1984). Although he did admit that his new scheme is not an ideal system, Corliss's (1994) new classification of the Protista differs from Cavalier-Smith's (1993) scheme by avoiding the super-, infra-, and supra- taxa at kingdom, phylum,

Table 1.2. The classification of the protists within the **EMPIRE EUKARYA** as proposed by Cavalier-Smith (1993).

SUPERKINGDOM I. ARCHEZOA
KINGDOM I. ARCHEZOA
Phyla Archamoebae, Metamonada, and Microsporidia
SUPERKINGDOM II. METAKARYOTA
KINGDOM II. PROTOZOA
Subkingdom I. Adictyozoa
Phylum Percolozoa
Subkingdom II. Dictyozoa
Branch I. Parabasalia
Phylum Parabasalia
Branch II. Bikonta
Infrakingdom I. Euglenozoa
Phylum Euglenozoa
Infrakingdom II. Neozoa
Parvkingdom I. Ciliomyxa
Superphylum I. Opalomyxa
Phyla Opalozoa and Mycetozoa
Superphylum II. Choanozoa
Phylum Choanozoa
Parvkingdom II. Alveolata
Superphylum I. Miozoa
Phyla Dinozoa and Apicomplexa
Superphylum II. Heterokaryota
Phylum Ciliophora
Parvkingdom III. Actinopoda
Phyla Heliozoa and Radiozoa
Parvkingdom IV. Neosarcodina
Phyla Rhizopoda and Reticulosa
Parvkingdom V. Entamoebia
Phylum Entamoebea
Parvkingdom VI. Myxozoa
Phyla Myxosporidia, Haplosporidia, and Paramyxia
Parvkingdom VII. Mesozoa
Phylum Mesozoa
KINGDOM III. CHROMISTA
Subkingdom I. Chlorarachnia
Phylum Chlorarachniophyta
Subkingdom II. Euchromista
Infrakingdom I. Cryptista
Phylum Cryptista
Infrakingdom II. Chromobiota
Phylum Heterokonta
Phylum Haptophyta

class, and ordinal levels. His self-proclaimed "user friendly" hierarchical classification divides the Protista into five kingdoms (Archezoa, Protozoa, Chromista, Plantae, Fungi), within the Eukarya, with further divisions into six subkingdoms, 34 phyla, and 83 classes (Table 1.3).

The Alveolates

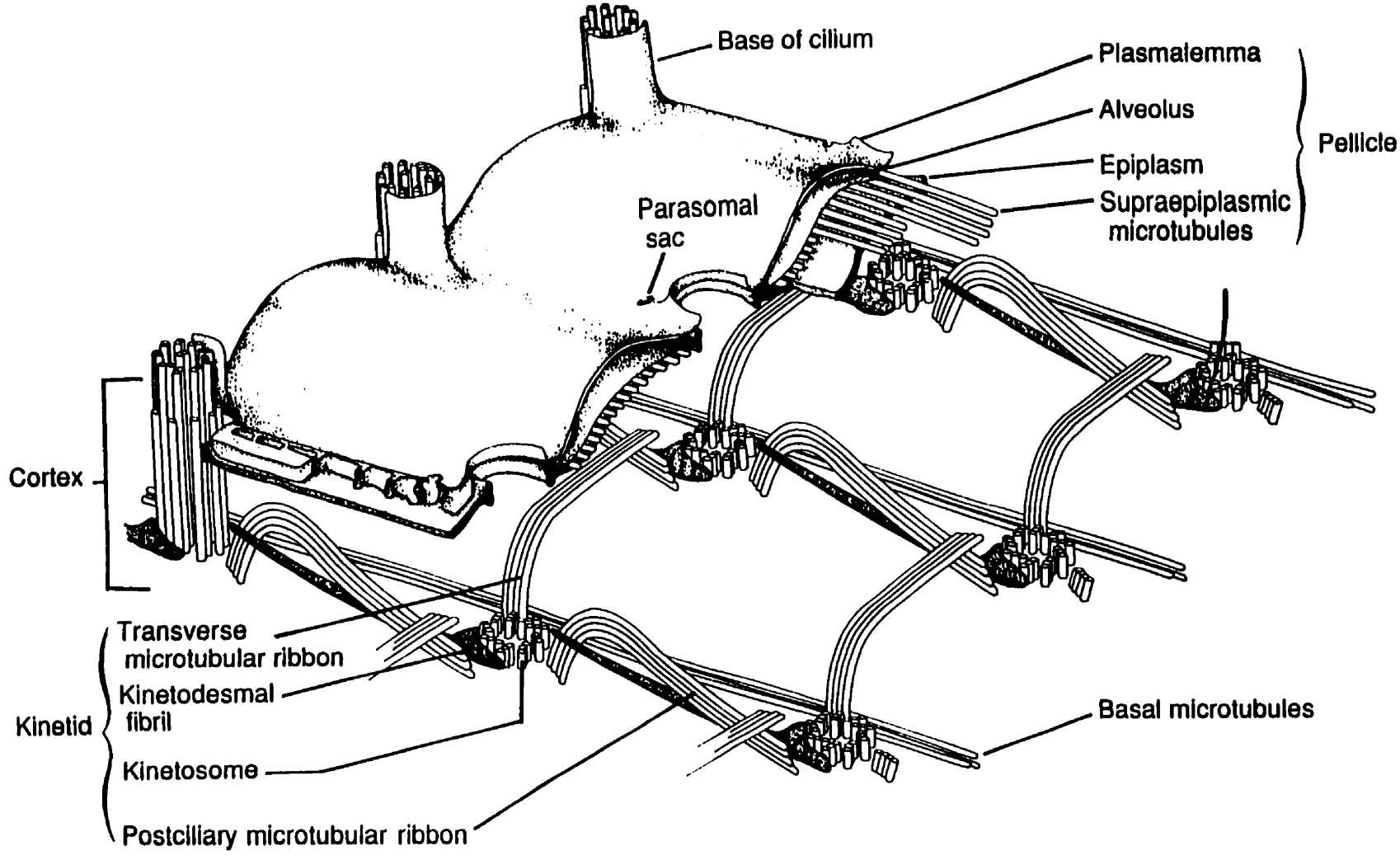
Cavalier-Smith (1993) placed the apicomplexans, ciliates, and dinoflagellates together within the parvkingdom Alveolata, an intermediate taxon between the new ranks infrakingdom and superphylum (see Table 1.2). These organisms are distinguished from all other protozoa by having cortical alveoli, single flattened membrane-bound sacs that usually occur beneath the plasma membrane and commonly have rows of microtubules underneath it (see Figure 1.5) (Corliss, 1979; Small and Lynn, 1985; Lee and Kugrens, 1992). Collectively called the alveolates, these organisms show the remarkable range of the structural complexity that can be attained within a single cell. Although the members of these three phyla have different ultrastructure and ecology, they consistently form a monophyletic group. Surprisingly, 20 years ago, Taylor (1976) presented an argument based on morphological data for the closeness of the ciliates and the dinoflagellates, but this was not widely accepted until the molecular data corroborated this close relationship.

Based on the GenBank database, the alveolates are the protist group for which there is the most amount of molecular data available with over 270 complete SSrRNA gene sequences. While the number of these gene sequences for the alveolates have more than quadrupled over the past ten years (*pers. obs.*), a third of these new sequences ($\approx$ 92) come from the phylum Ciliophora (*pers. obs.*).

Table 1.3. The classification of the protists within the **EMPIRE EUKARYA** as proposed by Corliss (1994).

KINGDOM I. ARCHEZOA		
Phylum Archamoebae	Phylum Microspora	Phylum Metamonada
KINGDOM II. PROTOZOA		
Phylum Percolozoa	Phylum Opalozoa	Phylum Parabasalia
Phylum Euglenozoa	Phylum Choanozoa	Phylum Mycetozoa
Phylum Ciliophora	Phylum Apicomplexa	Phylum Dinozoa
Phylum Radiozoa	Phylum Rhizopoda	Phylum Heliozoa
Phylum Ascetospora	Phylum Myxozoa	
KINGDOM III. CHROMISTA		
Subkingdom Heterokonta		
Phylum Bicosoecae	Phylum Dictyochae	Phylum Labyrinthomorpha
Phylum Diatomae	Phylum Phaeophyta	Phylum Raphidophyta
Phylum Pseudofungi		
Subkingdom Haptophyta		
Phylum Haptomonada		
Subkingdom Cryptophyta		
Phylum Cryptomonada		
Subkingdom Chlorarachniophyta		
Phylum Chlorarachniophyta		
KINGDOM III. PLANTAE		
Subkingdom Viridiplantae		
Phylum Prasinophyta	Phylum Chlorophyta	Phylum Ulvophyta
Phylum Charophyta		
Subkingdom Biliphyta		
Phylum Rhodophyta	Phylum Glaucophyta	
KINGDOM III. FUNGI		
Phylum Chytridiomycota		

Fig. 1.5. A detailed schematic drawing of the generalized somatic cytoarchitecture of a ciliate (copied from Lynn and Small, 1989). Nine kinetids are shown to illustrate the complexity of the microtubular arrangement within each cilium, as well as the fibrillar structures (a striated kinetodesmal fiber, a laterally-directed transverse ribbon, and a posteriorly-directed postciliary ribbon) associated with each kinetid that help provide structural support for the cell. The cell surface is covered by a plasma membrane (i.e. plasmalemma), which may contain indentations called parasomal sacs. Underlying the plasmalemma are the cortical alveoli, which are underlain by microtubules that lie on top of the dense epiplasm (i.e. supraepiplasmic microtubules). Basal microtubules may also be present running along side the kinetids, but not directly connected to them.


Base of cilium
Plasmalemma
Alveolus
Epiplasm
Supraepiplasmic microtubules
Pellicle
Parasomal sac
Cortex
Transverse microtubular ribbon
Kinetodesmal fibril
Kinetid
Kinetosome
Postciliary microtubular ribbon
Basal microtubules

The Ciliated Protozoa

The ciliated protists or ciliates are a very diverse assemblage of unicellular eukaryotes that comprise the phylum Ciliophora Doflein, 1901. With over 8,000 species (Lynn and Corliss, 1991), the ciliates have a wide variety of body shapes and sizes ranging from 10 μm to 4,500 μm. Three important criteria separate the ciliates from other protists: (1) the presence of a vegetative macronucleus and a germ-line micronucleus (dimorphic nuclei); (2) the process of sexual conjugation where two individuals temporarily join together to exchange gametic micronuclei; and (3) the presence of a complex infraciliature consisting of single, paired, or multiple kinetosomes with external ciliature covering most of the cell (Lynn and Corliss, 1991).

Ciliates can be found almost everywhere that water is located and they are the most conspicuous protists in aquatic ecosystems. They have been also found in soil, mud, desert sands, forest litter, tree holes, and in the digestive tracts of vertebrate and invertebrate hosts. Ciliates have been studied intensively by light and electron microscopy and have been routinely used as models because of their distribution and relative ease of culturing to study cytology, behaviour, biochemistry, morphogenesis, nutrition and most recently, molecular genetics (Small and Lynn, 1985). Moreover, their evolutionary history has been of interest to protistologists and evolutionary biologists because of their complex cytoarchitecture, diverse morphology, and habitat.

For almost two decades there have been at least two comprehensive and prominent classification schemes for the phylum Ciliophora, based primarily upon ultrastructural features of the ciliate cortex. One system is proposed by de Puytorac's group (de Puytorac *et al.*, 1984, 1987; de Puytorac, 1994) and the other scheme is proposed by Lynn

and his collaborators (Small and Lynn, 1981, 1985; Lynn and Corliss, 1991; Lynn and Small, 1998). In his most recent classification, de Puytorac (1994) divided the ciliates into four subphyla, with five super-classes containing 10 classes, 25 subclasses, and 70 orders. In contrast, Lynn and Small (1998) divide the ciliates into two subphyla, with 10 classes containing 16 subclasses and 54 orders. Although these two schemes appear quite similar, with both recognizing at least six classes (Colpodea, Heterotrichea, Karyorelictea, Litostomatea, Oligohymenophorea, and Phyllopharyngea), they differ greatly in the lumping or splitting of the major groups, relationships of higher taxa, and the relationships within each class. These differences arise primarily because of the researcher's interpretations of the significance of various structures (Lynn, 1991).

Although most ciliates are free-living, some species are obligate (e.g. *Ichthyophthirius*) and facultative (e.g. *Ophryoglena*) parasites of fish and invertebrates. Of the 10 monophyletic classes of ciliates proposed by Lynn and Small (1998) (see Table 1.1), only the class Litostomatea contains a group of ciliates (i.e. the subclass Trichostomatia), in which nearly all its members are endosymbionts of vertebrates (Table 1.4). Collectively known as the rumen ciliates, this agriculturally important group of endosymbionts was one of the few major groups of ciliates for which no SSrRNA gene sequence information was available.

The Rumen Ciliates

The rumen ciliates are the most abundant organisms that make up the rumen protozoa and are involved in host metabolism and digestion of plant material (Williams and Coleman,

Table 1.4. Distribution of some ciliate endosymbionts of vertebrate animals.

Species	Ruminant Host(s)
Balantidium coli	humans, pigs
Buetschlia parva *	cattle, sheep
Charonina ventriculi *	cattle, sheep
Cochliatoxum spp. *	horses
Cycloposthium spp.	kangaroos
Dasytricha ruminantium	almost all ruminants
Diplodinium dentatum	cattle, sheep
Diploplastron affine	cattle, goats, sheep
Enoploplastron confluens	musk-ox, reindeer
Entodinium caudatum	cattle, goats, sheep
E. dubardi species complex	blue duiker, cattle, goats, sheep
Eodinium monolobosum	cattle
Epidinium caudatum	cattle, sheep
Epidinium ecaudatum	cattle, sheep
Eremoplastron rostratum	cattle
Eudiplodinium maggii	bison
Isotricha intestinalis	almost all ruminants
Isotricha prostoma	almost all ruminants
Macropodinium spp.	kangaroos
Metadinium medium	cattle
Metadinium minorum	sheep
Microcetus lappus	bison, cattle
Ophryoscolex caudatus	cattle, goats, sheep
Ophryoscolex purkynjei	cattle, sheep
Ostracodinium mammosum	cattle
Parentodinium africanum *	brazilian cattle
Polyplastron mutivesiculatum	cattle, sheep

* indicates species that are rarely found.

1992). Rumen ciliates are found in the rumens of domesticated and wild ruminants (for a partial list see Table 1.5), in pseudoruminants (e.g. *Caloscolex* spp. are only found in old world camels), and in some non-ruminant animals (e.g. *Rhinozeta* spp. are only found in the black rhinoceros) (Williams and Coleman, 1992). Young ruminants isolated at birth do not contain rumen protozoa (Eadie, 1962; Dehority, 1978). However, these young animals become faunated either when infected adults regurgitate food and rumen fluid back into the mouth during rumination and salivate on feed which is then consumed by the young animal, or the protozoa are passed on by the mother to its offspring during grooming (Dehority, 1993).

The concentration of ciliates has been well documented in the rumen fluid of cattle and sheep, ranging between 6×10^4 and 4×10^6 ml^{-1} (Hungate, 1966). Albeit, higher concentrations of rumen ciliates, 5.77×10^6, 7.25×10^6 and 33.88×10^6 ml^{-1}, have been reported in the rumen contents from reindeer, white-tailed deer and blue duikers respectively (Westerling, 1970; Dehority, 1990, 1994). Thus it appears that the anaerobic environment within the rumen (i.e. 39° C with a pH near 6.5) is ideal for the formation of extremely dense populations of rumen protozoa, bacteria, and fungi that have evolved and adapted to this particular environment.

In the older literature (e.g. Kudo, 1947), the rumen ciliates were divided into two groupings: the holotrichs (order Holotricha) and the oligotrichs (order Spirotricha). When a revised classification of the phylum Protozoa was published by Honigberg *et al.* (1964), the orders Holotricha and Spirotricha were elevated to subclass status and the rumen oligotrichs were placed within the order Entodiniomorphida. In 1980, Levine *et al.* (1980) eliminated the subclass Holotricha and placed the rumen ciliates into two new orders, the Trichostomatida and the Prostomatida, within the Class Kinetogragminophorea.

Table 1.5. Rumen ciliates of some North American ruminants.

CATTLE
Entodinium bicarinatum
E. bursa
E. caudatum
E. costatum
E. dilobum
E. elongatum
E. exiguum
E. indicum
E. laterospinum
E. quadricuspis
E. rostratum
E. simulans
Eodinium bilobosom
E. monolobosum
Diplodinium flabellum
D. laeve
Eremoplastron bovis
E. rostratum
Ostracodinium clipeolum
O. dilobum
O. gracile
O. mammosum
O. obtusum
O. trivesculatum
Polyplastron multivesiculatum
Metadinium medium
Epidinium caudatum
E. ecaudatum
E. tricaudatum
Ophryoscolex caudatus
O. purkynjei
Isotricha prostoma
I. intestinalis
Dasytricha ruminantium

MOOSE
Entodinium alces
E. dubardi
E. enguum

SHEEP
Entodinium bursa
E. caudatum
E. nanum
Eremoplastron bovis
Ostracodinium minorum
Polyplastron multivesiculatum
Metadinium minorum
M. tauricum
Epidinium parvicaudatum
E. quadricaudatum
Ophryoscolex buissoni
O. bicinctus
O. bicoronatus
O. caudatus
O. inermis
Isotricha prostoma
I. intestinalis
Dasytricha ruminantium

REINDEER
Entodinium anteronucleatum
E. bicornutum
E. damae
E. dilobum
E. quadricuspis
Diplodinium dogieli
D. rangiferi
Eremoplastron tarandi
E. spectabile
Ostracodinium obtusum
Polyplastron articum
Metadinium magnum
Epidinium ecaudatum
E. gigas
Enoploplastron confluens

BIGHORN SHEEP
Entodinium montanum
E. nanum
E. ogimotoi
E. orbicularis
E.. protuberans
E. sierrae
Polyplastron californiense
Metadinium tauricum
Isotricha prostoma
Dasytricha ruminantium

MUSK-OXEN
Entodinium ovibos
Metadinium banksi
M. caudatum
M. magnum
Epidinium bicaudatum
E. cattanei
E. ecaudatum
E. gigas
E. parvicaudatum
E. quadricadatum
E. tricaudatum
Enoploplastron confluens

DEER
Entodinium abruptum
E. cervi
E. convexum
E. costatum
E. dubardi
E. medium
E. rhomboideum
Isotricha prostoma
I. intestinalis
Dasytricha ruminantium

Although the term holotrich is no longer strictly appropriate, it is still used in the current literature to refer to the non-entodiniomorphid ciliates. At present, Grain (1994b) recognizes three orders (Trichostomatida, Entodiniomorphida, and Blepharocorythida) of rumen ciliates within his class Vestibuliferea, the sister group to the class Litostomatea. In contrast, Lynn and his colleagues (Small and Lynn, 1985; Lynn and Small, 1998) recognize two orders of rumen ciliates (Vestibuliferida and Entodiniomorphida), within the subclass Trichostomatia, a sister group to the free-living haptorians (subclass Haptoria) within the class Litostomatea.

However, when Lipscomb and Riordan (1992) examined 46 morphological and ultrastructural characters for 21 genera of litostomes, they concluded that Small and Lynn's (1985) classification of the rumen ciliates was paraphyletic. In their study, the two vestibuliferid families, Isotrichidae and Balantidiidae, and the entodiniomorphid family, Buetschliidae, grouped within the subclass Haptoria and not within the subclass Trichostomatia. However, some characters used in their analysis may not be valid, such as the distribution of chromatin, which is dependent on the cell's physiological state. In addition, there is a considerable amount of missing data (10-16 missing or unknown characters out of 46) for those taxa whose phylogenetic position was under investigation.

Research Objectives

Since the mid-1950's relatively little phylogenetic research has been carried out on the rumen ciliates. Consequently, little is known about the phylogenetic relationships within and among these diverse and unique ciliates. Further, because most of these organisms are

morphologically similar and have overlapping size ranges, there is also uncertainty over the number of recognized species and genera. In the following section, I will briefly discuss my original research and major objectives for the next three chapters.

Chapter Two. Firstly, for almost 75 years, researchers have speculated on the evolution of members belonging to the largest family of rumen ciliates, the Ophryoscolecidae (Crawley, 1923; Dogiel, 1925, 1947; Lubinsky, 1957b, 1957c). Using morphological characters, such as the degree of torsion and shape of the cell, position of the nuclei and organelles, and the number of skeletal plates, Lubinsky (1957c) constructed a cladogram of the ophryoscolecids. On the basis of his analysis he divided the family into three subfamilies to reflect degrees of complexity. For example, (1) *Entodinium* was placed by itself in the subfamily Entodiniinae because it has only one ciliary band and no skeletal plates; (2) *Diplodinium*, *Eudiplodinium*, and *Polyplastron* were placed within the subfamily Diplodiniinae because they have two bands of cilia on the same transverse plane; and (3) *Epidinium* and *Ophryoscolex* were placed within the subfamily Ophryoscolecinae because they have one of their two ciliary bands near the midpoint of the cell surface. With our present knowledge of phylogenetic concepts, Lubinsky's (1957c) actual tree depicts the diplodiniinines as paraphyletic.

Secondly, Leipe and Hausmann (1989) replaced the subclass Haptoria with a new subclass, the Ditransversalia to include only those organisms with two transverse microtubular ribbons. As a result, their new subclass did not include traditional haptorians, such as *Didinium*, *Dileptus*, *Helicoprorodon*, *Mesodinium*, and *Monodinium* (i.e. they were believed to have only a single transverse microtubular ribbon). Furthermore, they removed the order

Vestibuliferida (*Balantidium*, *Isotricha*) from the subclass Trichostomatia and placed it within the Ditransversalia. Despite Lipscomb and Riordan's (1992) opposition to this new subclass, they agreed with Leipe and Hausmann's (1989) assessment that Small and Lynn's (1985) classification of the Haptoria was paraphyletic. In addition, Lipscomb and Riordan's (1992) cladistic analysis of 21 litostomes using morphological and ultrastructural characters showed that the vestibuliferids (*Isotricha* and *Balantidium*) grouped within the haptorian clade. Despite this, Grain (1994a) did not include the vestibuliferids (*Isotricha*, *Balantidium*) within his new classification of the "haptorian" litostomes. Moreover, Lynn and his collaborators (Small and Lynn, 1985; Lynn and Small, 1998) have always maintained that the vestibuliferids are the sister group to the entodiniomorphids within the subclass Trichostomatia and not within the subclass Haptoria.

Finally, in addition to the rumen ciliates belonging to the families Isotrichidae (order Vestibuliferida) and Ophryoscolecidae (order Entodiniomorphida), there are 12 additional families of ciliates within the subclass Trichostomatia *sensu* Small and Lynn that inhabit the forestomach and large intestine of a variety of vertebrate animals (e.g. camels, gorillas, elephants, hippopotamuses, horses, kangaroos, rhinoceroses, rodents, and wart-hogs). Two of the 12 families, Balantidiidae and Paraisotrichidae belong within the Vestibuliferida and the other ten families, Buetschliidae, Blepharycorythidae, Cycloposthiidae, Ditoxidae, Macropodiniidae, Polydiniellidae, Rhinozetidae, Spirodiniidae, Telamodiniidae, and Troglodytellidae belong within the Entodiniomorphida. In contrast, Grain (1994b) has suggested that there are six other families in his order Trichostomatida, which is the taxon comparable to the order Vestibuliferida *sensu* Small and Lynn. Thus,

the taxonomy of these ciliates is inconsistent. In fact, some of these families may have been arbitrarily placed within the Entodiniomorphida because of the similar gastrointestinal habitats they occupy.

This chapter presents for the first time the use of DNA sequence analysis to examine phylogenetic relationships of the rumen protozoa. SSrRNA genes from the ciliates, *Balantidium coli*, *Cycloposthium* sp., *Dasytricha ruminantium*, *Didinium nasutum*, *Dileptus* sp., *Diplodinium dentatum*, *Enchelyodon* sp., *Entodinium caudatum*, *Epidinium caudatum*, *Eudiplodinium maggii*, *Isotricha intestinalis*, *Isotricha prostoma*, *Macropodinium yalanbense*, *Ophryoscolex purkynjei*, and *Polyplastron multivesiculatum* are introduced and used in a phylogenetic analysis with other ciliates. Evolutionary relationships within the Ophryoscolecidae and the phylum Ciliophora are examined to determine: (1) if the rumen ciliates still form a monophyletic group, (2) if Lubinsky's (1957c) three subfamilial divisions of the family Ophryoscolecidae are supported by molecular data, and (3) if *En. caudatum* represents the earliest branching rumen ciliate, (4) if the rumen ciliates are the sister group to the free-living haptorian ciliates, (5) if the vestibuliferids belong within the haptorian clade and not with the entodiniomorphids as proposed by Leipe and Hausmann (1989) and Lipscomb and Riordan (1990, 1992), and (6) if the haptorians and trichostomes (i.e. vestibuliferids plus entodiniomorphids) form monophyletic groups, thereby suggesting that vertebrate endosymbiosis possibly occurred only once in the evolution of the ciliates, and (7) if the class Litostomatea *sensu* Small and Lynn (1985), to which the rumen ciliates belong, is monophyletic.

Chapter Three. The identification of "sibling species" or "species complexes" has always proven difficult. From an evolutionary perspective, analysis of the detailed genetic structure of strains and sibling species may help increase our understanding of the evolutionary mechanisms giving rise to this genetic differentiation. The ITS-1 and ITS-2 regions are considered to be quite variable and have been useful for identifying and discriminating interspecific and intraspecific genetic variation (White *et al.*, 1990; Allard and Honeycut, 1991; Hillis and Dixon, 1991; Schlötterer *et al.*, 1994). Thus, ITS spacers could be potentially useful for discovering patterns of evolution of the rumen ciliates.

Cells of *I. prostoma* are found in a variety of vertebrate animals worldwide and are morphologically the same. *Isotricha*-like organisms have also been reported in marsupials (Obendorf, 1984; Dellow *et al.*, 1988) and in the hoatzin (Domínguez-Bello *et al.*, 1993), a unique foregut fermenting bird of South America. Even though *I. prostoma* has a global distribution, little information is known about the extent of genetic divergence among isolates (i.e. do populations of *I. prostoma* in the same host species on different continents differ, or do populations of *I. prostoma* in different hosts on the same continent differ?). In this chapter, eight isolates of *I. prostoma* from two continents (North America and Australia) are examined to discover the extent of variation in their ITS-1 and ITS-2 regions, as well as their 5.8S LSrRNA.

Chapter Four. Because genetic distances are roughly linearly correlated with divergence time (i.e. clock-like), the use of divergence data to estimate the origin of groups, like ciliates, for which there is little or no fossil record is a common application in molecular systematics (Hillis *et al.*, 1996). Moreover, it can provide useful insights into a

group's evolution (Saunders and Druehl, 1992, Doolittle *et al.*, 1996; Hillis *et al.*, 1996; Wray *et al.*,, 1996). This chapter presents the first molecular chronometer to be calibrated for any protozoan group and will suggest two possible SSrRNA molecular clocks for the ciliates (i.e. a slow clock for the free-living ciliates, and a faster clock for the endosymbionts).

Ichthyophthirius is an obligate ectoparasite of freshwater teleosts. Its closest relative based upon morphological (Canella, 1964, Lynn *et al.*, 1991) and molecular data (Wright and Lynn, 1995) is the free-living, but histophagous ciliate *Ophryoglena*. From the fossil record the oldest known freshwater teleost is *Lycoptera*, a freshwater osteoglossomorph from the very late Jurassic/early Cretaceous of China, approximately 145 million years ago (Schaeffer and Patterson, 1984; Wilson and Williams, 1992; Patterson, 1993). From this information the rate of nucleotide substitution can be determined and the maximum age of divergence of the ciliates calculated. In this chapter, the pair-wise genetic distances of the SSrRNA sequences from most ciliates are examined and used to calibrate a molecular clock in order (1) to predict the time of divergence of the major ciliate lineages, and (2) to serve as an important model to researchers who may want to estimate the origin of other major lineages of protozoa for which there is little or no fossil record evidence available, except for their hosts.

CHAPTER TWO

Molecular Phylogeny Of The Endosymbiotic Ciliates Of Vertebrate Animals Inferred From Small Subunit Ribosomal RNA Sequences.

"To see a world in a grain of sand,
and heaven in a wild flower,
Hold infinity in the palm of your hand,
and eternity in an hour."

William Blake, Auguries of Innocence.

INTRODUCTION

Ciliates belonging to the subclass Trichostomatia *sensu* Small and Lynn are primarily endosymbionts of vertebrate animals. Discovered 155 years ago (Gruby and Delafond, 1843), these endosymbionts are collectively known as the rumen ciliates. They are the most abundant protozoa in the rumen and are involved in host metabolism and digestion of plant material. Over the past 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.

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 (for a review see Figure 1.5) both evident only during kinetosomal replication, and (3) microtubular bundles (nematodesmata) that extend into the cytoplasm from the bases of kinetids that surround the cytostome (Lynn and Corliss, 1991). At present, there are two classification schemes for the rumen ciliates based primarily upon ultrastructural features of the ciliate cortex. In Grain's (1994b) classification system, the rumen ciliates entirely comprise the class Vestibuliferea, with three orders (Trichostomatida, Entodiniomorphida and Blepharocorythida) (Table 2.1), and are a sister group to the class Litostomatea within the Subphylum Filicorticata. Lynn and his collaborators (Small and Lynn, 1981; Small and Lynn, 1985; Lynn and Corliss, 1991; Lynn and Small, 1998) recognized two orders of these endosymbionts, the Entodiniomorphida and the Vestibuliferida within the subclass Trichostomatia, which is the sister group to the subclass Haptoria within the class

Table 2.1. Classification schemes of the endosymbiotic ciliates of vertebrate animals as proposed by Lynn and Small (1998) and Grain (1994b). Not all taxa are shown.

Lynn and Small (1998)	Grain (1994b)
Class Litostomatea[1]	Class Vestibuliferea
Subclass Trichostomatia	Order Trichostomatida
Order Vestibuliferida	Family Balantidiidae
Family Balantidiidae	Family Coelosomididae
Family Isotrichidae	Family Conchostomatidae
Family Paraisotrichidae	Family Dasytrichidae
	Family Isotrichidae
	Family Paraisotrichidae
	Family Pycnotrichidae
	Family Protocaviellidae
	Family Trichospiridae
Order Entodiniomorphida	Order Entodiniomorphida
Suborder Entodiniomorphina	Family Cycloposthiidae
Family Cycloposthiidae	Subfamily Monoposthiinae
Family Ditoxidae	Subfamily Cycloposthiinae
Family Macropodiidae	Family Entodiniidae
Family Ophryoscolecidae	Family Ophryoscolecidae
Family Polydiniellidae	Subfamily Diplodiniinae
Family Rhinozetidae	Subfamily Epidiniinae
Family Spirodiniidae	Subfamily Opisthotrichinae
Family Telamodiniidae	Subfamily Ophryoscolecinae
Family Troglodytellidae	Subfamily Caloscolecinae
	Family Polydiniellidae
Suborder Archistomatina	Family Prototapirellidae
Family Buetschliidae[2]	Family Rhinozetidae
	Family Spriodiniidae
Suborder Blepharocorythina	Family Tripalmariidae
Family Blepharocorythidae	Family Troglodytellidae
	Order Blepharocorythida
	Family Blepharocorythidae

1. The class Litostomatea also contains the subclass Haptoria.

2. In Grain's (1994b) classification scheme, the family Buetschliidae is assigned to the order Haptorida within his class Litostomatea.

Litostomatea (see Table 2.1). 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.

The Entodiniomorphids

Generally, the entodiniomorphids are characterized by the presence of a rigid, often spiny, pellicle with cilia present mainly, or sometimes only in the oral region. Within the order Entodiniomorphida (Small and Lynn, 1985), more than half of the genera of rumen ciliates belong to the family Ophryoscolecidae, making it the largest family of rumen ciliates (see Table 2.1). These ciliates are abundant and easily distinguishable from other rumen ciliates by the presence of spines, skeletal plates (except for *Entodinium* and *Diplodinium*), and tufts of ciliature confined to the adoral and dorsal surfaces of the cell. Species of *Entodinium* only have an adoral zone of syncilia (AZS), whereas all other ophryoscolecids have an AZS and an additional dorsal zone[1] of syncilia (DZS). The DZS can be located either on the same

[1] The ventral surface of ciliates has classically been defined as the side of the cell on which the oral cavity opening is situated. Although some workers followed this convention for the rumen ciliates (Kofoid and MacLennan, 1930; Dogiel, 1947; Lubinsky, 1957b), Lubinsky (1958) and Dehority (1993) have deviated away from this approach and termed the side of the cell where the micronucleus is situated to the left of the macronucleus as the "upper" surface. This surface then determines the lower surface (opposite side) and the "left" and "right" sides. In order to avoid confusion, I will follow the classical definition and assume ventral is the surface onto which the oral cavity opens.

transverse plane as the AZS, between one quarter and one third of the way down the cell, or at the equator of the cell.

Crawley (1923), Dogiel (1925, 1947), and Lubinsky (1957a, 1957b, 1957c) have speculated on evolution within the Ophryoscolecidae. They believed that *Entodinium* species, referred to as "lower" ophryoscolecids, were more ancestral because they had only one AZS, one contractile vacuole, lacked skeletal plates, and had no more than one spine (Figure 2.1). They also concluded that *Entodinium*-like species were probably the first to colonize the rumen. Lubinsky (1957b, 1957c) produced a phylogeny of the ophryoscolecids using as characters: the degree of torsional displacement of the nuclei and vacuoles, the distance between the AZS and DZS, the development of the skeletal plates, the position of cellular organelles, and the size of the cell. From this analysis, he divided the Ophryoscolecidae into three subfamilies to reflect his "natural" groupings. (1) Because of the presumed ancestral characters of *Entodinium*, it was placed by itself into the subfamily Entodiniinae (Figure 2.1). (2) Ophryoscolecids with the DZS on the same transverse plane as the AZS (e.g. *Diplodinium*, *Eudiplodinium*, *Polyplastron*) were placed within the Diplodiniinae (Figure 2.2). (3) Ophryoscolecids with the DZS near the midpoint of the dorsal surface of the cell (e.g. *Epidinium*, *Ophryoscolex*) were placed within the Ophryoscolecinae (Figure 2.3).

This supported Lubinsky's (1957c) views on the evolution of the group. It is important to note that Lubinsky's (1957c) evolutionary tree depicts the Diplodiniinae as paraphyletic (Figure 2.4). I have reanalysed these relationships using a cladistic approach (Hennig, 1966) based on the limited morphological and ultrastructural data for rumen ciliates (Table 2.2). Indeed, there are no synapomorphies supporting the Diplodiniinae (Table 2.3, Figure 2.5). I

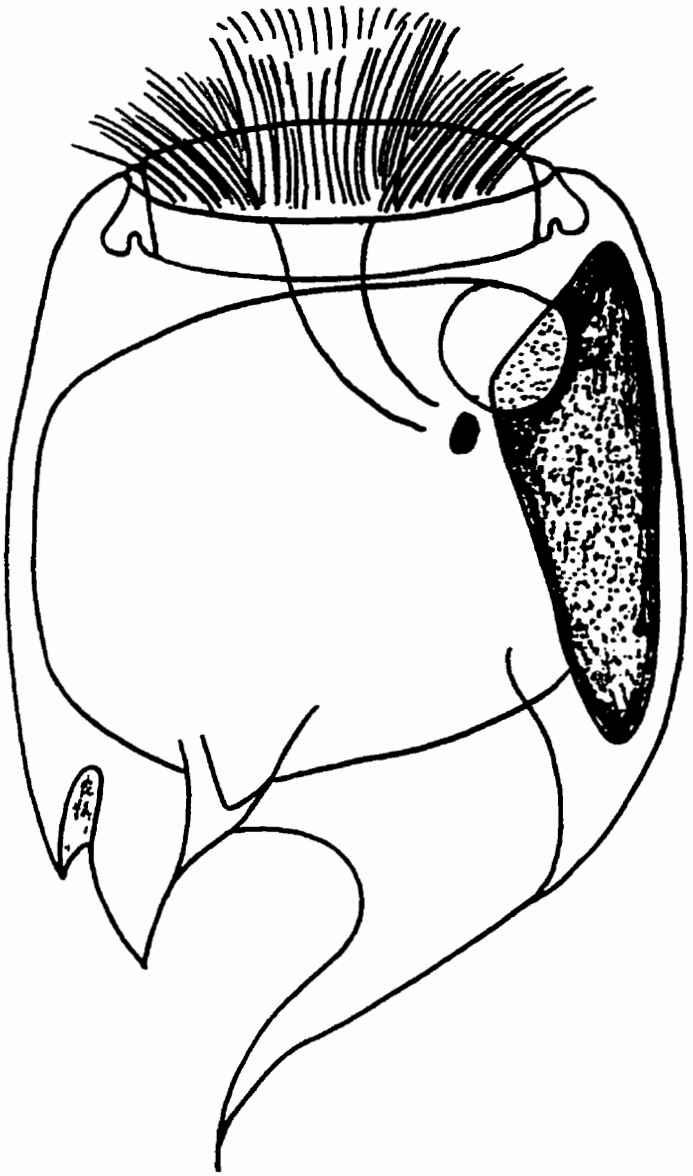


Fig. 2.1. A schematic drawing of the rumen ciliate *Entodinium caudatum* (taken from Dehority, 1993). According to Lubinsky (1957c), this ciliate is the ancestral form because of its single ciliary band and absence of skeletal plates. Based on these "primitive" features, he assigned *Entodinium* to the subfamily Entodiniinae.

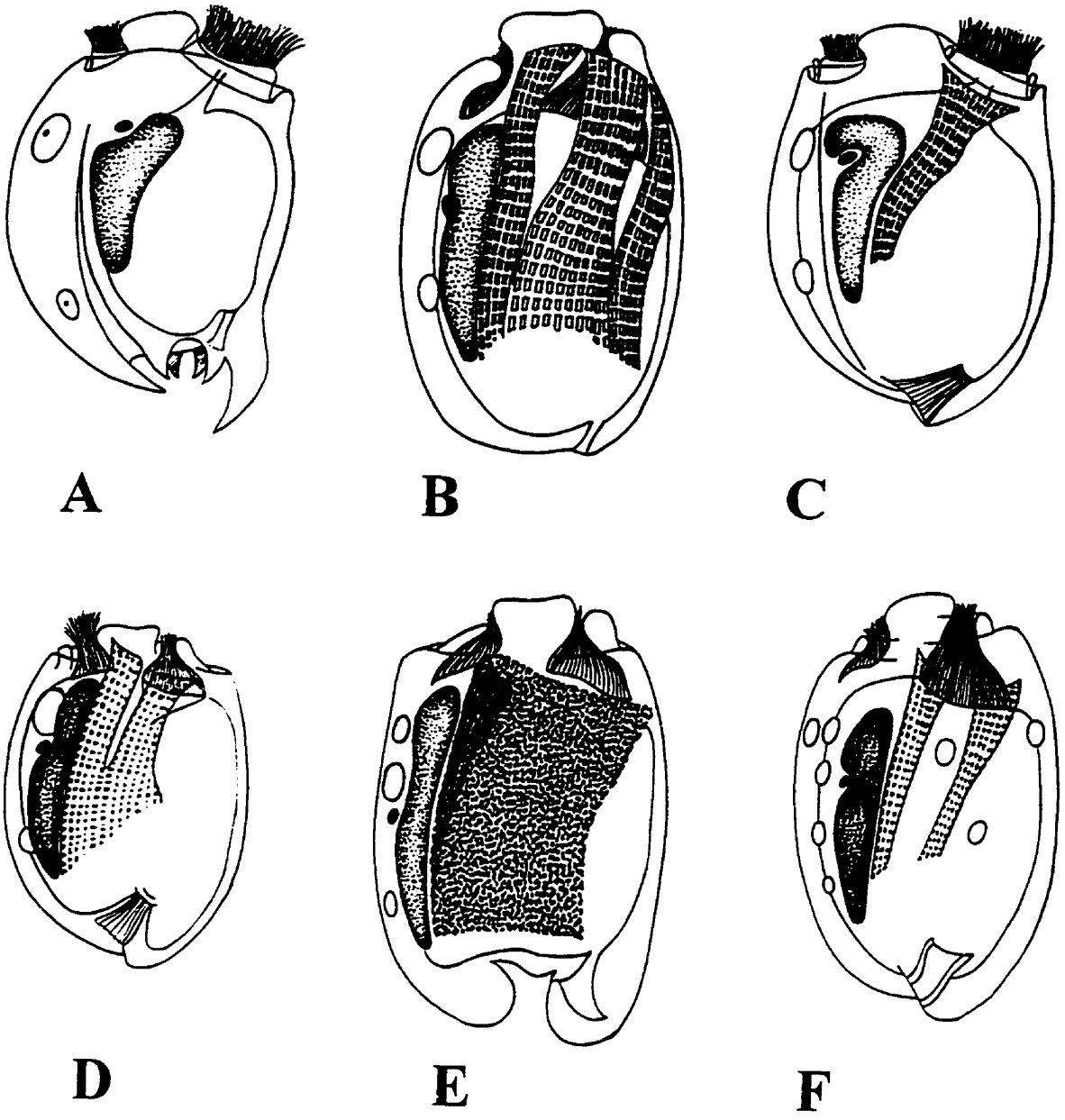


Fig. 2.2. Schematic drawings of the rumen ciliates (taken from Dehority, 1993) assigned to the subfamily Diplodiniinae. **(A)** *Diplodinium dentatum*, **(B)** *Enoploplastron confluens*, **(C)** *Eudiplodinium maggii*, **(D)** *Metadinium minorum*, **(E)** *Ostracodinium mammosum*, and **(F)** *Polyplastron multivesiculatum*. Notice the two ciliary bands, and with the exception of *Diplodinium*, all these cells have one or more skeletal plates (note that *Polyplastron* has five skeletal plates, although only two are visible from this side).

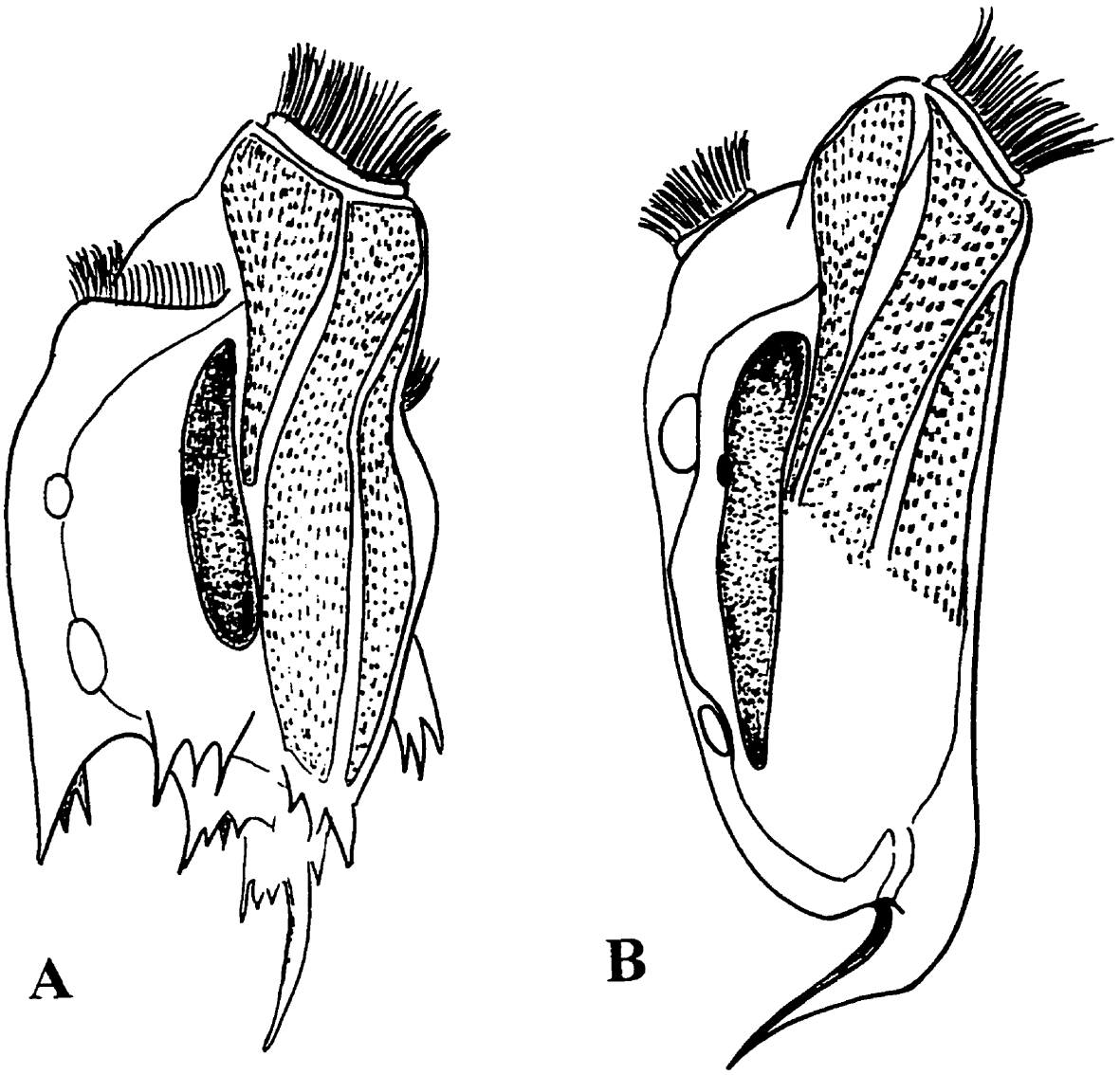


Fig. 2.3. Schematic drawings of the rumen ciliates (taken from Dehority, 1993) assigned to the subfamily Ophryoscolecinae. **(A)** *Ophryoscolex caudatus* and **(B)** *Epidinium caudatum.* Similar to the members of the subfamily Diplodiniinae, these cells also have two ciliary bands, but they are located on different cell planes. These cells are thought to be the most evolutionarily derived of the ophryoscolecid rumen ciliates. Notice the many spines.

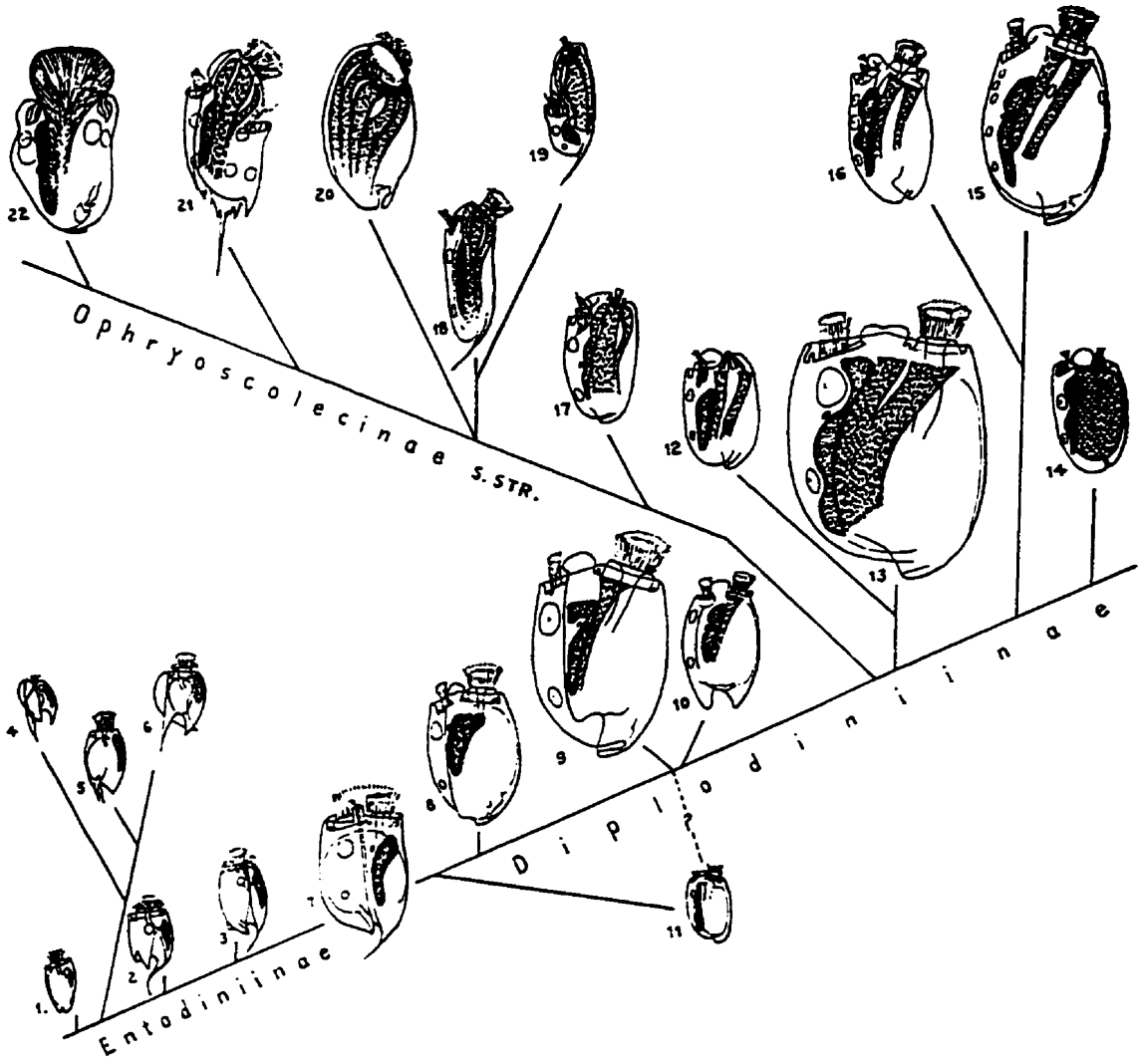


Fig. 2.4. A copy of Lubinsky's (1957b) evolutionary tree of the ophryoscolecid rumen ciliates based on morphological characters and the degree of torsion of the cell (see text for detail). According to Lubinsky's (1957b) tree, his subfamily Diplodiniinae is paraphyletic. Numbers are: **(1)** *Entodinium nanellum*, **(2)** *E. rectangulatum*, **(3)** *E. simulans*, **(4)** *E. indicum*, **(5)** *E. bifidum*, **(6)** *E. tricostatum*, **(7)** hypothetical ancestor of the higher Ophryoscolecidae, **(8)** *Diplodinium laeve*, **(9)** *Eudiplodinium maggii*, **(10)** *Eremoplastron dilobum*, **(11)** *Eodinium lobatum*, **(12)** *Diploplastron affine*, **(13)** *Metadinium tauricum*, **(14)** *Ostracodinium gracile*, **(15)** *Polyplastron multivesiculatum*, **(16)** *Elytroplastron bubali*, **(17)** *Enoploplastron triloricatum*, **(18)** *Epidinium caudatum*, **(19)** *Opisthotrichum janus*, **(20)** *Epiplastron africanum*, **(21)** *Ophryoscolex janus*, and **(22)** *Caloscolex camelinus*.

Table 2.2. Morphological characters of rumen ciliates based on data presented in Lubinsky (1957b) and Furness and Butler (1988).

1. Presence of lateral groove (1)*
2. At least one skeletal plate (1)
3. Micronucleus between macronucleus and nearest profile line of body (1)
4. Contractile vacuoles greater than one (1)
5. Esophageal sheath and vesicular phagoplasm, present (1)
6. Transverse crests supported by microtubules, present (1)
7. Phagoplasmic tongue or peristome, present (1)
8. Two anterior retractable zones of ciliature at different levels (1)
9. Two anterior retractable zones of ciliature at same level (1)
10. One retractable anterior zone of ciliature (1)

* (1) indicates derived state compared to *Blepharocorys* as outgroup (see Table 2.3).

Table 2.3. Character coding of morphological characters for rumen ciliates and the outgroup taxon *Blepharocorys*. Refer to **Table 2.2.** for character definitions.

Genus	Character 1 2 3 4 5	6 7 8 9 0
Blepharocorys	0 0 ? 0 ?	? ? 0 0 0
Entodinium	1 0 0 0 0	1 0 0 0 1
Diplodinium	1 0 1 1 ?	? 0 0 1 1
Eudiplodinium	1 1 1 1 0	0 0 0 1 1
Epidinium	0 1 1 1 1	0 1 1 1 1
Ophryoscolex	0 1 1 1 ?	0 ? 1 1 1
Ostracodinium	0 1 1 1 ?	? 0 0 1 1
Polyplastron	0 1 1 1 ?	? 0 0 1 1

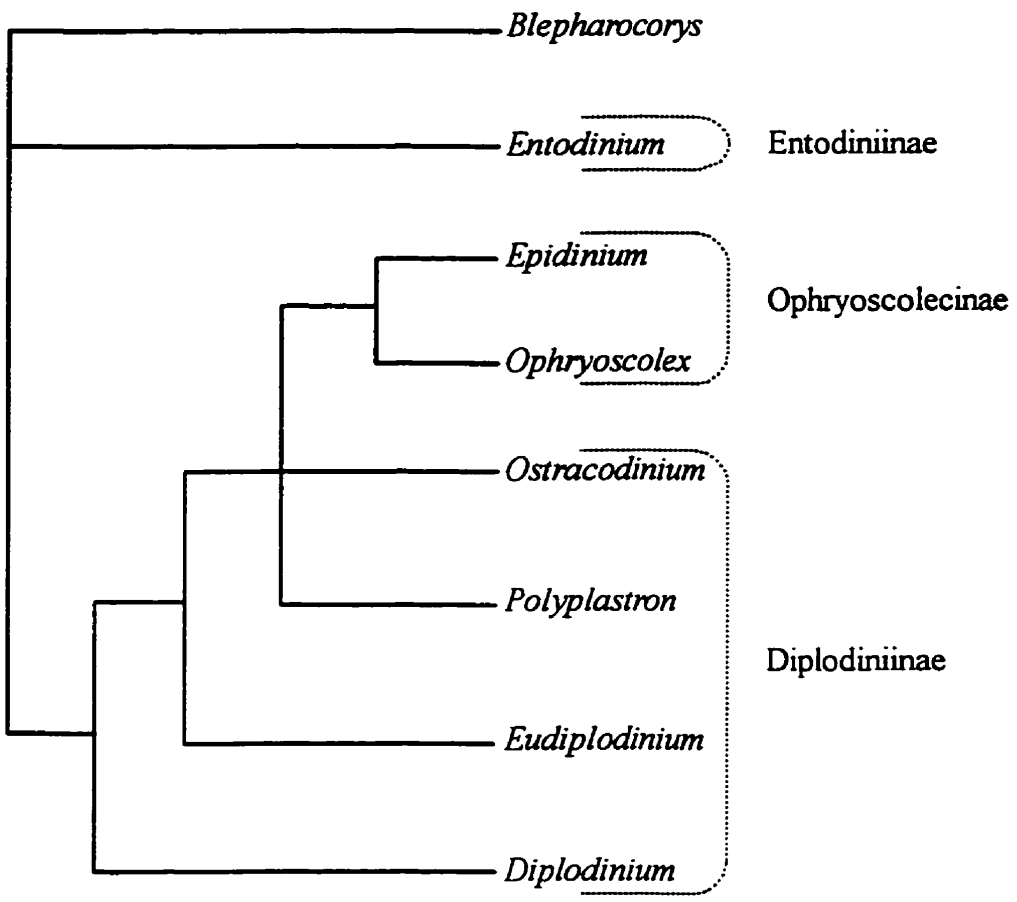


Fig. 2.5. One of three equally parsimonious cladograms for the entodiniomorphid rumen ciliates inferred from morphological and ultrastructural characters presented in Table 2.2 and coded as in Table 2.3. This tree shows an unresolved relationship amongst *Polyplastron*, *Ostracodinium*, and the Ophryoscolecinae (*Epidinium*, *Ophryoscolex*), whereas, the other two equally parsimonious trees depicted either *Polyplastron* or *Ostracodinium* as the sister group to the Ophryoscolecinae. Trees were produced using the branch and bound search program of PAUP (ver. 3.1) (Swofford, 1993), and have a tree length of 11, a consistency index of 0.91, and a retention index of 0.86.

have thus undertaken to discover whether molecular features of the 18S rRNA genes will support the monophyly of the Diplodiniinae.

Furness and Butler (1988) 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 (1957c), has a tube-like cytopharynx and shows the greatest development of cytoalimentary organization over *Polyplastron* (an evolutionarily 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 (1988), based on ultrastructural characters, corroborated the earlier work of Dogiel (1947) and Lubinsky (1957a, 1957b, 1957c).

There are 10 additional families of ciliates (Buetschliidae, Blepharocorythidae, Cycloposthiidae, Ditoxidae, Macropodiniidae, Polydiniellidae, Rhinozetidae, Spirodiniidae, Telamodiniidae, and Troglodytellidae) within the order the Entodiniomorphida that inhabit the forestomach and large intestine of a variety of vertebrate animals. For example, *Cycloposthium* (Cycloposthiidae) inhabits various vertebrate animals like hippopotamuses, elephants, horses, and its presence in kangaroos is a new host record for this genus.

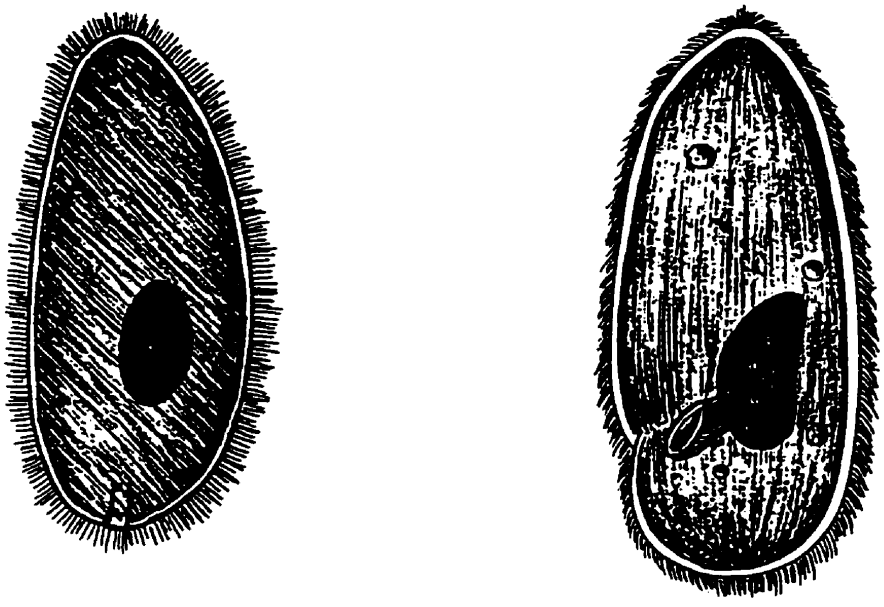
The taxonomy of these 10 families of entodiniomorphids is inconsistent (see Small and Lynn, 1985; Grain, 1994b; and Lynn and Small, 1998). In fact, some of these families may have been improperly placed within the Entodiniomorphida because of the similar

gastrointestinal habitats they occupy. For example, *Macropodinium* is a representative of the newest family of entodiniomorphids, Macropodiniidae Dehority, 1996, that inhabits the sacciform forestomach of macropod marsupials (wallabies and kangaroos) and is dissimilar to other members belonging to this large order. Thus, the elucidation of these relationships is important in understanding the evolution of the group and in determining whether vertebrate endosymbiosis evolved only once or more than once in the evolution of the ciliates.

The Vestibuliferids

The vestibuliferids are characterized by having more flexible pellicles with holotrichous ciliation (Figure 2.6). Small and Lynn (1985) recognized three families within their order Vestibuliferida: the Balantidiidae Reichenow in Doflein and Reichenow, 1929, Isotrichidae Bütschli, 1889, and Paraisotrichidae da Cunha, 1917 (Small and Lynn, 1985) (see Table 2.1). Balantidiids are typically found in the intestine of vertebrate animals and include *Balantidium coli*, the only ciliate that is known to be harmful to humans. Species belonging to the Paraisotrichidae are typically found in horses. The isotrichids, *Isotricha*, *Dasytricha*, and *Oligoisotricha*, are more widespread than the other vestibuliferids, being found in a wide variety of ruminants. In addition to these three families, Grain (1994b) has suggested that there are six other families in his order Trichostomatida (see Table 2.1), which is the taxon comparable to the order Vestibuliferida *sensu* Small and Lynn.

Dogiel (1947), Grain (1966), and Corliss (1979) have speculated on the evolution of the vestibuliferids. Grain (1966) proposed that these ciliates evolved from several evolutionary



A B

Fig. 2.6. A schematic drawing of **(A)** *Dasytricha ruminantium* and **(B)** *Isotricha intestinalis*. Notice the complete somatic ciliature and the absence of skeletal plates and spines (taken from Dehority, 1993).

lines of descent from a prostomial ancestral form that had oral nematodesmata, mucocysts, and toxicysts. Within the evolutionary line leading to the Isotrichidae, the prostomial ancestor lost toxicysts and mucocysts while its somatic kineties invaginated to form the vestibulum (Grain, 1966). With a gradual migration posteriorly and repositioning of the vestibulum, the genera *Isotricha* and *Dasytricha* evolved. Since Grain's (1966) evolutionary proposal, electron microscopy studies (see Lynn, 1991) have helped to define distinct clades, and now I can be more precise about putative ancestors. Thus, it is my view that because somatic kinetids are similar among litostomes (Lynn, 1991), the trichostome ciliates evolved from an haptorian-like ancestor that lost its oral toxicysts and oral dikinetids as it colonized the gastrointestinal environment of a variety of vertebrate species. I imagine that an ancestral holotrichous vestibuliferid, like *Balantidium*, evolved first, establishing symbiotic relationships with fishes (Grim, 1985, 1989, 1993). As vertebrate groups diversified, these ancestral vestibuliferids also diversified, giving rise to the other vestibuliferid genera and to the entodiniomorphids.

Monophyly Or Paraphyly Of The Rumen Ciliates

The subclass Haptoria *sensu* Small and Lynn contains three orders of ciliates, the Haptorida Corliss, 1974, the Pleurostomatida Schewiakoff, 1896, and the Pharyngophorida Small and Lynn, 1985. However, the relationships within this free-living group of predatory cells remain uncertain (Foissner and Foissner, 1988; Leipe and Hausmann, 1989; Lipscomb and Riordan, 1990; 1992). Generally, the haptorian somatic kinetid possesses two transverse microtubular ribbons, a convergent postciliary microtubular ribbon that extends posteriorly in double row stacks, and a laterally directed kinetodesmal fibre (refer to Figure 1.5) (Lynn and

Nicholls, 1985; Foissner and Foissner, 1988; Lipscomb and Riordan, 1990, 1992). However, Leipe and Hausmann (1989) argued that some species traditionally placed within the Haptoria do not have two transverse microtubular ribbons (e.g. *Didinium*), while some trichostomes have two transverse microtubular ribbons (e.g. *Isotricha*). Leipe and Hausmann (1989) replaced the subclass Haptoria with a new subclass, the Ditransversalia Leipe and Hausmann, 1989, to include only those organisms with two transverse microtubular ribbons. Thus, the Ditransversalia does not include traditional haptorians, such as *Didinium*, *Dileptus*, *Helicoprorodon*, *Mesodinium* and *Monodinium*, believed to have only a single transverse microtubular ribbon. Furthermore, because *Isotricha* and *Balantidium* have these two transverse microtubular ribbons, the order Vestibuliferida was removed from the subclass Trichostomatia and placed within the Ditransversalia.

Lynn (1991) and Lipscomb and Riordan (1990; 1992) opposed Leipe and Hausmann's (1989) suggestion of a new subclass for the following reasons: (1) several taxa having a single transverse microtubular ribbon may actually have a second set [e.g. *Helicoprorodon* (Lipscomb and Riordan, 1990) and *Didinium* (Lipscomb and Riordan, 1992)], which would invalidate Leipe and Hausmann's (1989) decision to separate them from the other haptorians; and (2) in species where a second set is not present, it may be more parsimonious to conclude that they were secondarily lost rather than primitively absent. Certainly, a putative second transverse ribbon microtubule appears transiently during somatic kinetid replication in the entodiniomorphid *Eudiplodinium* (Furness and Butler, 1986). Despite their opposition to a new subclass, Lipscomb and Riordan (1992) agreed with Leipe and Hausmann's (1989) assessment that Small and Lynn's (1985) classification of the Haptoria was paraphyletic: their

cladistic analysis of 21 litostomes using morphological and ultrastructural characters showed that the vestibuliferids (*Isotricha* and *Balantidium*) and the buetschliids (*Didesmis* and *Alloiozona*) grouped within the haptorian clade.

A few years ago, Grain (1994a) proposed a classification for his class Litostomatea, which does not include the subclass Ditransversalia Leipe and Hausmann, 1989. Moreover, Grain (1994a) discontinued the subclass Haptoria, transferred the subclass Trichostomatia *sensu* Small and Lynn to his class Vestibuliferea (see Table 2.1) (Grain, 1994b), and divided the litostomes (i.e. Haptoria) into five orders (Haptorida, Helicoprorodontida, Mesodiniida, Pleurostomatida, Spathidiida) and 17 families (Table 2.4). Although Grain (1994a) retained the buetschliids within the Haptorida and not within the entodiniomorphids, he did not include the vestibuliferids (i.e. *Isotricha* and *Balantidium*) within his new classification of the "haptorian" litostomes as suggested by Lipscomb and Riordan (1992).

For this study, I sequenced in both directions (see Figure 1.2B) the complete SSrRNA genes from six ophryoscolecids (*Diplodinium dentatum*, *Entodinium caudatum*, *Epidinium caudatum*, *Eudiplodinium maggii*, *Ophryoscolex purkynjei*, and *Polyplastron multivesiculatum)*, two putative entodiniomorphids (*Cycloposthium* sp. and *Macropodinium yalanbense)*, four vestibuliferids (*Balantidium coli*, *Dasytricha ruminantium*, *Isotricha prostoma*, and *Isotricha intestinalis)*, and three free-living haptorians (*Didinium nasutum*, *Dileptus* sp., and *Enchelyodon* sp.). These new sequences were compared to SSrRNA gene sequences from other ciliates and eukaryotes and phylogenetic analyses were used to determine: (1) if the ophryoscolecid rumen ciliates form a monophyletic group; (2) if Lubinsky's (1957c) three subfamilial divisions of the family Ophryoscolecidae are supported by

Table 2.4. Classification schemes of the Class Litostomatea.

Small and Lynn (1985)	Grain (1994a)
Subclass Haptoria	
Order Haptorida	**Order Haptorida**
Family Didiniidae	Suborder Acropisthiina
Didinium	Family Acropisthidae
Family Enchelyidae	Suborder Belonophryina
Enchelyodon	Family Actinobroliniidae
Homalozoon	Suborder Archistomatina
Family Lacrymariidae	Family Buetschliidae
Family Mesodiniidae	***Buetschlia***
Family Spathidiidae	**Order Spathidiida**
Spathidium	Suborder Spathidiina
Family Trachelophyllidae	Family Spathidiidae
	Spathidium
Order Pleurostomatida	Family Homalozoonidae
Family Amphileptidae	***Homalozoon***
Loxophyllum	***Enchelyodon***
	Family Pseudoholophryidae
Order Pharyngophorida	Suborder Didiniina
Family Actinobolinidae	Family Didiniidae
Family Helicoprorodontidae	***Didinium***
Family Tracheliidae	Suborder Lacrymariina
Dileptus	Family Lagynophryidae
	Family Lacrymariidae
	Suborder Trachelophyllina
	Family Trachelophyllidae
	Family Pseudotrachelocercidae
	Suborder Dileptina
	Family Tracheliidae
	Dileptus
	Suborder Enchelyina
	Family Enchelyidae
	Order Helicoprorodontida
	Family Helicoprorodontidae
	Order Pleurostomatidae
	Family Loxophyllidae
	Loxophyllum
	Family Amphileptidae
	Order Mesodiniida
	Family Mesodiniidae

molecular data; (3) if *Balantidium* and *Isotricha* spp. belong within the vestibuliferid clade rather than with the haptorians as proposed by Leipe and Hausmann (1989) and Lipscomb and Riordan (1990, 1992); (4) if the vestibuliferids (i.e. balantidiids and isotrichids) and entodiniomorphids (i.e. cycloposthiids, macropodiniids, and ophryoscolecids) form monophyletic groups consistent with the classification proposed by Small and Lynn (1985); (5) if the haptorians and trichostomes (i.e. vestibuliferids plus entodiniomorphids) form monophyletic groups, thereby suggesting that vertebrate endosymbiosis possibly occurred only once in the evolution of the ciliates; and (6) if the litostomes (i.e. haptorians plus trichostomes) form a monophyletic group consistent with the classification proposed by Small and Lynn (1985).

MATERIALS AND METHODS

Source of samples and culture conditions.

Genomic DNA and the SSrRNA gene from *Balantidium coli* (Figure 2.7A), an endosymbiotic ciliate isolated from the colon of a lowland gorilla (*Gorilla gorilla gorilla*), were gifts from Dr. C. Graham Clark (Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, London, ENGLAND). Genomic DNA from *Cycloposthium* sp. (Figure 2.7B). and *Macropodinium yalanbense* (Figure 2.7C), two ciliate endosymbionts isolated from the sacciform forestomachs of wallaby (*Macropus dorsalis*) and a Western grey kangaroo (*Macropus fuliginosus*) respectively, were gifts from Mr. Stephen Cameron (Department of Parasitology, University of Queensland, Brisbane, AUSTRALIA).

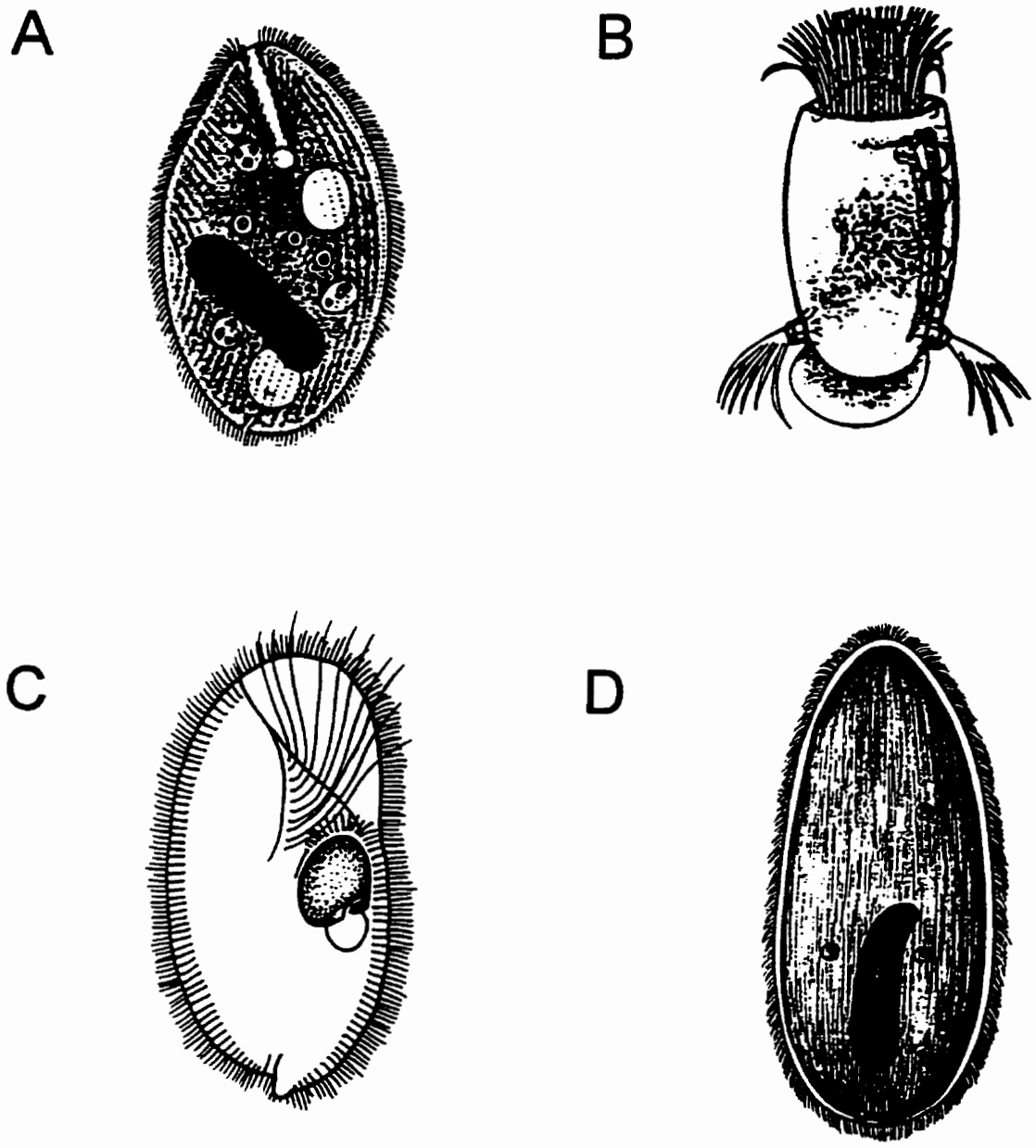


Fig. 2.7. Drawings of representatives from four different families of vertebrate endosymbionts. **(A)** *Balantidium coli* (Balantidiidae) (copied from Corliss, 1979), **(B)** *Cycloposthium* sp. (Cycloposthiidae) (copied from Corliss, 1979), **(C)** *Macropodinium yalanbense* (Macropodiniidae) (copied from Dehority, 1996), and **(D)** *Isotricha prostoma* (Isotrichidae) (copied from Dehority, 1993).

Cells of *E. maggii* (Figure 2.2C) were isolated from a mixed culture of rumen ciliates and fixed in 70% ethanol and were a gift by Dr. Tadeusz Michalowski (Department of Animal Science, University of Warsaw, Poland). Cells of *D. ruminantium* (Figure 2.6A), *D. dentatum* (Figure 2.2A), *En. Caudatum* (Figure 2.1), and *I. intestinalis* (Figure 2.6B) were collected from rumen-fistulated monofaunated sheep and were a 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* (Figure 2.3B), *I. prostoma* (Figure 2.7D), *O. purkynjei* (see *O. caudatus*, Figure 2.3A), and *P. multivesiculatum* (Figure 2.2F) was collected from a rumen-fistulated sheep, filtered through 100 μm Nitex mesh, and was a gift by Dr. Burk Dehority (Ohio Agricultural Research and Development Centre, Ohio State University, Wooster, Ohio, USA). Cells of *I. prostoma*, *O. purkynjei*, and *P. multivesiculatum* were fixed in 70% ethanol and isolated from other rumen ciliates using a hand-drawn Pasteur pipette under a microscope. *Epidinium caudatum* was cultured from single cells isolated from the 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 $CaCl_2.2H_2O$, 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. 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 x 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 was prepared as follows: air-dried orchard grass and whole kernels of wheat were ground to pass a 40-mesh screen. Then, 1.5 g ground wheat and 1.0 g orchard grass were 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 four weeks, cells were filtered through cheese cloth, collected by centrifugation, and fixed in 70% ethanol.

Cells of *D. nasutum* were obtained from Carolina Biological Supply Company (Burlington, North Carolina) and subcultured from single cells. Cultures were maintained using bacterized Cerophyl (ATCC medium 802) with *Paramecium caudatum* as a food source. Cells of *Didinium* with empty food vacuoles were isolated from *P. caudatum* using a hand-drawn Pasteur pipette under a dissecting scope. Cells of *Dileptus* sp. were obtained from a pond near Guelph, Ontario, Canada and were a gift from Dr. Michaela Strüder-Kypke (Department of Zoology, University of Guelph, Guelph, Ontario, CANADA). Cells of *Enchelyodon* sp. were obtained from a pond near Tübingen,

Germany, fixed in 70% ethanol, and were a gift from Dr. Christian Bardele (Zoologisches Institut der Universität Tübingen, GERMANY).

DNA extraction and sequencing.

A DNA extraction procedure using the non-ionic detergent, cetyltrimethylammonium bromide (CTAB) (Murray and Thompson, 1980; Reichardt and Rogers, 1994; Wilson, 1994), commonly used for bacterial and plant DNA extraction and purification, was slightly modified as follows: after picking individual cells (e.g. *P. multivesiculatum*) or harvesting rumen culture or fluid (e.g. *En. Caudatum* or *Ep. caudatum*, respectively) ciliates were pelleted in 1.5-ml microfuges tube by centrifugation at 6,000 *g* for 2 min. Cells were resuspended in 500 µl 1x 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 ml of 5 M NaCl were added and thoroughly mixed before the addition of 80 µl CTAB/NaCl [2% (w/v), 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 ml of dH_2O.

Otherwise, long-term (>12 months) fixed-cells were centrifuged in a 1.5 ml microcentrifuge tube at 14,000 rpm for 2 min, rinsed in 1 ml of dH_20 and centrifuged again at 14,000 rpm for 2 min. The cells were then resuspended in 500 µl of dH_20, stored at 4° C for 24 h, and centrifuged at 14,000 rpm for 2 min. Cells were resuspended in 570 µl of dH_20 and 30 µl proteinase K (20 mg/ml), incubated for 1 h at 37° C, and then centrifuged at 14,000 rpm for 2 min. The cells were then resuspended in 500 µl of digestion buffer (50 mM Tris pH 7.5; 1 mM EDTA; 0.5 % Tween 20) and 0.1-10 mg/ml of proteinase K. Depending on several factors (i.e. the number of cells, size of pellet, and the duration of fixation), the cells were incubated at 48° C for 2 to 5 days to slowly release the higher molecular weight DNA. The lysate was centrifuged at 14,000 rpm for 5 min and the aqueous layer was transferred to a new microcentrifuge tube and incubated at 95° C for 7 min to inactivate the proteinase K. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the lysate. The sample was mixed by inverting several times and centrifuged at 14,000 rpm for 5 min. The top phase was transferred to a new microfuge tube with 0.1 vol of 2 M $NaC_2H_3O_2$ and 2.0 volumes of 100% ethanol for at least 1.0 hr. at -20° C. The sample was centrifuged for 10 min. at 14,000 rpm, the supernatant was carefully removed, and the pellet was washed in 400 µl of 70% ethanol at -20° C. After 20 min, the pellet was centrifuged for 2 min at 14,000 rpm, air dried for 15 min, and resuspended in 50 µl of dH_2O.

Universal SSrRNA primers (Table 2.5) (Medlin *et al.*, 1988) were used in a polymerase chain reaction (PCR) amplification using a PTC-100™ thermal cycler (MJ Research Inc., Watertown, Massachusetts, USA). The following parameters constituted one PCR cycle: 30 sec denaturation at 94° C, 30 sec primer annealing at 55° C, and 90 sec primer extension at 72° C. On the 35th and last cycle, the primer extension stage was extended for 3 min. The resulting PCR product was run on a 1% agarose gel and quickly visualized under long-wave ultraviolet light (365 nm) (< 30 sec exposure). PCR product was excised under long-wave ultraviolet light (< 60 sec exposure) and purified using the GeneClean® kit (BIO/CAN, Mississauga, ON). The purified fragment was sequenced directly using an ABI Prism 377 Automated DNA Sequencer (Applied Biosystems Inc, Foster City, CA) using dye terminator and Taq FS (Figure 2.8) with three forward and three reverse internal universal 18S primers (Table 2.6) (Elwood *et al.*, 1985) and the M13 vector forward and reverse primers (Table 2.6).

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 (Hammerschmidt *et al.*, 1996), *Colpidium campylum* X56532 (Greenwood *et al.*, 1991a), *Coleps* sp. (Stechmann *et al.*, 1998), *Colpoda inflata* M97908 (Greenwood *et al.*, 1991b, *Discophrya collini* L26446 (Leipe *et al.*, 1994a), *Eufolliculina uhligi* U47620 (Hammerschmidt *et al.*, 1996), *Euplotes aediculatus* X03949, M14590 (Sogin *et al.*, 1986b), *Furgasonia blochmanni* X65150 (Bernhard *et al.*, 1995), *Glaucoma chattoni* X56533

Table 2.5. Universal PCR primers for the small subunit rRNA gene and the ITS-1/5.8S/ITS-2 DNA region.

Medlin Primer A[1]
5'-- AAC CTG GTT GAT CCT GCC AGT--3'
Medlin Primer B[1]
5'-- TGA TCC TTC TGC AGG TTC ACC TAC --3'
Jerome Primer C[2]
5'-- TTG GTC CGT GTT TCA AGA CG --3'

1. Medlin primers (Medlin *et al.*, 1988) were used to amplify the 18S SSrRNA gene

2. Jerome primer C (Jerome and Lynn, 1996) anneals 500 bp within the 28S LSrRNA gene and was used with Medlin primer A to amplify a segment of DNA consisting of the 18S SSrRNA gene, the ITS-1, 5.8S LSrRNA gene, ITS-2, and a portion of the 28S LSrRNA gene

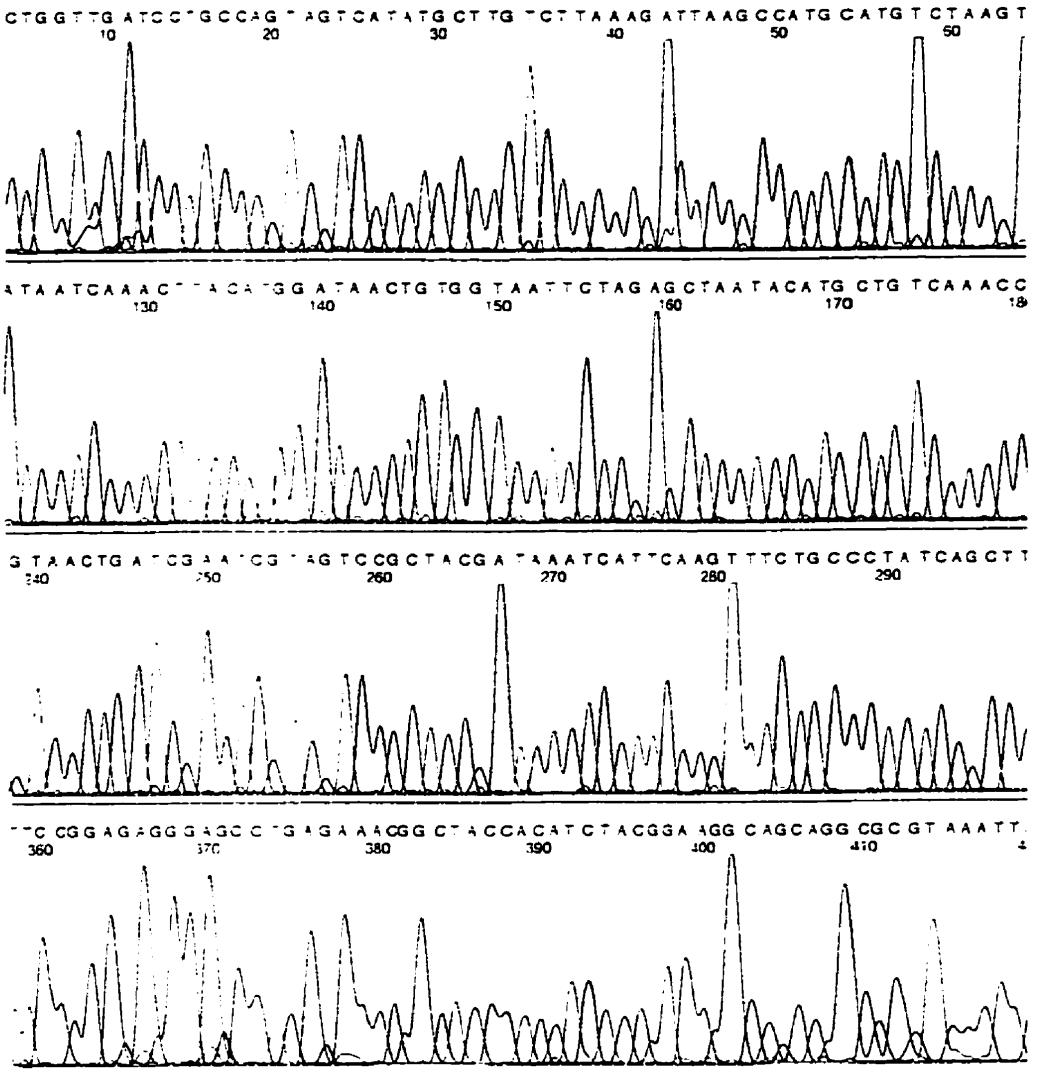


Fig. 2.8. A representative section of an autoradiograph showing a partial sequence of the SSrRNA. These sequences were produced using an ABI Prism 377 Automated DNA Sequencer (Applied Biosystems Inc, Foster City, CA) using dye terminator and Taq FS and each nucleotide is assigned a specific colour: T is red, C is blue, A is green, and G is black.

Table 2.6. Universal primers for the small subunit rRNA gene and vector DNA

Forward Internal Primers

Primer Code	Eukaryote Location[1]	Prokaryote Location[2]	Primer Sequence 5'-----> 3'
1F	4 - 20	9 - 25	CTG GTT GAT CCT GCC AG
*82F	79 - 94	--------	GAA ACT GCG AAT GGC TC
*300F	361 - 377	298 - 314	AGG GTT CGA TTC CGG AG
528F	569 - 584	528 - 543	CGG TAA TTC CAG CTC C
*690F	875 - 889	686 - 700	(T/C)AG AGG TGA AAT TCT
920F	1103 - 1119	906 - 922	GAA ACT TAA A(G/T)G AAT TG
*1055F	1239 - 1253	1047 - 1061	GGT GGT GCA TGG CCG
1200F	1397 - 1412	1195 - 1209	CAG GTC TGT GAT GCT C
1400F	1600 - 1616	1391 - 1407	TG(T/C) ACA CAC CGC CCG TC

Reverse Internal Primers

Primer Code	Eukaryote Location[1]	Prokaryote Location[2]	Primer Sequence 5'-----> 3'
108R	95 - 80	--------	GAG CCA TTC GCA GTT C
*300Ra	388 - 372	323 - 307	TCT CAG GCT CCC TCT CC
*300R	390 - 374	325 - 309	TCA GGC TCC CTC TCC GG
516R	556 - 541	516 - 502	ACC AGA CTT GCC CTC C
*690R	889 - 875	700 - 686	AGA ATT TCA CCT CTG
920R	1117 - 1103	920 - 906	ATT CCT TT(G/A) AGT TTC
*1055R	1253 - 1239	1061 - 1047	CGG CCA TGC ACC ACC
1200R	1412 - 1397	1209 - 1195	GGG CAT CAC AGA CCT G
1400R	1615 - 1601	1406 - 1392	ACG GGC GGT GTG T(G/A)C

pUC18 Primers

Forward Primer: 5'-- CCC AGT CAC GAC GTT GTA AAA CG --3'
Reverse Primer: 5'-- AGC GGA TAA CAA TTT CAC ACA GG --3'

1. Eukaryotic primer site represented by *Tetrahymena thermophila.*
2. Prokaryotic primer site represented by *Escherichia coli.*

* primers used for automated DNA sequencing

(Greenwood *et al.*, 1991a), *Homalozoon vermiculare* L26447 (Leipe *et al.*, 1994a), *Ichthyophthirius multifiliis* U17354 (Wright and Lynn, 1995), *Labyrinthuloides minuta* L27634 (Leipe *et al.*, 1994b), *Loxodes striatus* U24248 (Hammerschmidt *et al.*, 1996), *Loxophyllum utricullariae* L26448 (Leipe *et al.*, 1994a), *Metopus contortus* Z29516 (Embley *et al.* unpubl. data), *Metopus palaeformis* M86385 (Embley *et al.*, 1992), *Ophryoglena catenula* U17355 (Wright and Lynn, 1995), *Oxytricha granulifera*, X53486 (Schlegel *et al.*, 1991), *Paramecium tetraurelia* X03772 (Sogin and Elwood, 1986), *Prorocentrum micans* M14649 (Herzog and Maroteaux, 1986), *Prorodon teres* (Stechmann *et al.*, 1998), *Protocruzia* sp. X65153 (Hammerschmidt *et al.*, 1996), *Pseudomicrothorax dubius* X65151 (Bernhard *et al.*, 1995), *Sarcocystis muris* M64244, M34846 (Gajadhar *et al.*, 1991), *Spathidium* sp. Z22931 (Dyal *et al.*, 1995), *Symbiodinium pilosum* X62650, S44661 (Sadler *et al.*, 1992), *Tetrahymena corlissi* U17356 (Wright and Lynn, 1995), *Theileria buffeli* Z15106 (Allsopp *et al.*, 1994), *Tracheloraphis* sp. L31520 (Hirt *et al.*, 1995), *Trithigmostoma steini* X71134 (Leipe *et al.*, 1994a).

Theileria buffeli, *Sarcocystis muris*, *Prorocentrum micans*, *Symbiodinium pilosum*, and *Labyrinthuloides minuta* were used as the outgroups for the ciliates (Watrous and Wheeler, 1981; Maddison *et al.*, 1984; Smith, 1994; Stackebrandt and Ludwig, 1994). All sequences were globally aligned using the Dedicated Comparative Sequence Editor (DCSE) program (de Rijk and de Wachter, 1993) and further refined by considering secondary structural features of the 18S molecule.

PHYLIP (ver. 3.51C) (Felsenstein, 1993) was used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using the Kimura (1980)

two-parameter model. A distance-matrix tree was then constructed using the Fitch and Margoliash (1967) least squares (LS) method and the neighbour-joining (NJ) method (Saitou and Nei, 1987). The branch and bound method of PAUP (ver 3.1) (Swofford, 1993) was used for maximum parsimony (MP) analysis. Sequence data were reduced from 1,831 sites to 785 phylogenetically informative sites. Both parsimony and distance data were bootstrap resampled (Felsenstein, 1985) 1,000 times.

RESULTS

The lengths of the complete SSrRNA gene with GenBank/EMBL accession numbers in parentheses were as follows: *Balantidium coli* – 1,640 nucleotides (AF029763) (Figure 2.9); *Cycloposthium* sp. – 1,641 nucleotides (AF042485) (Figure 2.10); *Dasytricha ruminantium* – 1,638 nucleotides (U57769) (Figure 2.11); *Didinium nasutum* – 1,643 nucleotides (U57771) (Figure 2.12); *Dileptus* sp. – 1,641 nucleotides (AF029764) (Figure 2.13); *Diplodinium dentatum* – 1,638 nucleotides (U57764) (Figure 2.14); *Enchelyodon* sp. – 1,637 nucleotides (U80313) (Figure 2.15); *Entodinium caudatum* – 1,639 nucleotides (U57765) (Figure 2.16); *Epidinium caudatum* – 1,638 nucleotides (U57763) (Figure 2.17); *Eudiplodinium maggii* – 1,637 nucleotides (U57766) (Figure 2.18); *Isotricha intestinalis* – 1,639 nucleotides (U57770) (Figure 2.19); *Isotricha prostoma* – 1,641 nucleotides (AF029762) (Figure 2.20); *Macropodinium yalanbense* – 1,639 nucleotides (AF042486) (Figure 2.21); *Ophryoscolex purkynjei* – 1,636 nucleotides (U57768) (Figure 2.22); and *Polyplastron multivesiculatum* – 1,6390 nucleotides (U57767) (Figure 2.23).

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   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacacag taaaactgcg aatggctcat
 101 taaaacagtt atagtttatt tgatacatta aatggataac tgtagaaaaa
 151 ctagagctaa tacatgccga ggccgtaagg tcgtatttat tagatattcc
 201 aattaaggtg aatcataata acttcgcaaa tcgcgatttt gtcgcgataa
 251 atcatccaag tttctgccct atcatgcttt cgatggtagt gtattggact
 301 accatggctt tcacgggtaa cggggaatta gggttcgatt ccggagaagg
 351 agcctgagaa acggctacta catctacgga aggcagcagg cgcgtaaatt
 401 acccaatcct gactcaggga ggtggtgaca agatataacg acgcaattta
 451 ttttgtgatt gtagtgaggg tattccaaac cgaaccacta gtacgattag
 501 agggcaagtc tggtgccagc agccgcggta attccagctc taatagcgta
 551 tattaaagtt gttgcagtta aaaagctcgt agttggattt caaggcgtgt
 601 atactctttt tgagtatgct acctactagt ctctgactgt tactgtgaga
 651 aaattagagt gtttcaagca ggcttttgca agaatacatt agcatggaat
 701 aacgaatgtg tctagaatct tggttaattc tagattgcga ttaataggga
 751 cagttggggg cattagtatt taattgtcag aggtgaaatt cttggatttg
 801 ttaaagacta acgtatgcga aagcatttgc caaggatgtt ttcattaatc
 851 aagaacgaaa gataggggat caaagacaat cagatactgt cgtagtccta
 901 tctataaact atgccgacta gggattggaa tggttataac gccgtttcag
 951 taccttatga gaaatcaaag tctttgggtt ctgggggggag tatggtcgca
1001 agactgaaac ttaaagaaat tgacggaagg gcaccaccag gagtggagcc
1051 tgcggcttaa tttgactcaa cacggggaaa cttaccaggt ccagacatag
1101 taaggattga cagattgata gctctttctt gattctatgg gtggtggtgc
1151 atggccgttc ttagttggtg gagtgatttg tctggttaat tccgataacg
1201 aacgagacct taacctgcta actagtctaa tccattttat ggaatatgac
1251 ttcttagagg gactatgtat ttaaatacat ggaagtttga ggcaataaca
1301 ggtctgtgat gcccttatat gtcctgggct gcacgcgtgc tacactgatg
1351 catacaacaa gtgcctagcc cgccagggta tggcaatctc gaatatgcat
1401 cgtgatgggg atagatcttt gcaattatag atcttgaacg aggaattcct
1451 agtaagtgca agtcatcatc ttgcattgat tatgtccctg ccctttgtac
1501 acaccgcccg tcgctcctac cgataccggg tgatccggtg aacctttttgg
1551 accgcgatgc ggaaaaataa gtaaaccata tcacctagag gaaggagaag
1601 tcgtaacaag gtttccgtag gtgaacctgc ggaaggatca
```

Fig. 2.9. The complete SSrRNA gene sequence of ***Balantidium coli***. The total number of nucleotide positions = 1,640. Base count: 480 a, 301 c, 396 g, 463 t.

```
   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacacag tgaaactgcg aatggctcat
 101 taaaacagtt atagtttatt tgatacatta aatggataac tgtagaaaaa
 151 ctagagctaa tacatgccga gacctcacgg tcgtatttat tagatattcc
 201 aattaaggtg aatcataata acttagcaaa tcgcaatttt gttgcgataa
 251 atcatccaag tttctgccct atcatgcttt cgatggtagt gtattggact
 301 accatggctt tcacgggtaa cagggaatta gggttcgatt ctggagaagg
 351 agcttgagaa acggctacta catctacgga aggcagcagg cgcgtaaatt
 401 acccaatcct gactcaggga ggtggtgaca agatataaca acacgattaa
 451 aatcgcgatt gtagtgaggg tattctaaac cgaaccacta gtacgattag
 501 agggcaagtc tggtgccagc agccgcggta attccagctc taatagcgta
 551 tattaaagtt gctgcagtta aaaagctcgt agttggattt caaggacagt
 601 aaaccctctc gggaatactt cctactagtc tatgactgtt actgtgagaa
 651 aattagagtg tttcaagcag gcttttgcaa gaatacatta gcatggaata
 701 acgaatgtat atagaatatt ggttaattct atattacgag taatagagac
 751 agttgggggc aatagtattt aattgtcaga ggtgaaattc ctggatttgt
 801 taaagactaa cgtttgcgaa agcatttgcc aaggatgttt tcattaatca
 851 aggacgaaag ataggggatc aaagacaatc agatactgtc gtagtcctat
 901 ctataaacta tgccgactag ggattggagt gggaatacac catttcagta
 951 ccttatgaga aatcaaagtc tttgggttct ggggggagta tggtcgcaag
1001 actgaaactt aaagaaattg acggaagggc accaccagga gtggagcctg
1051 cggcttaatt tgactcaaca cggggaaact taccaggtcc agacatagta
1101 aggattgaca gattgatagc tctttcttga ttctatgggt ggtggtgcat
1151 ggccgttctt aattggtgga gtgatttgtc tggttaattc cgataacgaa
1201 cgagacctta acctgctaac tagtttattc catttcgatg gtttacaact
1251 tcttagaggg actatgtaaa acaaatgcat ggaagtttga ggcaataaca
1301 ggtctgtgat gcccttatat gtcctgggct gcacgcgtgc tacactgatg
1351 catacaacaa gtgcctagcc agatatggta tggcaatctc gaatatgtat
1401 cgtgatgggg attgatcttt gcaattatag atcatgaacg aggaattcct
1451 agtaagtgca agtcatcatc ttgcattgat tatgtccctg ccctttgtac
1501 acaccgcccg tcgctcctac cgataccggg tgatccggtg aacctttttgg
1551 acctcgtacg ggggaagata agtaaaccat atcacctaga ggaaggagaa
1601 gtcgtaacaa ggtttccgta ggtgaacctg cggaaggatc a
```

Fig. 2.10. The complete small subunit rRNA gene sequence of *Cycloposthium* sp. The total number of nucleotide positions = 1,641. Base count: 496 a, 300 c, 390 g, 455 t.

```
   1 aacctggtta atcctgccag tagtcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacacag taaaactgcg aatggctcat
 101 taaaacagtt atagtttatt tgatataaca aatggataac tgtagaaaaa
 151 ctagagctaa tacatgccgt aaccgcaagg ttgtatttat tagatattcc
 201 aattaggtga atcatgataa ctttgtcaaa tctcggtttt gccgagataa
 251 atcatccaag tttctgccct atcatgcttt cgatggtagt gtattggact
 301 accatggctt tcacgggtaa cagggaatta gggttcgatt ctggagaagg
 351 agcctgagaa acggctacta catctacgga aggcagcagg cgcgtaaatt
 401 acccaatcct gactcaggga ggtggtgaca agatataaca acgcaattta
 451 attgtgattg tagtgagggt tttccaaacc gaaccactag tacgattaga
 501 gggcaagtct ggtgccagca gccgcggtaa ttccagctct aatagcgtat
 551 attaaagttg ctgcagttaa aaagctcgta gttggatttc aaggattgtg
 601 tactcttcta gggtatgcac cctactagtc tttgactgtt actgtgagaa
 651 aattagagtg tttaaagcag gcaattgcaa gaatacatta gcatggaata
 701 acgaatgtat ctagaatctt ggttaattct aggtttcgat taatagagac
 751 agttgggggc attagtattt aattgtcaga ggtgaaattc ttggatttgt
 801 taaagactaa cgtatgcgaa agcatttgcc aaggatgttt tcattaatca
 851 agaacgaaag atagggggatc aaagacaatc agatactgtc gtagtcctat
 901 ctataaacta tgccgactag ggattggagt ggaatattca ccatttcagt
 951 accttatgag aaatcaaagt ctttgggttc tgggggggagt atggtcgcaa
1001 gactgaaact taaagaaatt gacggaaggg caccaccagg agtggagcct
1051 gcggcttaat ttgactcaac acggggaaac ttaccaggtc cagacatagt
1101 aaggattgac agattgatag ctctttcttg attctatgag tggtggtgca
1151 tggccgttct tagttggtgg agtgatttgt ctggttaatt ccgataacga
1201 acgagacctt aacctgctaa ctagactttt tcattttatg attaagtctt
1251 cttagaggga ctatatgctt taagtatatg gaagtttgag gcaataacag
1301 gtctgtgatg cccttatatg tcctgggctg cacgcgtgct acactgatgt
1351 atacaacaag tgcctagcta gatatagtat ggcaatctcg aatacgcatc
1401 gtgatgggga tagatctttg caattataga tcttgaacga ggaattccta
1451 gtaagtgcaa gtcatcatct tgcattgatt atgtccctgc cctttgtaca
1501 caccgcccgt cgctcctacc gataccgggt gatccggtga acctgttgga
1551 cacttttgag aaaaacaagt aaatcatatc acctagagga aggagaagtc
1601 gtaacaaggt ttccgtaggt gaacctgcag aaggatca
```

Fig. 2.11. The complete small subunit rRNA gene sequence of ***Dasytricha ruminantium***. The total number of nucleotide positions = 1,638. Base count: 491 a, 292 c, 384 g, 471 t.

```
   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agattaagcc
  51 atgcatgtct aagtataaat aactacacag taaaactgcg aatggctcat
 101 taaaacagtt atagtttatt tgatacatat taaatggata actgtagaaa
 151 cactagagct aatacatgcc aagaccgtaa ggttgtattt attagatatt
 201 ccaagtaggt gattcataat aacttcgcaa atctcagtta tactgagata
 251 aatcattcaa gtttctgccc tatcatgctc tcgatggtag tgtattggac
 301 taccatggct ctcacgggta acggggaatt agggttcgat tccggagaag
 351 gagcctgaga aacggctact acatctaagg aaggcagcag gcgcgtaaat
 401 tacccaatcc tgaatcaggg aggtagtgac aagatataac aacacgatta
 451 aaagtcgtga ttgtagtgag ggtattccaa accgaacttc gagtacgatt
 501 ggagggcaag tctggtgcca gcagccgcgg taattccagc tccaatagcg
 551 tatattaaag ttgttgcagt taaaaagctc gtagttggat ttctagataa
 601 gtggctattc gtagtttgct tgtctaccag tcttagactg ttactgtgag
 651 aaaattagag tgttcaaagc aggctattgc aagaatacat tagcatggaa
 701 taacgaatgt gtctagaata ttggttaatt ctagattacg attaataggg
 751 acagttgggg gcattagtat ttaattgtca gaggtgaaat tcttggattt
 801 attaaagact aacgtatgcg aaagcatttg ccaaggatgt tttcattaat
 851 caagaacgaa agatagggga tcaaagacga tcagataccg tcgtagtcct
 901 atctataaac tatgccgact agggattggt atggattcgt accatatcag
 951 taccttatga gaaatcaaag tctttgggtt ctgggggggag tatggtcgca
1001 agactgaaac ttaaagaaat tgacggaagg gcaccaccag gagtggagcc
1051 tgcggcttaa tttgactcaa cacggggaaa cttaccaggt ccagacatag
1101 taaggattga cagattgata gctctttctt gattctatgg gtggtggtgc
1151 atggccgttc ttagttggtg gagtgatttg tctggttaat tccgataacg
1201 aacgagacct taacctgcta actagatatg ctcattatat gggtacattc
1251 ttcttagagg gactatgtgt cgataagcgc atggaagttt gaggcaataa
1301 caggtctgtg atgcccttat atgtcctggg ctgcacgcgt gctacactga
1351 tgcatacaac aagtatccaa gcccgccagg gtgaggtaat ctcgaatatg
1401 catcgtgatg gggatagatc tttgcaatta tagatcttga acgaggaatt
1451 cctagtaaat gcaagtcatc atcttgcatt gattatgtcc ctgccctttg
1510 tacacaccgc ccgtcgctcc taccgatacc gggtgatccg gtgaaccttt
1551 tggaccgttt tacggaaaga taagtaaacc taatcaccta gaggaaggag
1601 aagtcgtaac aaggtttccg taggtgaacc tgcagaagga tca
```

Fig. 2.12. The complete small subunit rRNA gene sequence of ***Didinium nasutum***. The total number of nucleotide positions = 1,643. Base count: 492 a, 297 c, 395 g, 459 t.

```
   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacacag taaaactgcg aatggctcat
 101 taaaacagtt atagtttatt tgatataaca aatggataac tgtagaaaaa
 151 ctagagctaa tacatgccat aaccgtaagg ttgtatttat tagatattcc
 201 tggtaggtga ttcataataa cttagcgaat cgtgactcgc tcacgataaa
 251 tcattcaagt ttctgcccta tcatgctttc gatggtagtg tattggacta
 301 ccatggctct cacgggtaac ggagaattag ggttcgattc cggagaagga
 351 gcctgagaaa cggctactac atctaaggaa ggcagcaggc gcgtaaatta
 401 cccaatcctg actcagggag gtagtgacaa gatataacaa cgtgaagtca
 451 aattcgcgat tgtagtgagg ttattccaaa ccgaaattcg agtacgattg
 501 gaggacaagt cttggtgcca gcacccgcgg taattccagc tccaatagcg
 551 tatattaaag ttgttgcagt taaaaagctc gtagttggat ttctagggag
 601 aggcctcaaa accaatccct actagtccct tccgggacag ttactgtgag
 651 aaaattagag tgtttcaagc aggcgtttgc aggaatacat tagcatggaa
 701 taacgaatgt ttctagaatc ttggttaatt ctagacaacg attaataggg
 751 acagttgggg gcattagtat ttaactgtca gaggtgaaat tcttggattt
 801 gttaaagact aacgtatgcg aaagcatttg ccaaggatgt tttcattaat
 851 caagaacgaa agatagggga tcaaagacaa tcagatactg tcgtagtcct
 901 atctataaac tatgccgact agggattggg gaaattaaag tttcctcagt
 951 accttatgag aaatcaaagt ctttgggttc tgggggagt atggtcgcaa
1001 gactgaaact taaagaaatt gacggaaggg caccaccagg agtggagcct
1051 gcggcttaat ttgactcaac acggggaaac ttaccaggtc cagacatagt
1101 aaggattgac agattgatag ctctttcttg attctatggg tggtggtgca
1151 tgcccgttct tagttggtgg agtgatttgt ctggttaatt ccgataacga
1201 acgagacctt aacctgctaa ataatctgtt ccattttatg gaatttgatt
1251 tcttagaggg actatgcgac tataagcgca tggaagtttg aggcaataac
1301 aggtctgtga tgcccttata tgtcctgggc tgcacgcgtg ctacactgat
1351 acatacaaca agtgcctagt ccgctaggat atggcaatct acaatatgta
1401 tcgtgatggg gatagatctt tgcaattata gatcttgaac gaggaattcc
1451 tagtaaatgc aagtcatcat cttgcgttga ttatgtccct gccctttgta
1501 cacaccgccc gtcgctccta ccgataccgg gtgatccggt gaaccttta
1551 gaccgtttat cggaaaaata agtaaacctt atcacctaga ggaaggagaa
1601 gtcgtaacaa ggtttccgta ggtgaacctg cggaaggatc a
```

Fig. 2.13. The complete small subunit rRNA gene sequence of ***Dileptus*** sp. The total number of nucleotide positions = 1,641 Base count: 492 a, 306 c, 389 g, 454 t.

```
   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacacag taaaactgcg aatggctcat
 101 tacaacggtt attgtttatt tgatacaaca aatggataac tgtagaaaaa
 151 ctagagctaa tacatgcttt aaccgcaagg ttgtatttat tagatattcc
 201 ggattggtga atcataataa cttcgcaaat ctcgtttatg acgagataaa
 251 tcatccaagt ttctgcccta tcatgctttc gatggtagtg tattggacta
 301 ccatggctct cacgggtaac agggaattag ggttcgattc tggagaagga
 351 gcctgagaaa cggctactac atctacggaa ggcagcaggc gcgtaaatta
 401 cccaatcctg actcagggag gtggtgacaa gatataacaa cgcgatcaaa
 451 atcgcgattg tagtgagggt attctaaaca gaacctatag tacgattaga
 501 gggcaagtct ggtgccagca gccgcggtaa ttccagctct aatagcgtat
 551 attaaagttg ctgcagttaa aaagctcgta gttggatttc aaggactgta
 601 gacccctctg gggaatacat cctactagtc attgactgtt actgtgagaa
 651 aattagagtg tttcaagcag gctttcgcaa gaatacatta gcatggaata
 701 acgaatgtat ttagaatctt ggttaattct aaattacgat taatagagac
 751 agttgggggc attagtattt aattgtcaga ggtgaaattc ttggatttgt
 801 taaagactaa cgtatgcgaa agcatttgcc aaggatgttt tcattaatca
 851 aggacgaatg ataggggatc aaagacaatc agatactgtc gtactcctat
 901 ctataaacta tgccgactag ggattggagt gggaatacac catttcagta
 951 ccttatgaga aatcaaagtc tttgggttct ggggggagta tggtcgcaag
1001 actgaaactt aaagaaattg acggaagggc accaccagga gtggagcctg
1051 cggcttaatt tgactcaaca cggggaaact taccaggtcc agacatagta
1101 aggattgaca gattgatagc tctttcttga ttctatgggt ggtggtgcat
1151 ggccgttctt agttggtgga gtgatttgtc tggttaattc cgataacgaa
1201 cgagacctta acctgctaat tagatccttt tatcttataa tcggtatctt
1251 cttagaggga ctatgtaaaa caaatacatg gaagtttgag gcaataacag
1301 gtcagtgatg cccttatatg tcctgggctg cacgcgtgct acactgatgt
1351 atacaacaag tgcctaacca gacatggtat ggcaatctcg aatatgcatc
1401 gtgatgggga tagatctttg caattataga tcttgaacga ggaattccta
1451 gtaagtgcaa gtcatcatct tgcattgatt atgtccctgc cctttgtaca
1501 caccgtccgt cgctcctacc gataccgggt gatccggtga accttttgga
1551 ccttaactgg gaagataagt aaaccatatc acctagagga aggagaagtc
1601 gtaacaaggt ttccgtaggt gaacctgcag aaggatca
```

Fig. 2.14. The complete small subunit rRNA gene sequence of ***Diplodinium dentatum.*** The total number of nucleotide positions = 1,638. Base count: 494 a, 301 c, 386 g, 457 t.

```
   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agattaagcc
  51 atgcatgtct aagtataagt aactacacag taaaactgcg aatggctcat
 101 taaaacagtt atagtttatt tgatacataa aatggataac tgtagaaaaa
 151 ctagagctaa tacatgcctt aaccgtaagg ttgtatttat tagatatacc
 201 aagtgggtga ttcataataa ctttgcaaat cgctgcgaag cagtgataaa
 251 tcattcaagt ttctgcccta tcatgctttc gatggtagtg tattggacta
 301 ccatggctct cacgggtaac ggagaattag ggttcgattc cggagaagga
 351 gcctgagaaa cggctactac atctaaggaa ggcagcaggc gcgtaaatta
 401 cccaatcctg actcagggag gtagtgacag gatataacaa cgcgattaaa
 451 ttcgtgattg tagtgagggt attctaaacc gaacttcgag tacgattaga
 501 gggcaagtct ggcgccagca gccgcggtaa ttccagctct aatagcgtat
 551 attaaagttg ttgcagttaa aaagctcgta gttggatttc aagagtcgca
 601 atgtgtccgc acattgctct ctatcagtca ttgactgtta ctgtgagaaa
 651 attagagtgt ttcaagcagg ctattgcagg aatacattag catggaataa
 701 cgaatgtcta gaatcttggt taattctaga tgaagattaa tagggacagt
 751 tgggggcatt agtatttaat tgtcagaggt gaaattcttg gattatttaa
 801 agactaacgt atgcgaaagc atttgccaag gatgttttca ttaatcaaga
 851 acgaaagata ggggatcaaa gacgatcaga taccgtcgta gtcctatcta
 901 taaactatgc cgactaggga ttagggtgga aaagtaccat cttagtacct
 951 tatgagaaat caaagtcttt gggttctggg gggagtatgg tcgcaagact
1001 gaaacttaaa gaaattgacg gaagggcacc accaggagtg gagcctgcgg
1051 cttaatttga ctcaacacgg ggaaacttac caggtccaga cgtagtaagg
1101 attgacagat tgatagctct ttcttgattc tatgggtggt ggtgcatggc
1151 cgttcttagt tggtggagtg atttgtctgg ttaattccgt taacgaacga
1201 gaccttaacc tgctaactag acttgcttat tttataggta cagtcttctt
1251 agagggacta tgttgcgatg agcacatgga agtttgaggc aataacaggt
1301 ctgtgatgcc cttatatgtc ctgggctgca cgcgtgctac actgatgcat
1351 acaacgagtt cctagcccga tagggtaagg caatctttaa tatgcatcgt
1401 gatggggata gatctttgca attatagatc ttgaacgagg aattcctagt
1451 aagtgcaagt catcatcttg cattgattat gtccctgccc tttgtacaca
1501 ccgcccgtcg ctcctaccga taccgggtga taaggtgaat ttgttggacc
1551 gttttacggg aagataagta aaccttatca cctagaggaa ggagaagtcg
1601 taacaaggtt tccgtaggtg aacctgcaga aggatca
```

Fig. 2.15. The complete small subunit rRNA gene sequence of ***Enchelyodon*** sp. The total number of nucleotide positions = 1,637 Base count: 484 a, 292 c, 402 g, 459 t.

```
   1 aacctggttg atcctgccag taatcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacatag taaaactgcg aatggctcat
 101 tacaacagtt attgtttatt tgatacatta aatggataac tgtataaaaa
 151 ctagagctaa tacatgctaa ggccgcaagg ttgtatttat tagatattcc
 201 gataaggtga atcataataa cttcgcaaat ctcatctatg atgggataaa
 251 tcatccaagt ttctgcccta tcatgctttc gttggtagtg tattggacta
 301 ccatggctct cacgggtaac agggaattag ggttcgattc tggagaacga
 351 gcctgagaaa cggctactac atctacggaa ggcagcaggc gcgtaaatta
 401 cccaatcctg aatcagggag gtggtgacaa gatataacaa cgagacctta
 451 aatttcgatt gtagtgaggg ttttttaaat agaacctata gtacgattag
 501 agggcaagtc tggcgccagc agccgcggta attccagctc taatagcgta
 551 tactaaagtt gctgcagtta aaaagctcgt agttggattt caaggtttgt
 601 attctttacc gggaatacac cctactagtc attgactgtt actgtgagaa
 651 aattagagtg tttcaagcag gcttttgcaa gaatacatta gcatggaata
 701 acgaatgtat ttagaatcgt ggtttaattc taaataacga ttcatagaga
 751 cagttggggg cattagtatt taattgtcag aggtgaattt cttggatttg
 801 ttaaagacta acgtatgcga aagcatttgc caaggatgtt ttcattaatc
 851 aaggacgaaa gataggggat caaagacaat cagatactgt cgtagtccta
 901 tctataaact atgccgacta gggattggag tgggcatata ccatttcagt
 951 accttatgag aaatcaaagt ctttgggttc tgggggggagt atggtcgcaa
1001 gactgaaact taaagaaatt gacggaaggg caccaccagg agtggagcct
1051 gcggcttaat ttgactcaac acggggaaac ttaccaggtc cagacgtagt
1101 aaggattgac agattgatag ctctttcttg attctatggg tggtggtgca
1151 tggccgttct tagttggtgg agtgatttgt ctggttaatt ccgataacga
1201 acgagacctt aacctgctaa ctagattctt ctatactata gatgatatct
1251 tcttacaggg actatgttat acaaatacat ggaagtttga ggcaataaca
1301 ggtcagtgat gcccttatat gtcctgggct gcacgcgtgc tacactgatg
1351 catacaacaa gtgcctaacc agacatggta tggcaatctc gaatatgcat
1401 cgtgatgggg atagatcttt gcaattatag atcttgaacg aggaattcct
1451 agtaagtgca agtcatcatc ttgcattgat tatgtccctg ccctttgtac
1501 acaccgcccg tcgctcctac cgataccggg tgatccggtg aacctttttgg
1551 actcctttgg gaaagataag taaaccatat cacctagagg aaggagaagt
1601 cgtaacaagg tttccgtagg tgaacctgca gaaggaaca
```

Fig. 2.16. The complete small subunit rRNA gene sequence of ***Entodinium caudatum***. The total number of nucleotide positions = 1,639. Base count: 493 a, 298 c, 382 g, 466 t.

```
   1 aacctggttg atcctgccag taacatatgc ttgtctcaaa gattaagcca
  51 tgcatgtcta agtataaata actacacagt aaaactgcga atggctcatt
 101 acaacagtta ttgtttattt gatacattaa atggataact gtagaaaaac
 151 tagagctaat acatgctaag gcctcacggt cgtatttatt agatattcca
 201 gattggtgaa tcataataac ttcgcaaatc tcgtttatga cgagataaat
 251 catccaagtt tctgccctat catgctttcg atggtagtgt attggactac
 301 catggctctc acgggtaaca gggaattagg gttcgattct ggagaaggag
 351 cctgagaaac ggctactaca tctacggaag gcagcaggcg cgtaaattac
 401 ccaatcctga ctcagggagg tggtgacaag atataacaac gcgatttata
 451 tcgcgattgt agtgagggta ttctaaatag aacctatagt acgattagag
 501 ggcaagtctg gtgccggcag ccgcggtaat tccagctcta atagcgtata
 551 ttaatgttgc tgcagttaaa aagctcgtag ttggatttca aggactgtaa
 601 accctccggg gaatacatcc tactagtctt tgactgttac tgtgagtaaa
 651 ttagagtgtt tcaagcaggc tttcgcaaga atatattagc atggaataac
 701 gaatgtattt agaatcttgg ttaattctaa attacgatta atagagacag
 751 ttgggggcat tagtatttaa ttgtcagagg tgaaattctt ggatttgtta
 801 aagactaacg tatgcgaaag catttgccaa ggatgttttc attaatcaag
 851 gacgaaagat aggggatcaa agacaatcag atactgtcgt agtcctatct
 901 ataaactatg ccgactaggg attggagtgg gaatacacca tttcagtacc
 951 ttatgagaaa tcaaagtctt tgggttctgg ggggagtatg gtcgcaagac
1001 tgaaacttaa agaaattgac ggaagggcac caccaggagt ggagcctgcg
1051 gcttaatttg actcaacacg gggaaactta ccaggtccag acatagtaag
1101 gattgacaga ttgatagctc tttcttgatt ctatgggtgg tggtgcatgg
1151 ccgttcttag ttggtggagt gatttgtctg gttaattccg ataacgaacg
1201 agaccttaac ctgctaacta gttctcaata ctctgtattc tgcaacttct
1251 tagagggact atgtaaatca attacatgga agtttgaggc aataacaggt
1301 cagtgatgcc cttatatgtc ctgggctgca cgcgtgctac actgatgcat
1351 acaacaagtg cctagccaga catggtatgg caatctcgaa tatgcatcgt
1401 gatggggata gatctttgca attatagatc ttgaacgagg aattcctagt
1451 aagtgcaagt catcatcttg cattgattat gtccctgccc tttgtacaca
1501 ccgcccgtcg ctcctaccga taccgggtga tccggtgaac cttttggact
1551 ccgtacgggg gaagataagt aaaccatatc acctagagga aggagaagtc
1601 gtaacaaggt ttccgtaggt gaacctgcag aaggatca
```

Fig. 2.17. The complete small subunit rRNA gene sequence of ***Epidinium caudatum***. The total number of nucleotide positions = 1,638. Base count: 487 a, 303 c, 389 g, 459 t.

```
   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacacag taaaactgcg aatggctcat
 101 tacaacagtt attgtttatt tgatacataa aatggataac tgtagaaaaa
 151 ctagagctaa tacatgctat aaccgcaagg ttgtatttat tagatattcc
 201 aaattggtga atcataataa cttcgcaaat ctcgtttatg acgagataaa
 251 tcatccaagt ttctgcccta tcatgctttc gatggtagtg tattggacta
 301 ccatggctct cacgggtaac agggaattag ggttcgattc tggagaagga
 351 gcctgagaaa cggctactac atctacggaa ggcagcaggc gcgtaaatta
 401 cccaatcctg actcagggag gtggtgacaa gatataacaa cgcgatatta
 451 tcgcgattgt agtgagggta ttctaaacag aacctatagt acgattagag
 501 ggcaagtttg gtgccagcag ccgcggtaat tccagctcta atagcgtata
 551 ttaaagttgc tgcagttaaa aagctcgtag ttggatttca aggactgtaa
 601 accctctcgg gaatacatcc tactagtctc cggactgtta ctgtgagaaa
 651 attagagtgt ttcaagcagg ctttcgcaag aatacattag catggaataa
 701 cgaatgtatt tagaatcttg gttaattcta aattacggtt aatagagaca
 751 gttgggggca ttagtattta attgtcagag gtgaaattct tggatttgtt
 801 aaagactaac gtatgcgaaa gcatttgcca aggatgtttt cattaatcaa
 851 ggacgaaaga taggggatca aagacaatca gatactgtcg tagtcctatc
 901 tataaactat gccgactagg gattggagtg ggcatacacc atttcagtac
 951 cttatgagaa atcaaagttt ttgggttctg gggggagtat ggtcgcaaga
1001 ctgaaactta aagaaattga cggaagggca ccaccaggag tggagcctgc
1051 ggcttaattt gactcaacac ggggaaactt accaggtcca gacatagtaa
1101 ggattgacag attgatagct ctttcttgat tctatgggtg gtggtgcatg
1151 gccgttctta gttggtggag tgatttgtct ggttaattcc gataacgaac
1201 gagaccttaa cctgctaact agattctttt atcttataaa agttatcttc
1251 ttagagggac tatgtaaaac aaatacatgg aagtttgagg caataacagg
1301 tcagtgatgc ccttatatgt cctgggctgc acgcgtgcta cactgatgca
1351 tacaacaagt gcctaaccag acatggcatg gcaatctcga atatgcatcg
1401 tgatagggat agatctttgc aattatagat cttgaacgag gaattcctag
1451 taagtgcaag tcatcatctt gcattgatta tgtccctgcc ctttgtacac
1501 accgcccgtc gctcctaccg ataccgggtg atccggtgaa ccttttggac
1551 ctgtatgggg aagataagta aaccatatca cctagaggaa ggagaagtcg
1601 taacaaggtt tccgtaggtg aacctgcaga aggatca
```

Fig. 2.18. The complete small subunit rRNA gene sequence of ***Eudiplodinium maggii.*** The total number of nucleotide positions = 1,637. Base count: 497 a, 300 c, 384 g, 456 t.

```
   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacacag taaaactgcg aatggctcat
 101 taaaacagtt atagtttatt tgatacatta aatggataac tgtagaaaaa
 151 ctagagctaa tacatgccaa ggccgcaagg tcgtatttat tagataactc
 201 caatatgaat catagtaact tagcaaatct caatttcatt gagatatatc
 251 atccaagttt ctgccctatc agctttcgat ggtagtgtat tggactacca
 301 tggctttcac gggtaacagg gaattagggt tcgattctgg agaaggagcc
 351 tgagaaacgg ctactacatc tacggaaggc agcaggcgcg taaattaccc
 401 aatcctgact cagggaggtg gtgacaagat ataatgacgc tatgaaaaat
 451 agtgattata gtgagggtat tccaaaccga accacaagta cgattagagg
 501 gcaagtctgg tgccagcagc cgcggtaatt ccagctctaa tagcgtatat
 551 taaagttgct gcagttaaaa agctcgtagt tggatttcaa ggaacacgta
 601 ttccccccgga atatgtgccc tactagccct gggctgttac tgtgagaaaa
 651 ttagagtgtt taaagcaagc ttttgcaaga atacattagc atggaataac
 701 gaatgagtct agaatctagg tttaattcta gatctcgatt aatagagaca
 751 gttgggggca ttagtattta attgtcagag gtgaaattct tggatttgtt
 801 aaagactaac gtatgcgaaa gcatttgcca aggatgtttt cattaatcaa
 851 gaacgaaaga taggggatca aagacaatca gatactgtcg tagtcctatc
 901 tataaactat gccgactagg gattggaatg gaaattcacc atttcagtac
 951 cttatgagaa atcaaagtct ttgggttctg gggggagtat ggtcgcaaga
1001 ctgaaactta aagaaattga cggaagggca ccaccaggag tggagcctgc
1051 ggcttaattt gactcaacac ggggaaactt accaggtcca gacatagtaa
1101 ggattgacag attgatagct ctttcttgat tctatgggtg gtggtgcatg
1151 gccgttctta gttggtggag tgatttgtct ggttaattcc gataacgaac
1201 gagaccttaa cctgctaact agtctattac atttcatgta atttgacttc
1251 ttagagggac tatgtatatc aagtacatgg aagtttgagg caataacagg
1301 tctgtgatgc ccttatatgt cctgggctgc acgcgtgcta cactgatgca
1351 tacaacaagt gcctagctag acatagtatg gcaatctgga atatgcatcg
1401 tgatggggat agatctttgc aattatagat cttgaacgag gaattcctag
1451 taagtgcaag tcatcatctt gcattgatta tgtccctgcc ctttgtacac
1501 accgcccgtc gctcctaccg ataccgggtg atccggtgaa ccttttggac
1551 ctcgcaagag gaaaaataag taaaccatat cacctagagg aaggagaagt
1601 cgtaacaagg tttccgtagg tgaacctgca gaaggatca
```

Fig. 2.19. The complete small subunit rRNA gene sequence of ***Isotricha intestinalis***. The total number of nucleotide positions = 1,639. Base count: 503 a, 302 c, 384 g, 450 t.

```
   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacacag taaaactgcg aatggctcat
 101 taaaacagtt atagtttatt tgatacatta aatggataac tgtagaaaaa
 151 ctagagctaa tacatgccga gaccacaagg ttgtatttat tagatattgt
 201 aattaagatg aatcataata acttcacaaa tctcgatatc atcgagataa
 251 atcatccaag ttactgccct atcagctttc gatggtagtg tattggacta
 301 ccatggcttt cacgggtaac agggaattag ggttcgattc tggagaagga
 351 gcctgagaaa cggctactac atctacggaa ggcagcaggc gcgtaaatta
 401 cccaatcctg actcagggag gtggtgacaa gatataacga cccgattaat
 451 gtcgtgattg tagtgaggat attccaaaca gaatcacaag aacgattaga
 501 gggcaagtct ggtgccagca gccgcggtaa ttccagctct aatagcgtat
 551 attaaagttg ctgcagttaa aaagctcgta gttggatttc aaggattact
 601 cattcctatg gaatgtgtac cctactagcc agtattggct gttactgtga
 651 gaaaattaga gtgtttaaag caggctcatg caagaataca ttagcatgga
 701 ataacgaatg agtcatgaat cttggttaat tcttgtactc gattaataga
 751 gacagttggg ggcattagta tttaattgtc agaggtgaaa ttcttggatt
 801 tgttaaagac taacttatgc gaaagcattt gccaaggatg ttttcattaa
 851 tcaagaacga aagatagggg atcaaagaca atcagatact gtcgtagtcc
 901 tatctataaa ctatgccgac tagggattgg aatggcaatt taccatttca
 951 gtaccttatg agaaatcaaa gtctttgggt tctgggggga gtatggtcgc
1001 aagactgaaa cttaaagaaa ttgacggaag ggcaccacca ggagtggagc
1051 ctgcggctta atttgactca acacggggaa acttaccagg tccagacata
1101 gtaaggattg acagattgat agctctttct tgattctatg ggtggtggtg
1151 catggccgtt cttagttggt ggagtgattt gtctggttaa ttccgataac
1201 gaacgagacc ttaacctgct aattagtcgt cctcatatta tggggtatga
1251 cttcttagag ggactatgca tatcaagtgc atggaagttt gaggcaataa
1301 caggtctgtg atgcccttat atgtcctggg ctgcacgcgt gctacactga
1351 tgcatacaac aagtgcctag ctagacatag tatggcaatc tggaatatgc
1401 atcgtgatgg ggatagatct ttgcaattat agatcttgaa cgaggaattc
1451 ctagtaagtg caagtcatca tcttgcattg attatgtccc tgccctttgt
1501 acacaccgcc cgtcgctcct accgataccg ggtgatccgg tgaacctttt
1551 ggaccttaat aggaaaaata agtaaacctt atcacctaga ggaaggagaa
1601 gtcgtaacaa ggtttccgta ggtgaacctg cggaaggatc a
```

Fig. 2.20. The complete small subunit rRNA gene sequence of ***Isotricha prostoma***. The total number of nucleotide positions = 1,641. Base count: 500 a, 297 c, 387 g, 457 t.

```
   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacacag tgaaactgcg aatggctcat
 101 taaaacagtt atagtttatt tgatacatta aatggataac tgtagaaaaa
 151 ctagagctaa tacatgccga gacctcacgg tcgtatttat tagatattcc
 201 aattaaggtg aatcataata acttagcaaa tcgcaatttt gttgcgataa
 251 atcatccaag tttctgccct atcatgcttt cgatggtagt gtattggact
 301 accatggctt ttacgggtaa cggggaatta gggttcgatt ccggagaagg
 351 agcctgagaa acggctacta catctacgga aggcagcagg cgcgtaaatt
 401 acccaatcct gaatcaggga ggtggtgaca agatataacg gagtgaataa
 451 aatcgcgatc gtagtgaggg ttttctatac caaaccacta gtaccattag
 501 agggcaagtc tggtgccagc agccgcggta attccagctc taatagcgta
 551 tattaaagtt gctgcagtta aaaagctcgt agttggattt caaggaatat
 601 aatcacctac ggcgattata ccctactacc ctctcgggtg ttactttgag
 651 aaaattagag tgtttaaagc aggcatttgc aagaatacat tagcatggaa
 701 taacgaatgt gtttagaatc ttggttaatt ctagatgcgg ttaataggga
 751 cagttggggg cattagtatt taatagtcag aggtgaaatt cctggatttg
 801 ttaaagacta acttatgcga aagcatttgc caaggatgtt ttcattaatc
 851 aagaacgaaa gataggggat caaagacaat cagacactgt cgtagtccta
 901 tctataaact atgccgacta ggggttggag tgacattcat cacttcagta
 951 ccttatgaga aatcaaagtc tttgggttct ggggggagta tggtcgcaag
1001 actgaaactt aaagaaattg acggaagggc accaccagga gtggagcctg
1051 cggcttaatt tgactcaaca cggggaaact taccaggtcc agacatagta
1101 aggattgaca gattgatagc tctttcttga ttctatgggt ggtggtgcat
1151 ggccgttctt agttggtgga gtgatttgtc tggttaattc cgataacgaa
1201 cgagacctta acctgctaac taatctattc catcctatgg aatctgattt
1251 cttagaggga ctatgttttt aaatacatgg aagtttgagg caataacagg
1301 tctgtgatgc ccttatatgt cctgggctgc acgcgtgcta cactgataca
1351 tacaacaagt gcctagcccg ctagggtacg gcaatctcga atatgtatcg
1401 tgatggggat tgaactttgc aattatagtt catgaacgag gaattcctag
1451 taagtgcaag tcatcatctt gcgttgatta tgtccctgcc ctttgtacac
1501 accgcccgtc gctcctaccg ataccgggtg atccggtgaa ccttttggac
1551 tgctcacgcg gaaagataag taaaccacat cacctagagg aaggagaagt
1601 cgtaacaagg tttccgtagg tgaacctgcg gaaggatca
```

Fig. 2.21. The complete SSrRNA gene sequence of ***Macropodinium yalanbense***. The total number of nucleotide positions = 1,639. Base count: 486 a, 309 c, 390 g, 454 t.

```
   1 aacctggttg atcctgccag tagcatatgc ttgtctcaaa gactaagcca
  51 tgcatgtcta agtataaata actacacagt aaaactgcga atggctcatt
 101 acaacagtta ttgtttattt gatacattaa atggataact gtagaaaaac
 151 tagagctaat acatgctaag gccgcaaggt cgtatttatt agatattcca
 201 aatcggtgaa tcataataac ttcgcaaatc tcatctatga tgagataaat
 251 catccaagtt tctgccctat catgctttcg atggtagtgt attggactac
 301 catggctctc acgggtaaca gggaattagg gttcgattct ggagaaggag
 351 cctgagaaac ggctactaca tctacggaag gcagcaggcg cgtaaattac
 401 ccaatcctga ctcagggagg tggtgacaag atataacaac gcgatttata
 451 tcgcgattgt agcgagggta ttctaaatag aacctatagt acgattagag
 501 ggcaagtctg gtgccagcag ccgcggtaat tccagctcta atagcgtata
 551 ttaatgttgc tgcagttaaa aagctcgtag ttggatttca aggactgtat
 601 accctcccgg gcatacaacc tactagtctc tgactgttac tgtgagtaaa
 651 ttagagtgtt tcaagcaggc ttttgcaaga atatattagc atggaataac
 701 gaatgtattt agaatcttgg ttaattctat attacgatta atagagacag
 751 ttgggggcat tagtatttaa ttgtcagagg tgaaattctt ggatttgtta
 801 aagactaacg tatgcgaaag catttgccaa ggatgttttc attaatcaag
 851 gacgaaagat aggggatcaa agacaatcag atactgtcgt agtcctatct
 901 ataaactatg ccgactaggg attggagtgg gaatacacca tttcagtacc
 951 ttatgagaaa tcaaagtctt tgggttctgg ggggagtatg gtcgcaagac
1001 tgaaacttaa agaaattgac ggaagggcac caccaggagt ggagcctgcg
1051 gcttaatttg actcaacacg gggaaactta ccaggtccag acatagtaag
1101 gattgacaga ttgatagctc tttcttgatt ctatgggtgg tggtgcatgg
1151 ccgttcttag ttggtggagt gatttgtctg gttaattccg ataacgaacg
1201 agaccttaac ctgctaacta gttgcttttg ctttgcgatt gctaacttct
1251 tagagggact atgtaaatca attacatgga agtttgaggc aataacaggt
1301 cagtgatgcc cttatatgtc ctgggctgca cgcgtgctac actgatgtat
1351 acaacaagtg cctagccaga catggtatgg caatctcgaa tatgcatcgt
1401 gatggggata gatctttgca attatagatc ttgaacgagg aattcctagt
1451 aagtgcaagt catcatcttg cattgattat gtccctgccc tttgtacaca
1501 ccgcccgtcg ctcctaccga taccgggtga tccggtgtac cttttggact
1551 cgcaagggaa agataagtaa accatatcac ctagaggaag gagaagtcgt
1601 aacaaggttt ccgtaggtga agctgcagaa ggatca
```

Fig. 2.22. The complete small subunit rRNA gene sequence of ***Ophryoscolex purkynjei***. The total number of nucleotide positions = 1,636. Base count: 486 a, 302 c, 389 g, 459 t.

```
   1 aacctggttg atcctgccag tagtcatatg cttgtctcaa agactaagcc
  51 atgcatgtct aagtataaat aactacacag taaaactgcg aatggctcat
 101 tacaacagtt attgtttatt tgatacaaca aatggataac tgtagaaaaa
 151 ctagagctaa tacatgcttt aatcgtaaga ttgtatttat tagatattct
 201 gaattggtga atcataataa cttcgcaaat ctcgtttatg acgagataaa
 251 tcatccaagt ttctgcccta tcatgctttc gatggtagtg tattggacta
 301 ccatggctct cacgggtaac aggaattagg gttcgattct ggagaaggag
 351 cctgagaaac ggctactaca tctacggaag gcagcaggcg cgtaaattgc
 401 ccaatcctga ctcagggagg tggtgacaag atataacgac gcggttatta
 451 tcgcgattgt agtgagggta ttctaaacag aacctatagt acgattagag
 501 ggcaagtctg gtgccagcag ccgcggtaat tccagctcta atagcgtata
 551 ttaaagttgc tgcagttaaa aagctcgtag ttggatttca aggattgtaa
 601 aacctccacg gggatacatc ctactagtct tcggactgtt actgtgagaa
 651 aattagagtg tttcaagcag gctttcgcaa gaatacatta gcatggaata
 701 acgaatgtat ttagaatctt ggttaattct aaattacgat taatagagac
 751 agttgggggc attagtattt aattgtcaga ggtgaaattc ttggatttgt
 801 taaagactga cgtatgcgaa agcatttgcc aaggatgttt tcattaatca
 851 aggacgaaag ataggggatc aaagacaatc agatactgtc gtagtcctat
 901 ctataaacta tgccgactag ggattggagt gggttataca ccatttcagt
 951 accttatgag aaatcaaagt ctttgggttc tggggggagt atggtcgcaa
1001 gactgaaact taaagaaatt gacggaaggg caccaccagg agtggagcct
1051 gcggcttaat ttgactcaac acggggaaac ttaccaggtc cagacatagt
1101 aaggattgac agattgatag ctctttcttg attctatggg tggtggtgca
1151 tggccgttct tagttggtgg agtgatttgt ctggttaatt ccgataacga
1201 acgagacctt aacctgctaa ctagattcta tcatcttatg attgatatct
1251 tcctagaggg actatgttaa acaaatacat ggaagtttga ggcaataaca
1301 ggtcagtgat gcccttatat gtcctgggct gcacgcgtgc tacactgatg
1351 tatacaacaa gtgcctaacc agacatggta tggcaatctc gaatatgcat
1401 cgtgataggg atagatcttt gcaattatag atcttgaacg aggaattcct
1451 agtaagtgca agtcatcatc ttgcattgac tatgtccctg ccctttgtac
1501 acaccgcccg tcgctcctac cgataccggg tgatccggtg aacctttttgg
1551 acctgtaagg ggaagataag taaaccatat cacctagagg aaggagaagt
1601 cgtaacaagg tttccgtagg tgaacctgca gaaggatca
```

Fig. 2.23. The complete small subunit rRNA sequence of ***Polyplastron multivesiculatum.*** The total number of nucleotide positions = 1,639. Base count: 493 a, 298 c, 388 g, 460 t.

Molecular phylogenies

The complete SSrRNA gene sequences of these 15 ciliates were examined and compared against other SSrRNA gene sequences from ciliates, representing most of the major lineages within the phylum Ciliophora, and those from other eukaryotes. Bootstrap values of both parsimony (Figure 2.24) and distance-matrix (Figure 2.25) trees strongly support (93% MP, 100% LS, 100% NJ) the ciliates as a monophyletic group and as the sister group 100% of the time to the apicomplexan-dinoflagellate clade (c.f. Figures 2.24, 2.25), forming a cluster known as the alveolates (Parvkingdom Alveolata) (Cavalier-Smith, 1993).

All major lineages within the phylum Ciliophora (i.e. classes Litostomatea, Spirotrichea, Prostomatea, Colpodea, Oligohymenophorea, Nassophorea, Phyllopharyngea, Karyorelictea, and Heterotrichea) are monophyletic with bootstrap analyses strongly supporting the karyorelicteans-heterotrichs as the sister group to all other ciliates. These two major lineages comprise the subphylum Postciliodesmatophora Gerassimova and Seravin, 1976 with the remaining ciliates making up the subphylum Intramacronucleata Lynn, 1996.

Bootstrap data strongly support the vertebrate endosymbionts (subclass Trichostomatia) as a monophyletic group 100% of time. However, parsimony and distance–matrix analyses could not resolve the phylogenetic positions of the putative entodiniomorphid ciliate *M. yalanbense* (Macropodiniidae) and the putative vestibuliferid ciliate *B. coli* (c.f. Figures 2.24, 2.25). As a result, branches were collapsed to produce a polychotomy with *Macropodinium* and *Balantidium* on a branch with a clade consisting of the vestibuliferids *Isotricha* spp. and *Dasytricha* (order Vestibuliferida) and a clade consisting of the

Fig. 2.24. 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 1,000 trees. Branches with less than 50% bootstrap support were collapsed. No significance is placed on the lengths of the branches connecting the species. Names for suprageneric taxa appear in boldface.

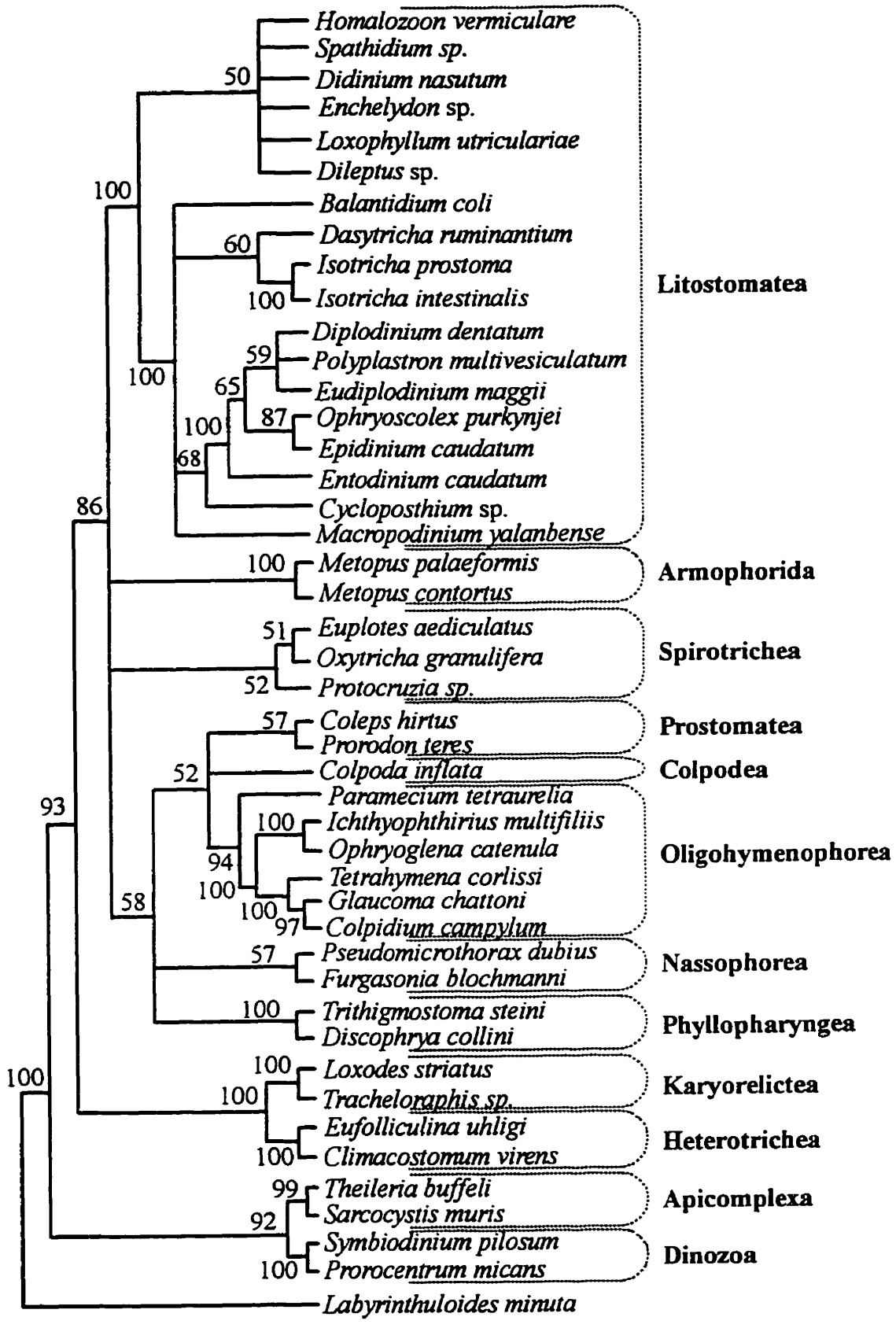
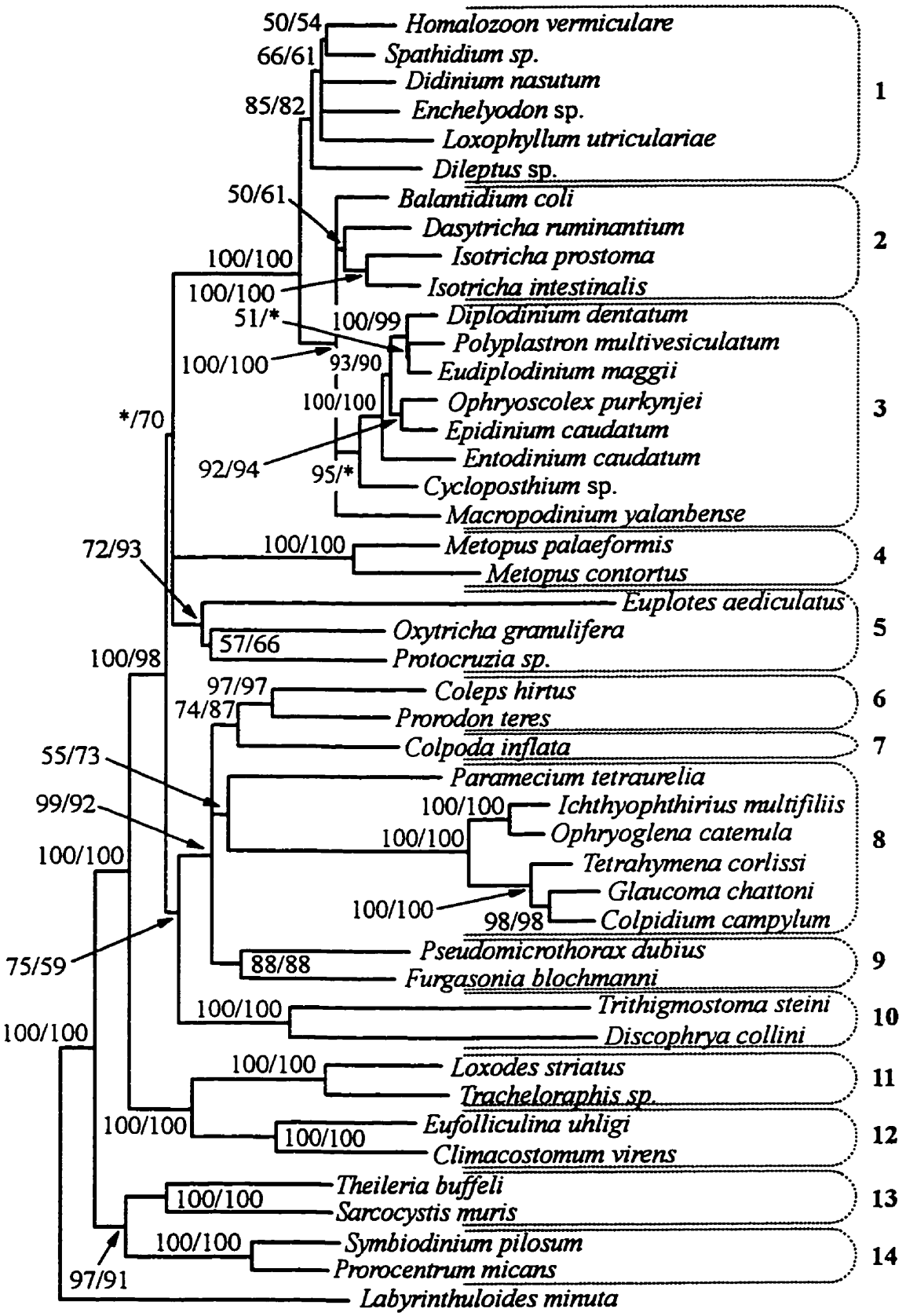

Homalozoon vermiculare
Spathidium sp.
Didinium nasutum
Enchelydon sp.
Loxophyllum utriculariae
Dileptus sp.
Balantidium coli
Dasytricha ruminantium
Isotricha prostoma
Isotricha intestinalis
Diplodinium dentatum
Polyplastron multivesiculatum
Eudiplodinium maggii
Ophryoscolex purkynjei
Epidinium caudatum
Entodinium caudatum
Cycloposthium sp.
Macropodinium yalanbense
Litostomatea
Metopus palaeformis
Metopus contortus
Armophorida
Euplotes aediculatus
Oxytricha granulifera
Protocruzia sp.
Spirotrichea
Coleps hirtus
Prorodon teres
Prostomatea
Colpoda inflata
Colpodea
Paramecium tetraurelia
Ichthyophthirius multifiliis
Ophryoglena catenula
Tetrahymena corlissi
Glaucoma chattoni
Colpidium campylum
Oligohymenophorea
Pseudomicrothorax dubius
Furgasonia blochmanni
Nassophorea
Trithigmostoma steini
Discophrya collini
Phyllopharyngea
Loxodes striatus
Tracheloraphis sp.
Karyorelictea
Eufolliculina uhligi
Climacostomum virens
Heterotrichea
Theileria buffeli
Sarcocystis muris
Apicomplexa
Symbiodinium pilosum
Prorocentrum micans
Dinozoa
Labyrinthuloides minuta

Fig. 2.25. A SSrRNA distance tree of the ciliates derived from evolutionary distances produced by the Kimura 2-parameter (1980) correction model and constructed using the Fitch and Margoliash (1967) least squares (LS) method and the neighbor-joining (NJ) method (Saitou and Nei, 1987). The consensus trees for 1,000 bootstrap resamplings of the data set (LS and NJ) were computed independently and are indicated as a percentage at the base of each branch. The LS bootstrap percentage is followed by the NJ bootstrap percentage. 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. Numbers in boldface are as follows: **(1)** Haptoria, **(2)** Vestibuliferida, **(3)** Entodiniomorphida, **(4)** Armophorida, **(5)** Spirotrichea, **(6)** Prostomatea, **(7)** Colpodea, **(8)** Oligohymenophorea, **(9)** Phyllopharyngea, **(10)** Nassophorea, **(11)** Karyorelictea, **(12)** Heterotrichea, **(13)** Apicomplexa, and **(14)** Dinozoa.


50/54
66/61
85/82
50/61
100/100
100/100
51/*
100/100
*/70
92/94
72/93
100/100
57/66
100/98
97/97
74/87
55/73
99/92
100/100
75/59
100/100
100/100
100/100
100/100
100/100
100/100
100/99
93/90
95/*
100/100
100/100
98/98
88/88
97/91
Homalozoon vermiculare
Spathidium sp.
Didinium nasutum
Enchelyodon sp.
Loxophyllum utriculariae
Dileptus sp.
Balantidium coli
Dasytricha ruminantium
Isotricha prostoma
Isotricha intestinalis
Diplodinium dentatum
Polyplastron multivesiculatum
Eudiplodinium maggii
Ophryoscolex purkynjei
Epidinium caudatum
Entodinium caudatum
Cycloposthium sp.
Macropodinium yalanbense
Metopus palaeformis
Metopus contortus
Euplotes aediculatus
Oxytricha granulifera
Protocruzia sp.
Coleps hirtus
Prorodon teres
Colpoda inflata
Paramecium tetraurelia
Ichthyophthirius multifiliis
Ophryoglena catenula
Tetrahymena corlissi
Glaucoma chattoni
Colpidium campylum
Pseudomicrothorax dubius
Furgasonia blochmanni
Trithigmostoma steini
Discophrya collini
Loxodes striatus
Tracheloraphis sp.
Eufolliculina uhligi
Climacostomum virens
Theileria buffeli
Sarcocystis muris
Symbiodinium pilosum
Prorocentrum micans
Labyrinthuloides minuta
1
2
3
4
5
6
7
8
9
10
11
12
13
14

ophryoscolecids and *Cycloposthium*.

The two species of *Isotricha*, which paired together 100% of the time, were aligned against each other and 75 nucleotide differences ($d \approx 4.5\%$) were observed between them (Figure 2.26). The transition/transversion ratio was 1:1 and there were six insertions/deletions for *I. prostoma* and eight for *I. intestinalis*. Further, the isotrichid clade, including *Dasytricha*, is weakly supported by bootstrap values (60% MP, 50% LS, 61% NJ). The ophryoscolecids formed a monophyletic group (100% MP, LS, NJ) consistent with their placement in the order Entodiniomorphida with *Cycloposthium* (family Cycloposthiidae) as the sister group to the ophryoscolecid rumen ciliates (family Ophryoscolecidae). Within this monophyletic group, *Entodinium* (subfamily Entodiniinae) is the earliest branching ciliate (100% MP, LS, NJ), basal to a clade consisting of the more derived rumen ciliates, *Epidinium* and *Ophryoscolex* (subfamily Ophryoscolecinae), and the morphologically "intermediate" rumen ciliates, *Polyplastron*, *Diplodinium*, and *Eudiplodinium* (subfamily Diplodiniinae). *Epidinium* and *Ophryoscolex* consistently group together 87% (MP), 92% (LS), and 94% (NJ) of the time (Figures 2.24, 2.25) and are the sister group to the monophyletic diplodiniines (59% MP, 100% LS, and 99% NJ). Both MP and NJ analyses could not resolve the phylogenetic relationships within the Diplodiniinae (Figure 2.24, 2.25).

The free-living haptorians *Dileptus*, *Enchelyodon*, *Homalozoon*, *Loxophyllum*, *Didinium*, and *Spathidium* formed a monophyletic group (subclass Haptoria) (50% MP, 85% LS, 82% NJ), and despite their unresolved polychotomy, they remained the sister group to the vertebrate endosymbionts (subclass Trichostomatia) 100% of the time (Figures 2.24, 2.25). These two major lineages comprise the litostome ciliates (class Litostomatea). However,

Fig. 2.26. A pair-wise comparison of the SSrRNA gene sequence between two species of *Isotricha*. Missing data is indicated by a dash. There are 75 nucleotide differences between *I. prostoma* and *I. intestinalis*. The transition to transversion ratio is 1:1. Note: This alignment continues on the next page.

I. prostoma AACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGACTAAGCC
I. intestinalis .....

I. prostoma ATGCATGTCTAAGTATAAATAACTACACAGTAAAACTGCGAATGGCTCAT
I. intestinalis .....

I. prostoma TAAAACAGTTATAGTTTATTTGATACATTAAATGGATAACTGTAGAAAAA
I. intestinalis .....

I. prostoma CTAGAGCTAATACATGCCGAGACCACAAGGTTGTATTTATTAGATATTGT
I. intestinalis .....A..G..G.....C.....-----.

I. prostoma AATT--AAGATGAATCATAATAACTTCACAAATCTCGATATCATCGAGAT
I. intestinalis ..C.CC..T.....G.....AG.....A..T....T.....

I. prostoma AAATCATCCAAGTTACTGCCCTATCAGCTTTCGATGGTAGTGTATTGGAC
I. intestinalis .T.....T.....

I. prostoma TACCATGGCTTTCACGGGTAACAGGGAATTAGGGTTCGATTCTGGAGAAG
I. intestinalis .....

I. prostoma GAGCCTGAGAAACGGCTACTACATCTACGGAAGGCAGCAGGCGCGTAAAT
I. intestinalis .....

I. prostoma TACCCAATCCTGACTCAGGGAGGTGGTGACAAGATATAACGACCCGATTA
I. intestinalis .....T...G.T..G.

I. prostoma ATG-TCGTGATTGTAGTGAGGATATTCCAAACAGAATCACAAGAACGATT
I. intestinalis .AAA.A.....A.....G.....C...C.....T.....

I. prostoma AGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTAATAGCG
I. intestinalis .....

I. prostoma TATATTAAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCAAGGATT
I. intestinalis .....AC

I. prostoma ACTCATTCCTATGGAATGTGTACCCTACTAGCCAGTATTGGCTGTTACTG
I. intestinalis ..GT.....CCC.....A...G.....---C.G.....

I. prostoma TGAGAAAATTAGAGTGTTTAAAGCAGGCTCATGCAAGAATACATTAGCAT
I. intestinalis .....A...TT.....

I. prostoma GGAATAACGAATGAGTCATGAATCTTGGTT-AATTCTTGTACTCGATTAA
I. intestinalis .....TA.....A....T.....A.AT.....

I. prostoma TAGAGACAGTTGGGGGCATTAGTATTTAATTGTCAGAGGTGAAATTCTTG
I. intestinalis .....

I. prostoma GATTTGTTAAAGACTAACTTATGCGAAAGCATTTGCCAAGGATGTTTTCA
I. intestinalis .....G.....

I. prostoma TTAATCAAGAACGAAAGATAGGGGATCAAAGACAATCAGATACTGTCGTA
I. intestinalis .....

I. prostoma GTCCTATCTATAAACTATGCCGACTAGGGATTGGAATGGCAATTTACCAT
I. intestinalis .....A....C.....

I. prostoma TTCAGTACCTTATGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGG
I. intestinalis .....

I. prostoma TCGCAAGACTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGAGTG
I. intestinalis .....

I. prostoma GAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCCAGA
I. intestinalis .....

I. prostoma CATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGT
I. intestinalis .....

I. prostoma GGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGA
I. intestinalis .....

I. prostoma TAACGAACGAGACCTTAACCTGCTAATTAGTCGTCCTCATATTATGGGGT
I. intestinalis .....C.....TATTA...T.C...TAA.

I. prostoma ATGACTTCTTAGAGGGACTATGCATATCAAGTGCATGGAAGTTTGAGGCA
I. intestinalis T.....T.....A.....

I. prostoma ATAACAGGTCTGTGATGCCCTTATATGTCCTGGGCTGCACGCGTGCTACA
I. intestinalis .....

I. prostoma CTGATGCATACAACAAGTGCCTAGCTAGACATAGTATGGCAATCTGGAAT
I. intestinalis .....

I. prostoma ATGCATCGTGATGGGGATAGATCTTTGCAATTATAGATCTTGAACGAGGA
I. intestinalis .....

I. prostoma ATTCCTAGTAAGTGCAAGTCATCATCTTGCATTGATTATGTCCCTGCCCT
I. intestinalis .....

I. prostoma TTGTACACACCGCCCGTCGCTCCTACCGATACCGGGTGATCCGGTGAACC
I. intestinalis .....

I. prostoma TTTTGGACCTT--AATAGGAAAAATAAGTAAACCTTATCACCTAGAGGAA
I. intestinalis .....CGC..G.....A.....

I. prostoma GGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
I. intestinalis .....A.....

neither maximum parsimony analysis nor distance-matrix methods could resolve the sister group to the litostomes (Figures 2.24, 2.25).

DISCUSSION

Leipe *et al.* (1994a) noted that the SSrRNA gene sequences of the haptorian ciliates, *Homalozoon* and *Loxophyllum*, were approximately 120 nucleotides shorter than the average length of the ciliate 18S rRNA gene. The 12 new trichostome 18S sequences and the three new haptorian 18S sequences are the same length as the other haptorian 18S genes from *Homalozoon*, *Spathidium*, and *Loxophyllum*. From secondary structure analyses, I have concluded that the litostomes (i.e. trichostomes and haptorians) have "lost" helix E23-5 (De Rijk *et al.*, 1992; Neefs *et. al.*, 1993) and have a "reduction" in the length of other helices within Variable Region 4 (V4) (c.f. Figures 2.27, 2.28). Clearly, this is a diagnostic feature of the litostomes.

After the 18S rRNA sequences for *D. ruminantium* and *P. multivesiculatum* were elucidated, it was discovered that a SSrRNA gene sequence had already been submitted to GenBank for these two rumen ciliates. When these sequences were aligned against my sequences, 28 nucleotide differences were found between the *D. ruminantium* from Ottawa and that of *D. ruminantium* from Britain (Embley *et al.*, 1995) (Table 2.7), and eight nucleotide differences were found between the *P. multivesiculatum* from Ohio and that of *P. multivesiculatum* from Britain (Table 2.8). In comparison, the largest number of nucleotide differences between two morphologically similar species of *Tetrahymena*, *T. empidokyrea* and *T. malaccensis*, is 39 (*pers. obs.*).

Fig. 2.27. A secondary structural model of the SSrRNA gene (De Rijk *et al.*, 1992; Neefs *et. al.*, 1993) for *Tetrahymena thermophila* (Lynn, *unpubl. data*) showing the nine variable (V) regions. Within V4, helix E23-5 (see arrow) is missing in the litostomes (see Figure 2.28). In addition, the lengths of the other helices in V4 are shorter in the litostomes (c.f. Figures 2.27 and 2.28). This model is representative of most ciliates except the litostomes.

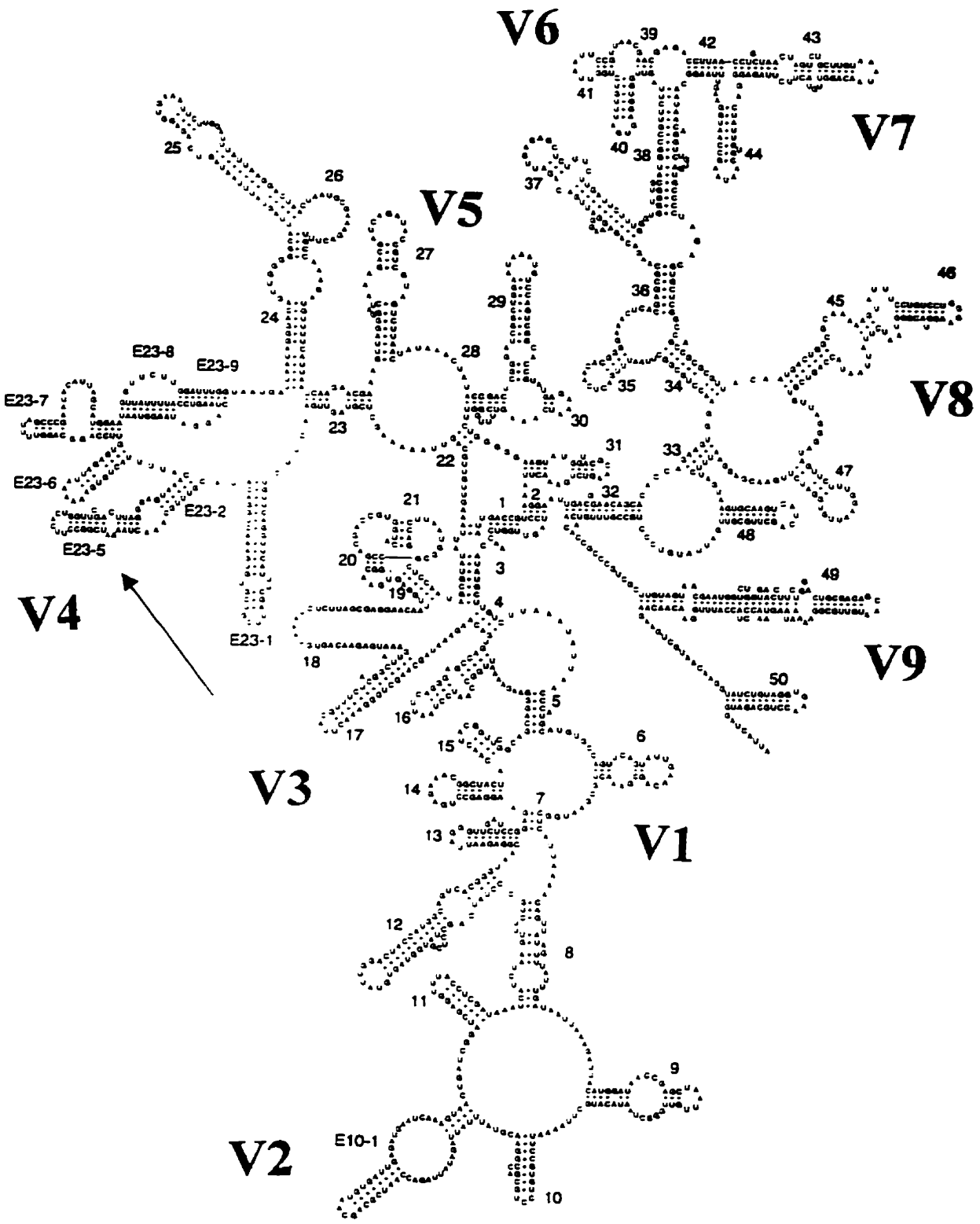

V6
V7
V5
V8
V4
V9
V3
V1
V2
E23-8
E23-9
E23-7
E23-6
E23-2
E23-5
E23-1
E10-1

Fig. 2.28. A secondary structural model of the SSrRNA gene (De Rijk *et al.*, 1992; Neefs *et. al.*, 1993) for the litostome *Homalozoon vermiculare* (Lynn, *unpubl. data*) showing the nine variable (V) regions. Within V4, the litostomes are missing helix E23-5 (it is present in all other ciliates, see Figure 2.27). Another diagnostic feature of the litostomes is that the other helices in that region are much shorter than those found in other ciliates.

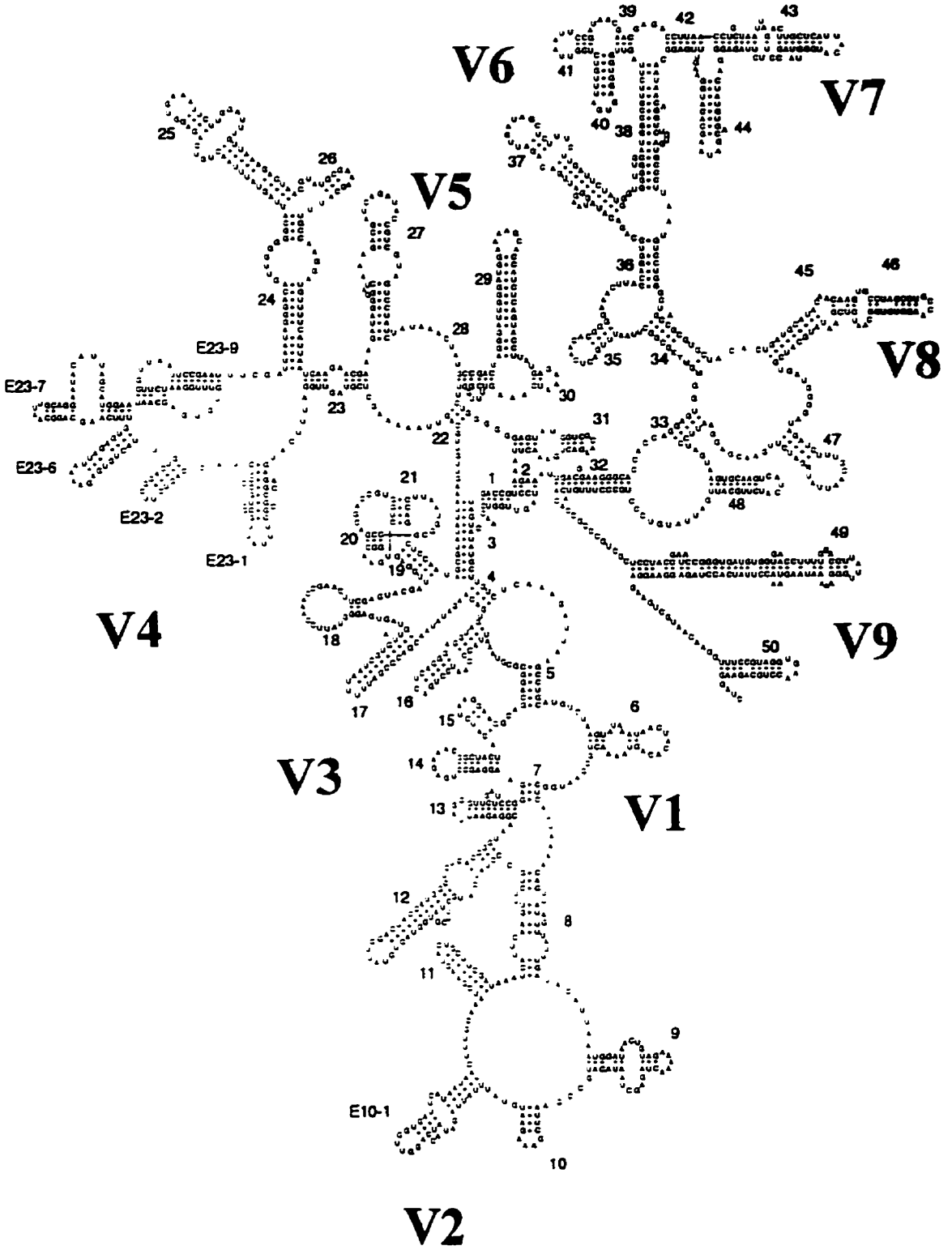

V6
V7
V5
V8
V4
V9
V3
V1
V2
E23-9
E23-7
E23-6
E23-2
E23-1
E10-1

Table 2.7. Nucleotide differences between *Dasytricha ruminantium* (Ottawa) and *D. ruminantium* (London)

Species	Sequence Position 5' --3'														
	56	128	129	169	170	172	173	216	224	310	346	360	471	607	609
D. ruminantium (Ottawa)	T	A	C	G	T	A	C	G	T	T	G	A	T	T	T
D. ruminantium (London)	C	T	T	A	A	G	T	A	C	C	C	T	A	C	C

Species	Sequence Position 5' --3'												
	612	773	845	937	938	1047	1048	1050	1051	1141	1244	1246	1397
D. ruminantium (Ottawa)	G	-	-	T	A	G	A	C	C	A	T	A	C
D. ruminantium (London)	A	C	C	A	T	-	-	N	N	G	A	T	T

Table 2.8. Differences between British and N. 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

There are three possible explanations for the large number of nucleotide differences between these sequences: (1) these are actually different species of *Dasytricha* and *Polyplastron* respectively, (2) there are sequencing errors, and/or (3) there is extreme intraspecific genetic variation. So far, only one other species of *Dasytricha* has been recognized, *D. hukuokaensis*. However, its occurrence has not been reported since it was first described more than 40 years ago by Hukui and Nisida (1954). I am confident that the cells I identified before DNA extraction were *D. ruminantium* and *P. multivesiculatum* respectively. I sequenced both DNA strands (see Figure 1.2b) while Embley *et al.* (1995) did not indicate if both strands of their *D. ruminantium* or *P. multivesiculatum* SSrRNA gene were sequenced to confirm their reading. If the identities of *D. ruminantium* (British) and *P. multivesiculatum* (British) are confirmed not to be other species, and if these nucleotide differences are confirmed by subsequent research not to be sequencing errors, there would be a significant amount of genetic divergence between North American and European populations of *D. ruminantium* and *P. multivesiculatum*. If genetic distance between *Tetrahymena* species is taken as a standard, this would warrant establishing new species status for North American *Dasytricha* "*ruminantium*" and *Polyplastron* "*multivesiculatum*".

Phylogeny Within The Phylum Ciliophora

All trees indicated, with very high bootstrap support (Figures 2.24, 2.25), 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 *Tracheloraphis*, and their sister group, the heterotrichs (class Heterotrichea), *Climacostomum* and

Eufolliculina, consistent with recent phylogenies inferred from large and small subunit rRNA data (Baroin-Tourancheau *et al.*, 1992, 1995; Hirt *et al.*, 1995; Hammerschmidt *et al.*, 1996). The classes Karyorelictea and Heterotrichea constitute the Subphylum Postciliodesmatophora Gerassimova and Seravin, 1976 *sensu* Small and Lynn (1985), which is characterized by strongly overlapping postciliary microtubular ribbons (Lynn and Corliss, 1991). Within the Postciliodesmatophora, the heterotrichs divide their macronucleus with extramacronuclear microtubules, while the karyorelicteans have non-dividing macronuclei (Raikov, 1982; Hammerschmidt *et al.*, 1996). Lynn (1996) 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.* (1996) 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 my analyses, all trees depicted *Protocruzia* in close association with the spirotrichs.

Phylogeny Of The Litostome Ciliates

Consistent with other studies (Leipe *et al.*, 1994a; Hammerschmidt *et al.*, 1996; Stechman *et al.*, 1998), the litostomes (class Litostomatea) formed a monophyletic group consisting of the haptorians and the trichostomes. As with previous studies using SSrRNA (Leipe *et al.*, 1994a; Embley *et al.*, 1995; Hirt *et al.*, 1995; Hammerschmidt *et al.*, 1996;

Wright and Lynn, 1997; Wright *et al.*, 1997a, 1997b) and the large subunit rRNA (Baroin-Tourancheau *et al.*, 1992, 1995), the sister group to the litostomes could not be unambiguously resolved.

The three new haptorian sequences (*Didinium*, *Dileptus*, *Enchelyodon*) did little to resolve the relationships within the subclass Haptoria in the parsimony and distance-matrix analyses. While parsimony analysis showed the haptorians as an unresolved clade, the distance-matrix trees depicted *Dileptus* (order Pharyngophorida) as the sister group to a polychotomy consisting of the four haptorids (order Haptorida) and *Loxophyllum* (order Pleurostomatida). It also showed the enchelyid *Homalozoon* grouping with *Spathidium* (a spathiidid) and not with the other enchelyid *Enchelyodon*. Monophyly of the haptorians is barely supported (50%) by parsimony analysis, but strongly supported (85% LS, 82% NJ) by distance-matrix methods. This is consistent with previous studies that indicated the haptorians to be a monophyletic group (Leipe *et al.*, 1994a; Hammerschmidt *et al.*, 1996; Wright and Lynn, 1997a, 1997b; Wright *et al.*, 1997).

The monophyly of the trichostomes is strongly supported by bootstrap data (100%) from both parsimony and distance-matrix methods. However, the addition of four new trichostome sequences created a polychotomy with the presumed vestibuliferid *Balantidium* and the putative entodiniomorphid *Macropodinium* not grouping with the other vestibuliferids or the entodiniomorphids respectively. *Cycloposthium* did group with the ophryoscolecids consistent with its placement in the order Entodiniomorphida by Small and Lynn (1985).

Phylogeny Of The Ophryoscolecid Rumen Ciliates

All trees depicted *Entodinium caudatum* as the earliest branching ophryoscolecid before a dichotomy containing *Epidinium* and *Ophryoscolex* on one branch (Ophryoscolecinae) and *Polyplastron*, *Diplodinium*, and *Eudiplodinium* (Diplodiniinae) on the other branch. *Entodinium*'s basal position to the other ophryoscolecids supports the idea that *Entodinium* is a representative of the ancestral entodiniomorphids (Crawley, 1923; Dogiel, 1925, 1947; Lubinsky, 1957b, 1957c).

Lubinsky (1957b, 1957c) used the lateral groove exhibited by members of the "*laterale*" group of *Entodinium* species and the diplodiniines, *Diplodinium* and *Eudiplodinium*, to place these latter genera closer to the base of his tree (Figure 2.4). My cladistic analysis of morphological characters supports this branching order (Figure 2.5). Moreover, in my cladistic analysis *Diplodinium* appears basal to all other ophryoscolecids (except *Entodinium*) because it lacks a skeletal plate. Lubinsky (1957c) regarded this feature of *Diplodinium* as significant in placing it as an early branch in his tree. He also observed that the right side of *Diplodinium* is remarkably similar to the left side of *Entodinium* and imagined that, with acquisition of skeletal plates, a torsional displacement of structures occurred across the left side of the cell repositioning the contractile vacuole, lateral groove, and nuclei, giving rise to the more derived diplodiniines, such as *Eudiplodinium* and *Polyplastron*. Finally, the DZS was imagined to have migrated posteriorly from the transverse plane of the AZS, leading to the derivation of the ophryoscolecines. Based upon the six SSrRNA sequences for ophryoscolecids, these ciliates do fall into three distinct groupings that correspond to Lubinsky's (1957c) subfamilial division of the Ophryoscolecidae. However, my molecular phylogenies show relationships within the

diplodiniines as unresolved (see Figures 2.24, 2.25).

Grain (1994b) has revised the taxonomy of the entodiniomorphids, elevating the Entodiniinae to family rank, the Entodininiidae (refer Table 2.1). This is consistent with my morphological and molecular analyses, although there is no unambiguous synapomorphy for this family. Grain (1994b) has retained the family Ophryoscolecidae and subfamily Diplodiniinae, but removed *Epidinium* from the subfamily Ophryoscolecinae Lubinsky, 1957 to the subfamily Epidiniinae Latteur, 1966 (refer Table 2.1). In my opinion, there is insufficient data to support these changes by Grain (1994b). I would prefer to await molecular sequence evidence from representatives of the other entodiniomorphid families, Ditoxidae Strelkow, 1939, Polydiniellidae Corliss, 1960, Rhinozetidae Van Hoven, 1988, Spirodiniidae Strelkow, 1939, Telamodiniidae Latteur and Dufey, 1967, and Troglodytellidae Corliss, 1979, before concluding that the genetic distance and topological position of *Entodinium* warrants recognition at the family rank. Further, four signature sequences (13 - 26 bp) for *En. caudatum*, two for *Ep. caudatum*, and three for *P. multivesiculatum* (Table 2.9) 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).

Phylogeny Of The Vestibuliferid Rumen Ciliates

Even though the two species of *Isotricha* paired together 100% of the time to form a clade, their pairing with *Dasytricha* was weakly supported by bootstrap data (60% MP, 50% LS, 61% NJ). Although this is in contrast to the recent study by Wright and Lynn (1997b)

Table 2.9. Signature sequences of *Entodinium*, *Epidinium* and *Polyplastron*.

Species	Signature Sequences	Seq. Pos. 5'--3'	Helix #
Entodinium caudatum	5'-- GAG ACC TTA AAT TTC --3'	442--456	17
	5'-- GAT TCT TCT ATA CTA TAG ATG ATA TC --3'	1224--1249	43
	5'-- TGT TAT ACA AAT A --3'	1265--1277	44
	5'-- CTC CTT TGG GAA AGA TA --3'	1552--1568	49
Epidinium caudatum	5'-- GTT CTC AAT ACT CTG TAT TCT GCA AC --3'	1221--1246	43
	5'-- CTC CGT ACG GGG AAG ATA --3'	1262--1274	49
Polyplastron multivesiculatum	5'-- GCG GTT ATT ATC GC --3'	442--455	17
	5'-- GAT TCT ATC ATC TTA TGA TTG ATA TC --3'	1225--1250	43
	5'-- CCT GTA AGG GGA AGA TA --3'	1553--1569	49

where bootstrap values were significantly higher, this lower bootstrap support is probably a result of the addition of sequences from *Balantidium* and *Macropodinium*. More importantly, my study clearly indicates that the vestibuliferids *Balantidium*, *I. intestinalis*, *I. prostoma*, and *Dasytricha* do not belong within the haptorian lineage, but form a sister group to the entodiniomorphids. Based on morphological data, de Puytorac *et al.* (1994) have also shown that another vestibuliferid, *Paraisotricha*, does not group with the haptorians, but rather groups with the phyllopharyngeans. However, they depict the buetschliid, *Alloiozona*, as a haptorian, relating it closer to *Monodinium* than to *Spathidium*. Contrary to this morphological analysis (de Puytorac *et al.*, 1994), my results show the free-living haptorians (subclass Haptoria) as the sister group to the trichostomes. Moreover, the haptorians are never found within the trichostomes (i.e. vestibuliferids or entodiniomorphids). This is consistent with my view that the trichostomes evolved from a haptorian-like ancestor that lost its toxicysts and oral dikinetids in concert with invagination of the vestibulum.

Lipscomb and Riordan (1992) suggested that Small and Lynn's (1985) classification of the rumen ciliates was paraphyletic. Lipscomb and Riordan (1992) used 46 morphological and ultrastructural characters (see Table 2.10) for 21 genera of litostomes in a cladistic analysis applying successive weighting to the data set. They concluded that the two vestibuliferid families, Isotrichidae and Balantidiidae, and the family Buetschliidae belonged to the subclass Haptoria and not the subclass Trichostomatia. Because of these differences with my SSrRNA trees, I undertook a reanalysis of this data set since Lipscomb and Riordan (1992) did not report which characters were more heavily weighted by their analysis nor did they describe the topology of the other equally parsimonious tree.

Table 2.10. Lipscomb and Riordan's (1992) characteristics for the Haptoria

1. Mucocysts: (0) +; (1) -; (2) doughnut -shaped

2. Clathrocysts: (0) -; (1) +

3. Conocysts: (0) -; (1) +

4. Nail-shaped toxicysts: (0) -; (1) +

* 5. Toxicyst distribution: (0)-; (1) + around mouth but not within the nematodesmata and inserted in the somatic cortex; (2) in tentacles surrounded by microtubules; (3) within the nematodesmata; (4) within the nematodesmata and inserted in the cortex

6. Outer tube of toxicyst shows striations in cross-section: (0)-; (1)+

* 7. Toxicyst types (0) one; (1) two; (2) two and smaller toxicyst is spindle-shaped

8. Space between the membrane surrounding the toxicyst and outer tube: (0) thin and clear; (1) filled with granular material; (2) filled with flocculent material

9. Micronuclear chromatin condensed into large clumps in the centre of the nucleus: (0) -; (1) +

10. Chromatin of macronucleus distributed: (0) evenly; (1) a layer under the membrane

11. Distribution of nucleoli in macronucleus: (0) peripheral; (1) evenly; (2) central

12. Kineties: (0) extend from suture; (1) bipolar rows; (2) from mouth to posterior end, but mouth is ventral; (3) girdles of cilia; (4) from posterior mouth to anterior pole

13. Postciliary microtubules overlap: (0) -; (1) + in stacks

14. Angle of postciliary microtubules: (0) divergent; (1) convergent

15. Direction of the kinetodesmal fibre (Kd): (0) anterior; (1) anterior and lateral

16. Kd length: (0) overlapping; (1) short

17. Transverse microtubules: (0) radial; (1) tangential

18. Transverse microtubules extend to adjacent kinety: (0) +; (1) -

19. Two sets of transverse microtubules: (0) -; (1) + one short and extends laterally, one longer and extends anteriolaterally

* 20. At proximal end of the kinesome, some microtubules of the first transverse ribbon and accompanying electron-dense material extend through the telacorticalis: (0) -; (1) +; (2) + and electron-dense material forms a cylinder

* 21. Left - right (or dorsal-ventral) differentiation of somatic ciliation: (0) -; (1) +

22. Cilia separated by extended cortical ridges: (0)- ; (1) +

* 23. Oral dikinetids: (0) + and only the one furthest from the oral opening is ciliated; (1) both ciliated

* 24. Microtubular roots of the nonciliated kinetosomes of dikinetid: (0) postciliary microtubules; (1) transverse and perhaps one to three very short postciliary microtubules

* 25. Microtubular roots of the ciliated kintosomes of dikinetid (0) -; (1) a single postciliary microtubule; (2) a posticilary ribbon (a very short tangential ribbon may also be present); (3) a postciliary ribbon an two transverse ribbons. This transformation series is linear

* 26. Distinct fibrous annulus: (0) -; (1) +

***indicates ordered characters that were recoded in my analysis (see Table 2.11).**

Table 2.10. (Continued). Lipscomb and Riordan's (1992) characteristics for the Haptoria

27. Nematodesmata originate: (0) -; (1) dense plate at base of both kinetosomes; (2) from nonciliated kineosomes of the oral dikenetid.

28. Nematodesmata shape: (0) -; (1) round bundle and a wedge-shaped group of mcrotubules; (2) rectangular to round groups; (3) triangular.

29. Nematodesmata paired: (0) -; (1) +

* 30. Oral monokinetids arise through loss of the posterior kinetosome of the oral pair: (0) -; (1) +

* 31. Oral monokinetids arise through the loss of the oral pair, leaving the oralized somatic kinetosomes as the source of the supporting microtubules: (0) -; (1)+

* 32. Cross-section through the oral area shows nematodesmata, accessory ribbons of microtubules that originate perpendicular to the transverse microtubules of the oral dikinetid and tranverse microtubules: (0)-; (1) +; (2) + and fibrous material is well developed and confluent around all the oral; (3) no buldge microtubules; (4) + and bulge microtubules coalesce to form nematodesmata-like bundles. The transformation series is not linear; states 2 and 4 are derived separatively from state 1

* 33. Position of the mouth: (0) ventral and circular; (1) apical and circular; (2) apical and domed; (3) apical and slit-like; (4) with proboscis; (5) with vestibule. The transformation series is not linear; states 2, 4 and 5 are derived separatively from state 1.

34. Microtubules lining cytopharynx: (0) postciliary microtubules; (1) independent ribbon of microtubules; (2) transverse microtubules; (3) set of microtubules arising adjacent to the insertion of the transverse microtubules.

* 35. Number of oral dikinetids equals the number of somatic kineties (0) oral dikinetids not associated with ends of somatic kineties; (1) +; (2) more oral dikinetids and they are perpendicular to kineties; (3) more oral dikinetids and they are parallel to the kineties; (4) somatic kinetosomes spiral around anterior end, oral dikinetids a closed ring. The transformation series is not linear; states 3 and 4 are derived from state 1, and state 2 is derived from state 3.

36. Right-left differentiation of the oral kinetosomes: (0) -; (1) +.

37. Epiplasm: (0) well developed; (1) thin; (2) apparently absent. The transformation series is linear.

38. Alveoli: (0) well developed; (1) tightly compressed.

39. Endoplasmic reticulum between cortex and endoplasm interrupted only at kinetosomes: (0) -; (1) +.

40. Dorsal brush of clavate cilia: (0) -; (1) + in straight rows; (2) + in zig-zag rows.

41. Dorsal brush is pairs of clavate cilia. The posterior kinetosome has normal infraciliature, anterior only nematodesmata: (0) -; (1) +; (2) anterior kinetosome lacks nematodesmata.

* 42. Oralized somatic kinetosomes at the anterior ends of the kineties: (0) -; (1) + with nematodesmata; (2) + with numerous nematodesmata forminf several rings a round the oral area, but no Kd on most aneterior kinetosome.

* 43. Somatic kinetosomes in a kinety connected: (0) -; (1) + by a striated fiber; (2) + by electron-dense material separated by a stripe that lies perpendicular to the kinety axis. This transformation series is linear.

44. General body shape: (0) rounded; (1) oral area flattened; (2) laterally compressed.

45. Concrement vacuole: (0) -; (1) +.

46. Tela corticalis: (0) +; (1) -.

*** indicates ordered characters that were recoded in my analysis (see Table 2.11).**

I recoded their eight ordered (non-linear and linear) multistate characters, 5, 12, 25, 32, 33, 35, 37, and 43 (Table 2.11) using additive binary coding (Wiley *et al.*, 1991) and provided this as input for PAUP (ver 3.1) (Swofford, 1993). When the data were examined using the branch and bound method of PAUP with all characters of equal weight, I obtained 24 equally parsimonious trees having a length of 95 steps with a consistency index (CI) of 0.84 and a retention index (RI) of 0.86. Even with the high number of missing characters (10-16 characters) for the trichostomes, all 24 trees showed the vestibuliferids (*Balantidium*, *Isotricha*) and the buetschliids (*Alloiozona*, *Didesmis*) as a monophyletic group (the trichostomes) and as a sister group to the haptorian clade (Figure 2.29).

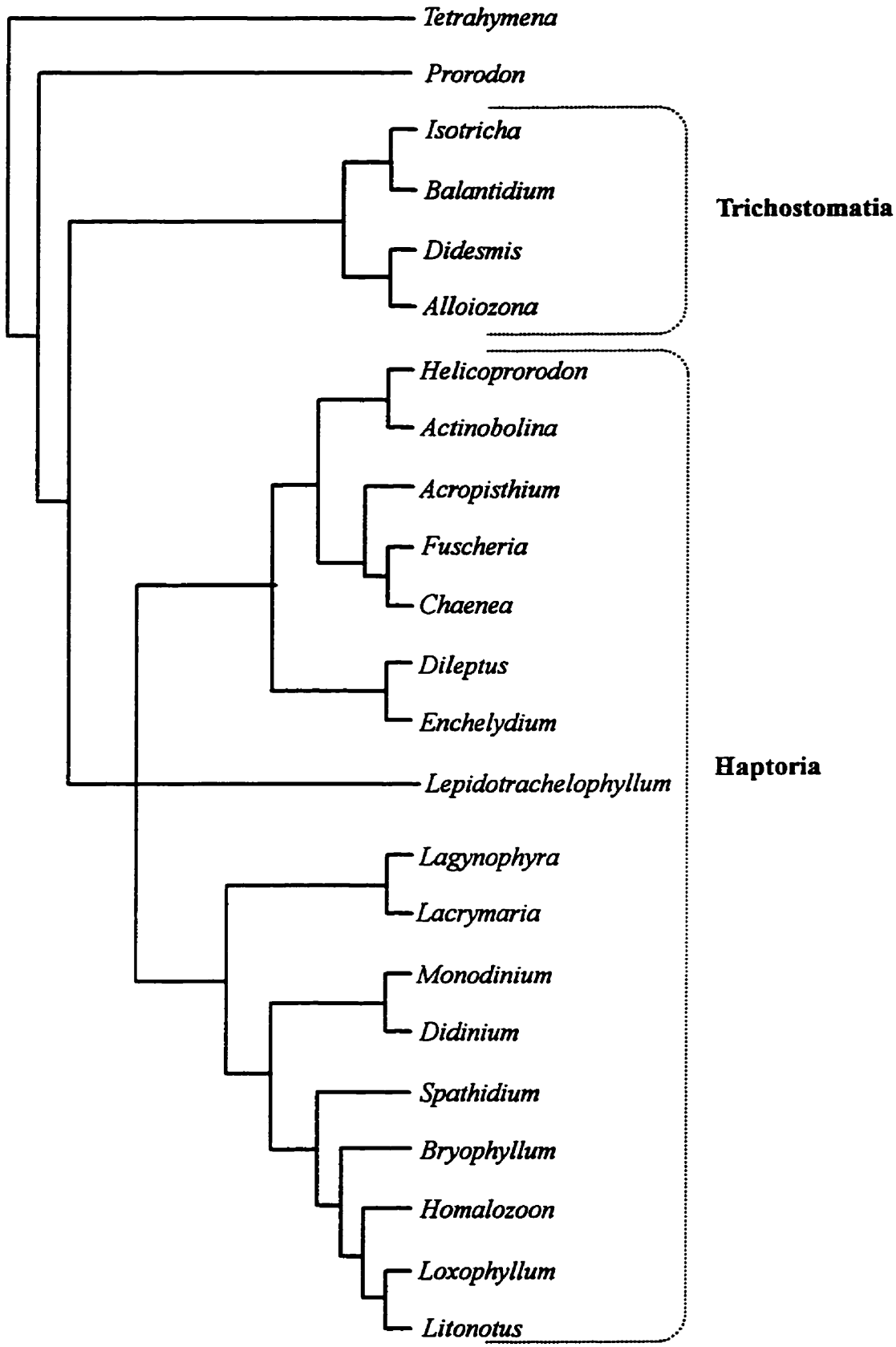
Lipscomb and Riordan (1992) used successive weighting to reduce the number of equally parsimonious trees to two, both having a length of 94 steps and a CI of 0.85 and a RI of 0.87. Lipscomb and Riordan's (1992) two trees differ from my 24 trees, based on the same data, by only one step. When I treated the eight ordered multistate characters as unordered, I reduced the number of equally parsimonious trees to 14. These trees had fewer steps (91) and a better CI (0.87) and RI (0.88), and they still depicted the trichostomes as a monophyletic group and as the sister group to the haptorians. Although successive weighting reduced the number of equally parsimonious trees, it apparently pulled the trichostomes (vestibuliferids and buetschliids) within the haptorian cluster. It is important to know which characters were weighted more heavily in the Lipscomb and Riordan (1992) analysis to determine whether these characters can truly be interpreted as "taxonomically stronger" than the 18S rRNA features. Because no sequences are available for the Buetschliidae, in my opinion, there are insufficient data to support their removal from the Entodiniomorphida to the Haptoria as

Table 2.11. Lipscomb and Riordan's (1992) character coding for the litostomes, and the outgroup taxa, *Tetrahymena* and *Prorodon*. Refer to **Table 2.10** for character definitions.

Genus	Characters 1234 5 678901 2 345678901234 5 678901 2 3 4 5 6 789012 3 456 (tens: 1, 2, 3, 4)[a]
Tetrahymena	000000000??000000000000000000000000000000000000000000000000000000000
Prorodon	0000100000000110000101110000111000110000000100001100001000000000000
Isotricha	?00000000??1011101111?1?1000?????0?2001001010001 2????01110??200000
Balantidium	?00000000??1011100111111?00?????0?200100101000012????011101?200000
Acropisthium	00001010?01???1000?1111??00001110022000100010000210000 11101?100001
Actinobolina	1000110000101110001111110000011100220001100100002100001 11?11100000
Alloiozona	?00000000???0110001??????000?????0?2001001010000210000 1110??200010
Bryophyllum	000010100100011000111111100001110022000100000100211100111011000200
Chaenea	0000101010 1?0?100011111110010111002200010001100021000011101?100001
Didinium	0100101101000110101111111200011111220001001110002101001110110 11000
Didesmis	?00000000??1011000111?1??000?????0?2001001010000210000 11101?200010
Dileptus	00001010000002100011111111?000111002201010001001021110011 1011?00000
Enchelydium	0000101002?00110001111111000?1???0?20101000100002???011112 1100000
Fuscheria	20011010001???100011111110010111002200010001000021000011101?100001
Helicoprorodon	000010000110111000111111110001110022000110010000310010111 0??100000
Homalozoon	001010100 1??01100011001110100111002200010000010021110011102 2000200
Lacrymaria	00001010 0??00110001111111000011100231001000110002100001110??010000
Lagynophrya	200010100 1?00110001 11?1??00001110023100100010000210000 11101?000000
Lepidotrach.	000010100 0????1000111111100001110 0?20001000100002100001 1101?000000
Litonotus	00?01010012?0?1000?1111??01001110022000100000100211101 11101?000200
Loxophyllum	00101011 0????10001111111010011100220001000001002111011110??000200
Monodinium	00001010 01??011010?111111 20001???02?00010011100021010011101?011000
Spathidium	00001010 0100011000111111100001110022000100000100211100111011?00100

a. Brackets indicate ordered character state transformations that were coded using additive binary coding (Wiley *et al.*, 1991).

Fig. 2.29. One of 24 equally parsimonious trees of the litostomes inferred from Lipscomb and Riordan's (1990, 1992) morphological and ultrastructural data (Table 2.11). All 24 trees showed the Trichostomatia as the sister group to the Haptoria. The branch and bound search program of PAUP (Swofford, 1993) was used to find this tree having 95 steps with a consistency index of 0.84 and a retention index of 0.86. No significance is placed on the lengths of the branches connecting the species. Names for suprageneric taxa appear in boldface.


Tetrahymena
Prorodon
Isotricha
Balantidium
Didesmis
Alloiozona
Trichostomatia
Helicoprorodon
Actinobolina
Acropisthium
Fuscheria
Chaenea
Dileptus
Enchelydium
Lepidotrachelophyllum
Haptoria
Lagynophyra
Lacrymaria
Monodinium
Didinium
Spathidium
Bryophyllum
Homalozoon
Loxophyllum
Litonotus

proposed by Foissner and Foissner (1988) and Grain (1994a). I would prefer to await molecular data from members of the Buetschliidae before concluding that they be transferred to the Haptoria. Furthermore, my reanalysis of Lipscomb and Riordan's (1992) data (see Figure 2.29), coupled with the overwhelming support from molecular data using various phylogenetic methods and software packages, convincingly demonstrates that the isotrichid vestibuliferids belong with the Trichostomatia and not within the Haptoria.

Outgroup Analysis Of The Trichostomes

Similar to previous parsimony analyses by Wright and his collaborators (Wright and Lynn, 1997a, 1997b; Wright *et al.*, 1997), the free-living haptorians were barely supported (50% MP) as a monophyletic group. I believed that the addition of more haptorian sequences (e.g. *Didinium*, *Dileptus*, *Enchelyodon*) might help to resolve relationships within the clade, but it did not. Most researchers recommend using the sister group as the outgroup to determine the topology of the ingroup (Maddison *et al.*, 1984; Brooks and McLennan, 1991; Wiley *et al.*, 1991), although Nixon and Carpenter (1993) have argued that it is not necessary for the outgroup to be, or to include, the sister group and criticized others for propagating this myth. They also suggested that one or more outgroups can be selected on the basis of the outgroup(s) sharing synapomorphies with the ingroup. In light of this, different lineages within the subphylum Intramacronucleata (see Lynn, 1996; Wright *et al.*, 1997) were used as outgroups to the haptorians. Because of the long branch lengths of the phyllopharyngeans (see Figure 2.25) *Trithigmostoma* and *Discophrya* were excluded from this analysis. Seven datasets were constructed, each containing only the

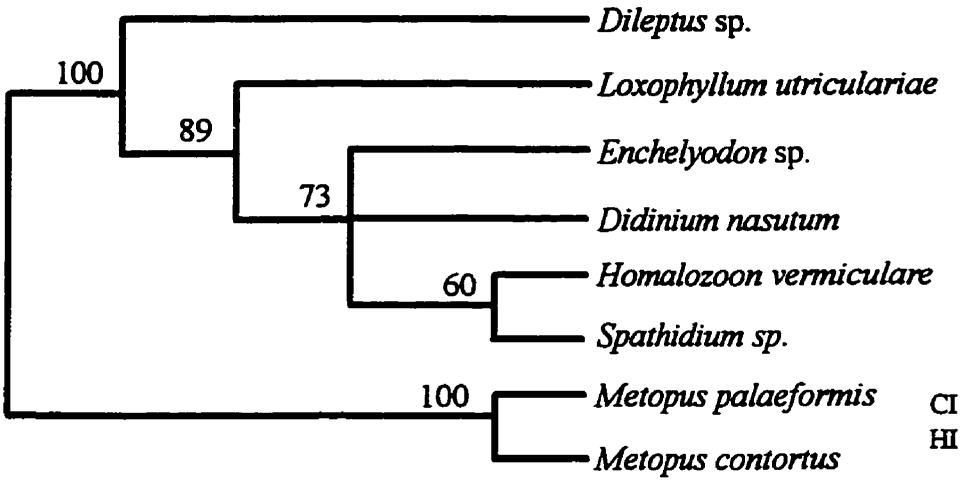
haptorians and one of the seven outgroup taxa (i.e. the armophorids, colpodeans, nassophoreans, oligohymenophoreans, prostomateans, spirotricheans, trichostomes).

When each of these seven outgroups was used in a parsimony analysis, three different trees were produced (Figure 2.30). Only one of the three trees resolved the relationships within the haptorians and it depicted *Dileptus* (order Pharyngophorida) as the earliest branching haptorian before a dichotomy containing *Loxophyllum* (order Pleurostomatida) on one branch and *Homalozoon*, *Enchelyodon*, *Spathidium* and *Didinium* (order Haptorida) on the other branch. The other two trees depicted the haptorians as an unresolved polychotomy similar to the trees from the original data set (c.f. Figures 2.24, 2.25, 2.30B, 2.30C). Polychotomies are usually attributed to simultaneous cladogenesis or to scarce and/or ambiguous data (Coddington and Scharff, 1996). In light of this, the six haptorians were aligned against each other and examined for phylogenetically informative sites. It was discovered that only 85 of the 1,660 sites were phylogenetically informative (i.e. 5.1% of the SSrRNA gene; see Figure 2.31) for the six diverse genera of haptorians. In comparison, there were 127 phylogenetically informative sites for 13 species belonging to the genus *Tetrahymena* (Wright and Lynn, 1995)

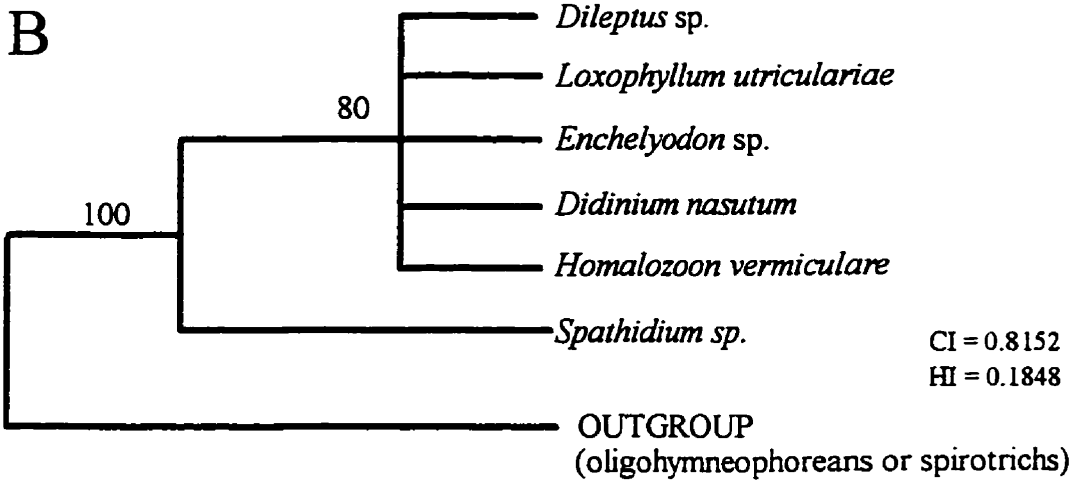
Lack of resolution among multiple outgroup taxa may affect the topology of the ingroup (Nixon and Carpenter, 1993). In order to determine what effect the unresolved haptorians might have on the topology of the trichostomes, all taxa except the haptorians and trichostomes were removed from the primary data set, and the six haptorians were used singly and together as the outgroup. Barta *et al.* (1991) used a similar approach when they used various outgroups (i.e. dinoflagellates, ciliates, yeast, slime mold) singly to individually root

Fig. 2.30. A comparison of maximum parsimony trees of the haptorian ciliates inferred from complete SSrRNA gene sequences with a bootstrap resampling of a reduced data set. The numbers at the nodes represent the percentage of times the group occurred out of 1,000 trees. Branches with less than 50% bootstrap support were collapsed. No significance is placed on the lengths of the branches connecting the species. **(A)** When the armophorids were used as the outgroup, some relationships with the haptorians are resolved. **(B)** When either the oligohymenophoreans or spirotrichs were used as the outgroup, the relationships amongst the haptorians remain unresolved. **(C)** When either the nassophoreans, colpodeans, or prostomateans were used as the outgroup, the phylogenetic relationships amongst the haptorians remain unresolved.

A


100
89
73
60
100
Dileptus sp.
Loxophyllum utriculariae
Enchelyodon sp.
Didinium nasutum
Homalozoon vermiculare
Spathidium sp.
Metopus palaeformis
Metopus contortus
CI = 0.8333
HI = 0.1667

B


80
100
Dileptus sp.
Loxophyllum utriculariae
Enchelyodon sp.
Didinium nasutum
Homalozoon vermiculare
Spathidium sp.
CI = 0.8152
HI = 0.1848
OUTGROUP
(oligohymneophoreans or spirotrichs)

C

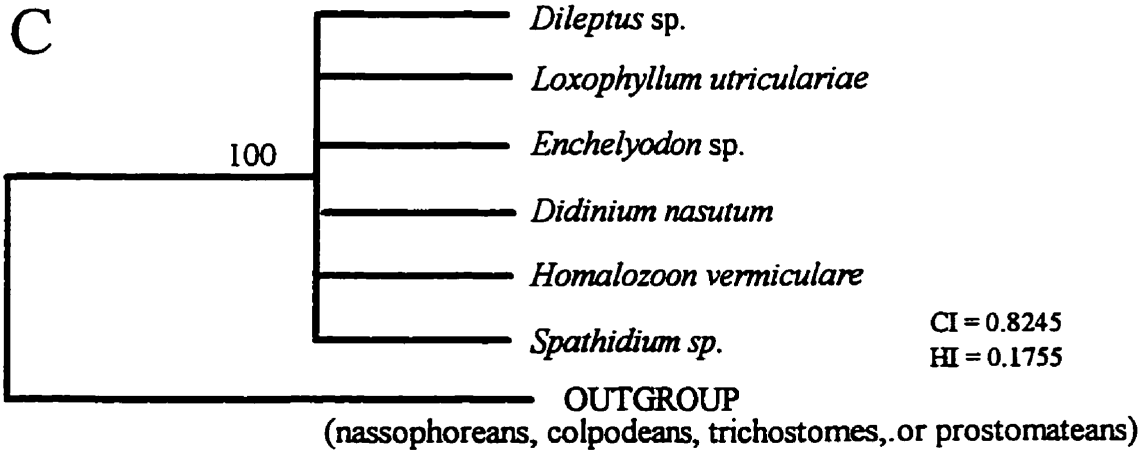
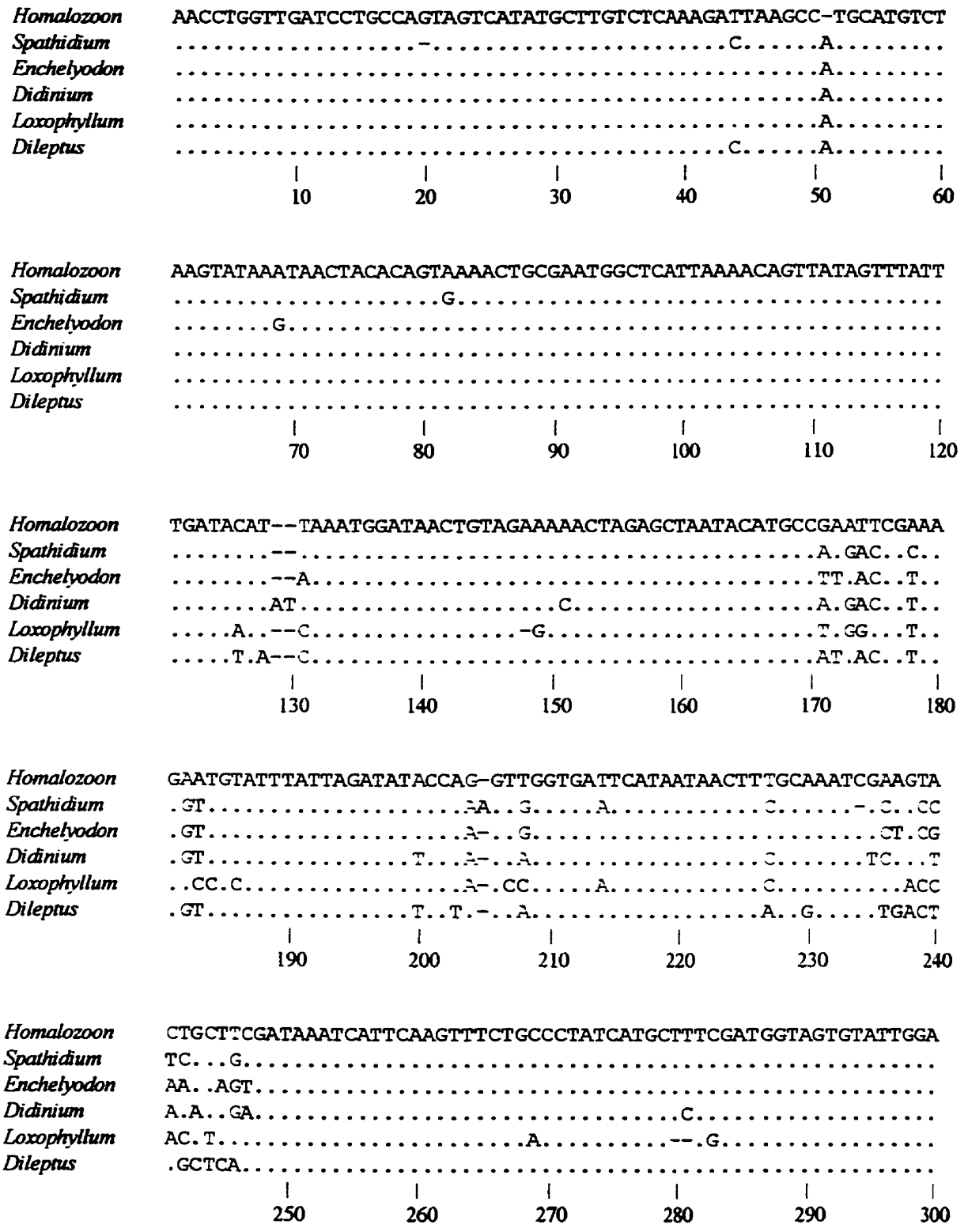
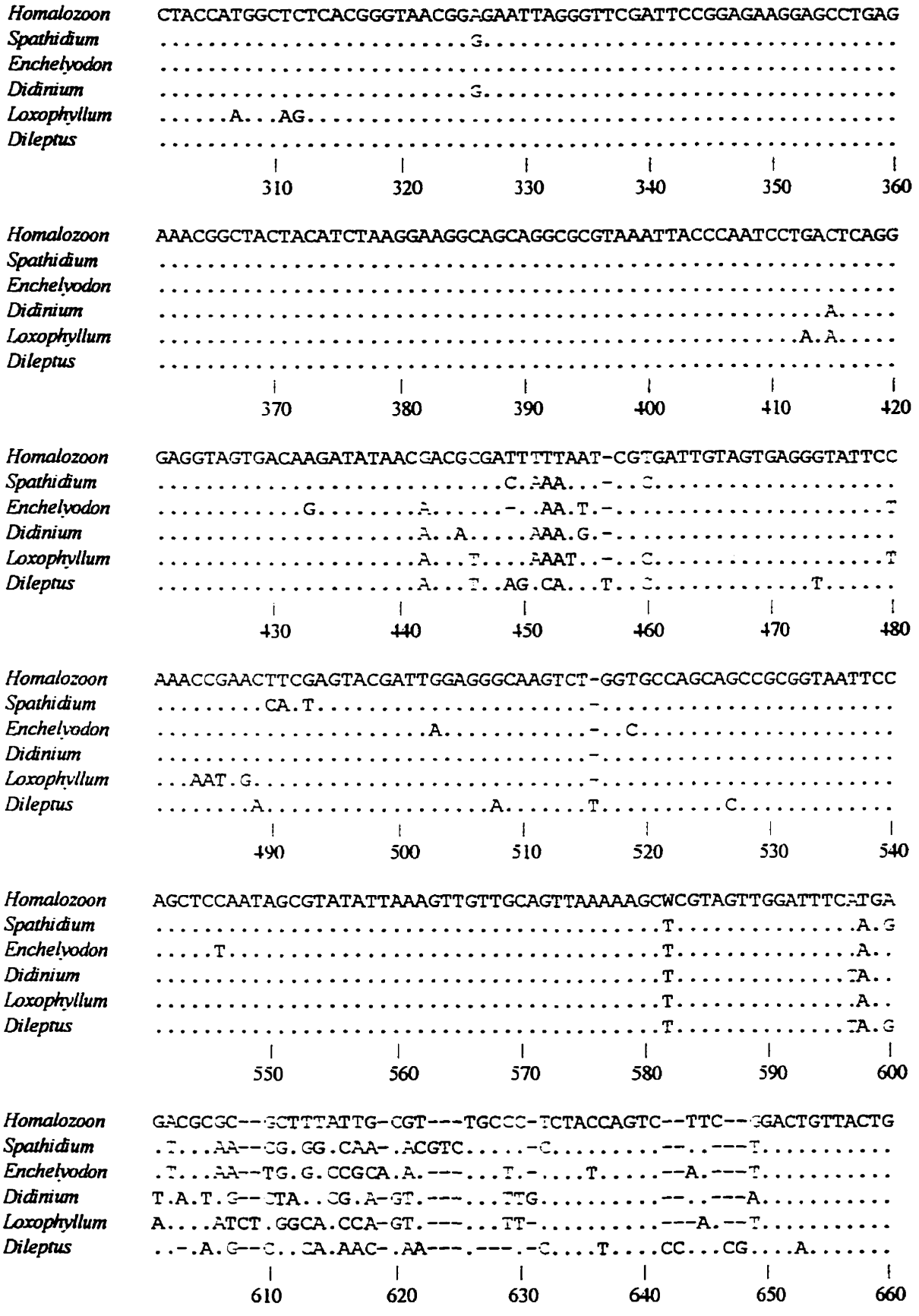
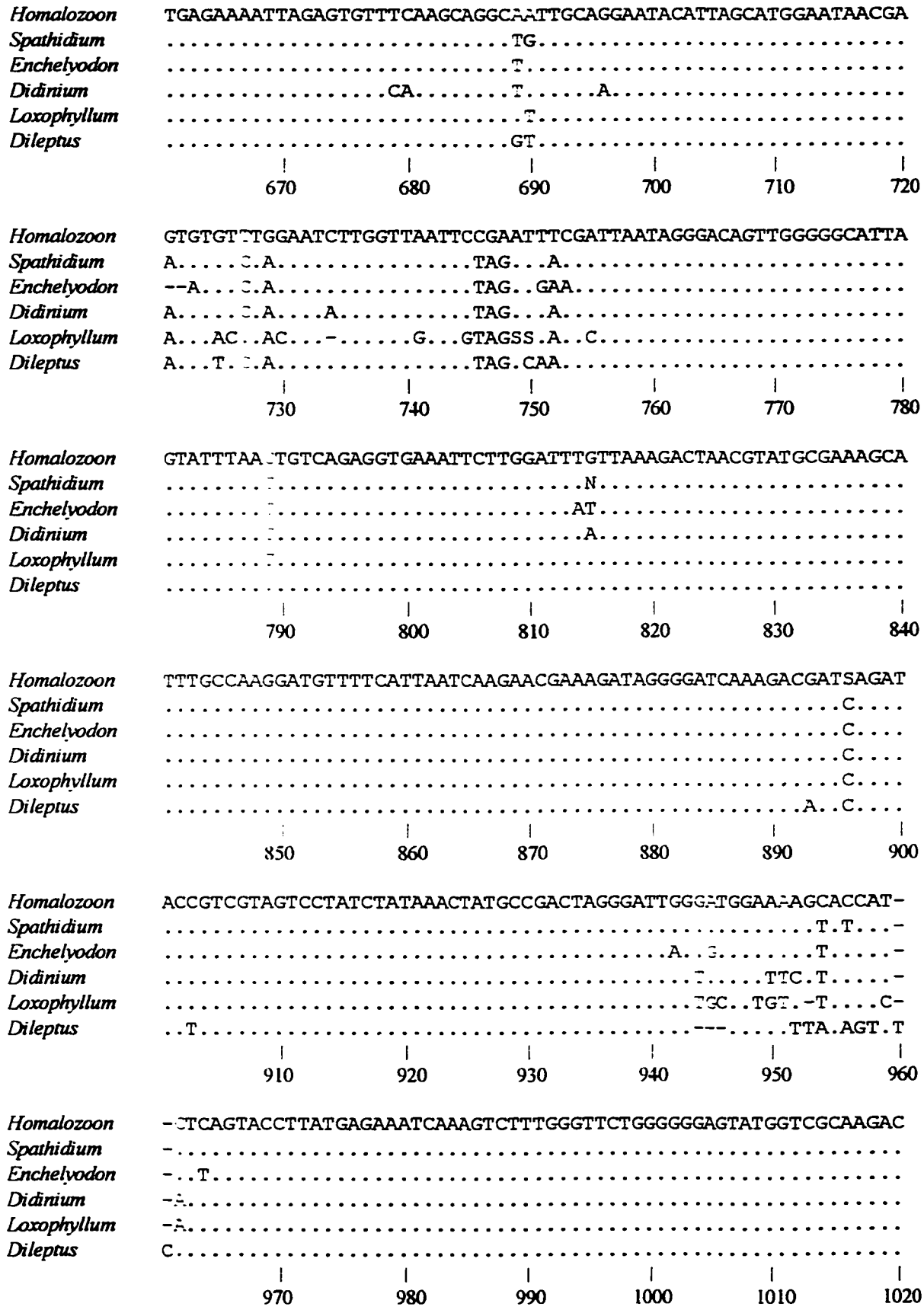
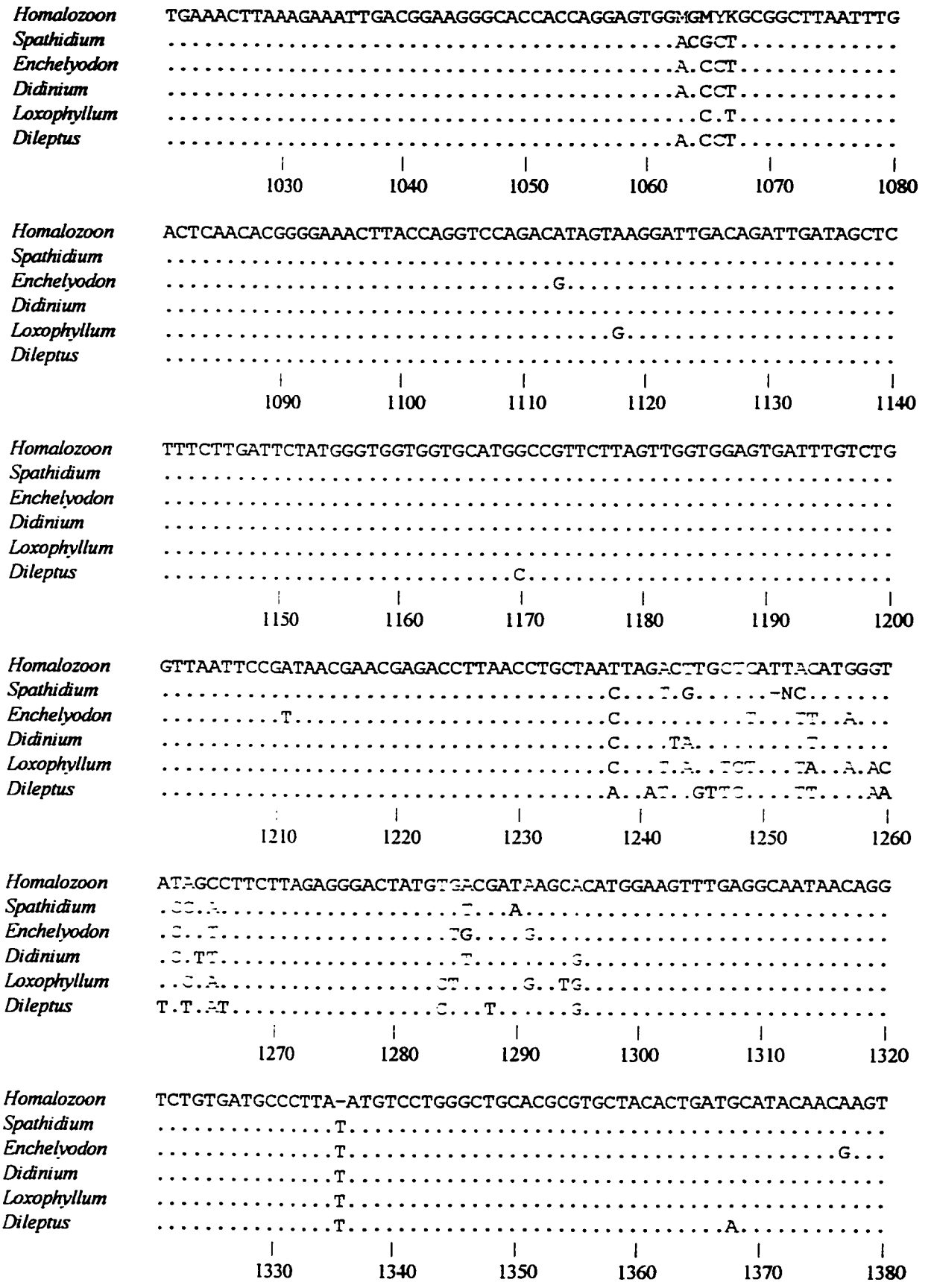

100
Dileptus sp.
Loxophyllum utriculariae
Enchelyodon sp.
Didinium nasutum
Homalozoon vermiculare
Spathidium sp.
CI = 0.8245
HI = 0.1755
OUTGROUP
(nassophoreans, colpodeans, trichostomes,.or prostomateans)

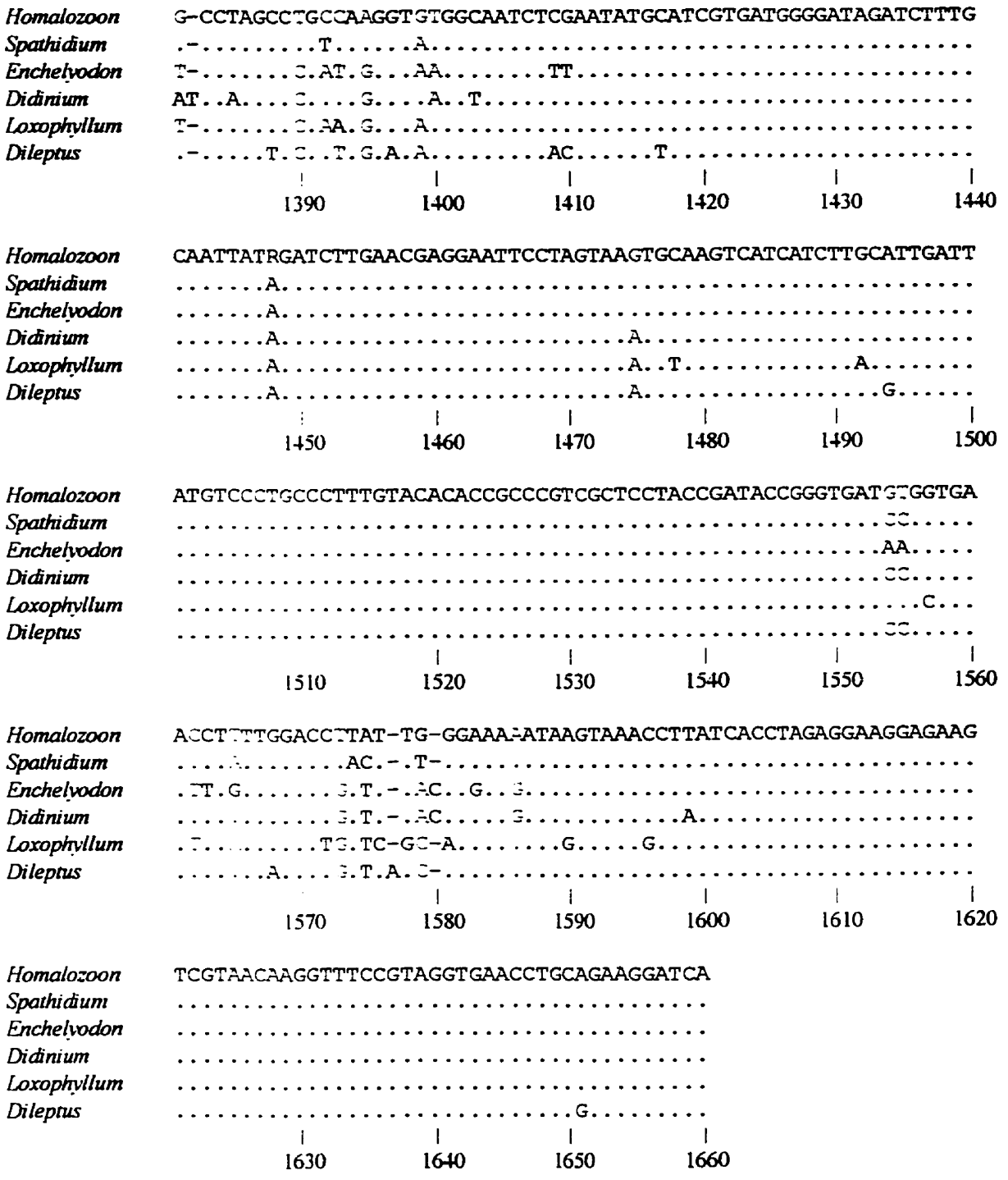
Fig. 2.31. Sequence alignment of the free-living haptorians. *Homalozoon* is used as the reference sequence and only nucleotide differences with the reference sequence are indicated for the other taxa. Red highlighted nucleotides represent phylogenetically informative sites. There are only 85 phylogenetically informative sites out of 1,660 positions amongst the six genera of haptorians. Note: This alignment continues for five pages.


Homalozoon AACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCC-TGCATGTCT
Spathidium .....-.....C.....A.....
Enchelyodon .....A.....
Didinium .....A.....
Loxophyllum .....A.....
Dileptus .....C.....A.....
10 20 30 40 50 60
Homalozoon AAGTATAAATAACTACACAGTAAAACTGCGAATGGCTCATTAAAACAGTTATAGTTTATT
Spathidium .....G.....
Enchelyodon .....G.....
Didinium .....
Loxophyllum .....
Dileptus .....
70 80 90 100 110 120
Homalozoon TGATACAT--TAAATGGATAACTGTAGAAAAACTAGAGCTAATACATGCCGAATTCGAAA
Spathidium .....--.....A.GAC..C..
Enchelyodon .....--A.....TT.AC..T..
Didinium .....AT.....C.....A.GAC..T..
Loxophyllum .....A..--C.....-G.....T.GG...T..
Dileptus .....T.A--C.....AT.AC..T..
130 140 150 160 170 180
Homalozoon GAATGTATTTATTAGATATACCAG-GTTGGTGATTCATAATAACTTTGCAAATCGAAGTA
Spathidium .GT.....AA..G.....A.....C.....-.C..CC
Enchelyodon .GT.....A-..G.....CT.CG
Didinium .GT.....T...A-..A.....C.....TC...T
Loxophyllum ..CC.C.....A-.CC.....A.....C.....ACC
Dileptus .GT.....T..T.-..A.....A..G.....TGACT
190 200 210 220 230 240
Homalozoon CTGCTTCGATAAATCATTCAAGTTTCTGCCCTATCATGCTTTCGATGGTAGTGTATTGGA
Spathidium TC...G.....
Enchelyodon AA..AGT.....
Didinium A.A..GA.....C.....
Loxophyllum AC.T.....A.....--.G.....
Dileptus .GCTCA.....
250 260 270 280 290 300


Homalozoon CTACCATGGCTCTCACGGGTAACGGAGAATTAGGGTTCGATTCCGGAGAAGGAGCCTGAG
Spathidium .....G.....
Enchelyodon .....
Didinium .....G.....
Loxophyllum .....A...AG.....
Dileptus .....
310 320 330 340 350 360
Homalozoon AAACGGCTACTACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTGACTCAGG
Spathidium .....
Enchelyodon .....
Didinium .....A.....
Loxophyllum .....A.A.....
Dileptus .....
370 380 390 400 410 420
Homalozoon GAGGTAGTGACAAGATATAACGACGCGATTTTTAAT-CGTGATTGTAGTGAGGGTATTCC
Spathidium .....C.AAA...-..C.....
Enchelyodon .....G.....A.....-..AA.T.-.....T
Didinium .....A..A.....AAA.G.-.....
Loxophyllum .....A...T....AAAT..-..C.....T
Dileptus .....A...T..AG.CA...T..C.....T.....
430 440 450 460 470 480
Homalozoon AAACCGAACTTCGAGTACGATTGGAGGGCAAGTCT-GGTGCCAGCAGCCGCGGTAATTCC
Spathidium .....CA.T.....-.....
Enchelyodon .....A.....-..C.....
Didinium .....-.....
Loxophyllum ...AAT.G.....-.....
Dileptus .....A.....A.....T.....C.....
490 500 510 520 530 540
Homalozoon AGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCWCGTAGTTGGATTTCATGA
Spathidium .....T.....A.G
Enchelyodon .....T.....T.....A..
Didinium .....T.....TA..
Loxophyllum .....T.....A..
Dileptus .....T.....TA.G
550 560 570 580 590 600
Homalozoon GACGCGC--GCTTTATTG-CGT---TGCCC-TCTACCAGTC--TTC--GGACTGTTACTG
Spathidium .T...AA--CG.GG.CAA-.ACGTC.....-C.....--..---T.....
Enchelyodon .T...AA--TG.G.CCGCA.A.---...T.-....T.....--A.---T.....
Didinium T.A.T.G--CTA..CG.A-GT.---...TTG.....--..---A.....
Loxophyllum A....ATCT.GGCA.CCA-GT.---...TT-.....---A.--T.....
Dileptus ..-.A.G--C..CA.AAC-.AA---.---.-C....T....CC...CG....A.....
610 620 630 640 650 660


Homalozoon TGAGAAAATTAGAGTGTTTCAAGCAGGCAATTGCAGGAATACATTAGCATGGAATAACGA
Spathidium .....TG.....
Enchelyodon .....T.....
Didinium .....CA.....T.....A.....
Loxophyllum .....T.....
Dileptus .....GT.....
670 680 690 700 710 720
Homalozoon GTGTGTTTGGAATCTTGGTTAATTCCGAATTTCGATTAATAGGGACAGTTGGGGGCATTA
Spathidium A.....C.A.....TAG...A.....
Enchelyodon --A...C.A.....TAG..GAA.....
Didinium A.....C.A....A.....TAG...A.....
Loxophyllum A...AC..AC...-.....G...GTAGSS.A..C.....
Dileptus A...T..C.A.....TAG.CAA.....
730 740 750 760 770 780
Homalozoon GTATTTAA.TGTCAGAGGTGAAATTCTTGGATTTGTTAAAGACTAACGTATGCGAAAGCA
Spathidium .....T.....N.....
Enchelyodon .....T.....AT.....
Didinium .....T.....A.....
Loxophyllum .....T.....
Dileptus .....
790 800 810 820 830 840
Homalozoon TTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGATAGGGGATCAAAGACGATSAGAT
Spathidium .....C....
Enchelyodon .....C....
Didinium .....C....
Loxophyllum .....C....
Dileptus .....A..C....
850 860 870 880 890 900
Homalozoon ACCGTCGTAGTCCTATCTATAAACTATGCCGACTAGGGATTGGGATGGAAAAGCACCAT-
Spathidium .....T.T...-
Enchelyodon .....A..G.....T.....-
Didinium .....T.....TTC.T.....-
Loxophyllum .....TGC..TGT.-T....C-
Dileptus ..T.....---.....TTA.AGT.T
910 920 930 940 950 960
Homalozoon -CTCAGTACCTTATGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGAC
Spathidium -.....
Enchelyodon -..T.....
Didinium -A.....
Loxophyllum -A.....
Dileptus C.....
970 980 990 1000 1010 1020


Homalozoon TGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGAGTGGMGMYKGCGGCTTAATTTG
Spathidium .....ACGCT.....
Enchelyodon .....A.CCT.....
Didinium .....A.CCT.....
Loxophyllum .....C.T.....
Dileptus .....A.CCT.....
1030 1040 1050 1060 1070 1080
Homalozoon ACTCAACACGGGGAAACTTACCAGGTCCAGACATAGTAAGGATTGACAGATTGATAGCTC
Spathidium .....
Enchelyodon .....G.....
Didinium .....
Loxophyllum .....G.....
Dileptus .....
1090 1100 1110 1120 1130 1140
Homalozoon TTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTG
Spathidium .....
Enchelyodon .....
Didinium .....
Loxophyllum .....
Dileptus .....C.....
1150 1160 1170 1180 1190 1200
Homalozoon GTTAATTCCGATAACGAACGAGACCTTAACCTGCTAATTAGACTTGCTCATTACATGGGT
Spathidium .....C...T.G.....-NC.....
Enchelyodon .....T.....C.....T...TT..A...
Didinium .....C....TA.....T.....
Loxophyllum .....C...T.A..TCT...TA..A.AC
Dileptus .....A..AT..GTTC....TT....AA
1210 1220 1230 1240 1250 1260
Homalozoon ATAGCCTTCTTAGAGGGACTATGTGACGATAAGCACATGGAAGTTTGAGGCAATAACAGG
Spathidium .CC.A.....T...A.....
Enchelyodon .C..T.....TG....G.....
Didinium .C.TT.....T.....G.....
Loxophyllum ..C.A.....CT.....G..TG.....
Dileptus T.T.AT.....C...T.....G.....
1270 1280 1290 1300 1310 1320
Homalozoon TCTGTGATGCCCTTA-ATGTCCTGGGCTGCACGCGTGCTACACTGATGCATACAACAAGT
Spathidium .....T.....
Enchelyodon .....T.....G...
Didinium .....T.....
Loxophyllum .....T.....
Dileptus .....T.....A.....
1330 1340 1350 1360 1370 1380


Homalozoon G-CCTAGCCTGCCAAGGTGTGGCAATCTCGAATATGCATCGTGATGGGGATAGATCTTTG
Spathidium .-.....T.....A.....
Enchelyodon T-.....C.AT.G...AA.....TT.....
Didinium AT..A....C....G....A..T.....
Loxophyllum T-.....C.AA.G...A.....
Dileptus .-.....T.C..T.G.A.A.....AC.....T.....
1390 1400 1410 1420 1430 1440
Homalozoon CAATTATRGATCTTGAACGAGGAATTCCTAGTAAGTGCAAGTCATCATCTTGCATTGATT
Spathidium .....A.....
Enchelyodon .....A.....
Didinium .....A.....A.....
Loxophyllum .....A.....A..T.....A.....
Dileptus .....A.....A.....G.....
1450 1460 1470 1480 1490 1500
Homalozoon ATGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATACCGGGTGATGTGGTGA
Spathidium .....CC.....
Enchelyodon .....AA.....
Didinium .....CC.....
Loxophyllum .....C...
Dileptus .....CC.....
1510 1520 1530 1540 1550 1560
Homalozoon ACCTTTTGGACCTTAT-TG-GGAAA-ATAAGTAAACCTTATCACCTAGAGGAAGGAGAAG
Spathidium ....A.....AC.-.T-.....
Enchelyodon .TT.G.....C.T.-.AC..G..G.....
Didinium .....C.T.-.AC.....G.....A.....
Loxophyllum .T.....TG.TC-GC-A.....G.....G.....
Dileptus .....A....C.T.A.C-.....
1570 1580 1590 1600 1610 1620
Homalozoon TCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA
Spathidium .....
Enchelyodon .....
Didinium .....
Loxophyllum .....
Dileptus .....G.....
1630 1640 1650 1660

several apicomplexan taxa. When the six haptorians were used together or singly as the outgroup, parsimony analysis with bootstrap resampling revealed five different tree topologies (Figure 2.32). When all six haptorians were used as the outgroup, the trichostome topology was similar to the initial parsimony tree (i.e. *Cycloposthium* paired with the ophryoscolecids and the positions of *Macropodinium* and *Balantidium* were unresolved) (c.f. Figures 2.24, 2.25) and the haptorian topology was similar to the distance-matrix trees (i.e. *Dileptus* was the sister group to the other haptorians) (c.f. Figures 2.25, 2.32A). *Spathidium* also produced the same tree topology. When *Didinium* was used as the outgroup (Figure 2.32B), *Cycloposthium* remained with the ophryoscolecids and the positions of *Macropodinium*, *Balantidium*, and now *Dasytricha* were unresolved (c.f. Figures 2.25, 2.32A, 2.32B). Similarly, when *Enchelyodon* was used as the outgroup (Figure 2.32C), the positions of *Cycloposthium*, *Dasytricha*, *Macropodinium*, and *Balantidium* were unresolved (c.f. Figures 2.24, 2.32A, 2.32B, 2.32C). In contrast, when *Homalozoon* was used as the outgroup (Figure 2.32D), *Cycloposthium* remained with the ophryoscolecids to form a clade that was the sister group to *Macropodinium*, but the positions of *Balantidium and Dasytricha* remained unresolved (c.f. Figures 2.24, 2.32A, 2.32B, 2.32C, 2.32D). *Loxophyllum* and *Dileptus* produced the same tree when there were used singly as the outgroup (Figure 2.32E). This tree closely resembled the one produced when either *Spathidium* or the haptorians were used as outgroups (c.f. Figures 2.32A, 2.32E). Barta *et al.* (1991) also noticed differences in the rooting point of ingroup taxa when different outgroups were used singly. Nixon and Carpenter (1993) note that character states assigned to the sister group may or may not be similar to those of the common ancestor of the ingroup; relationships within the ingroup may not be resolved because

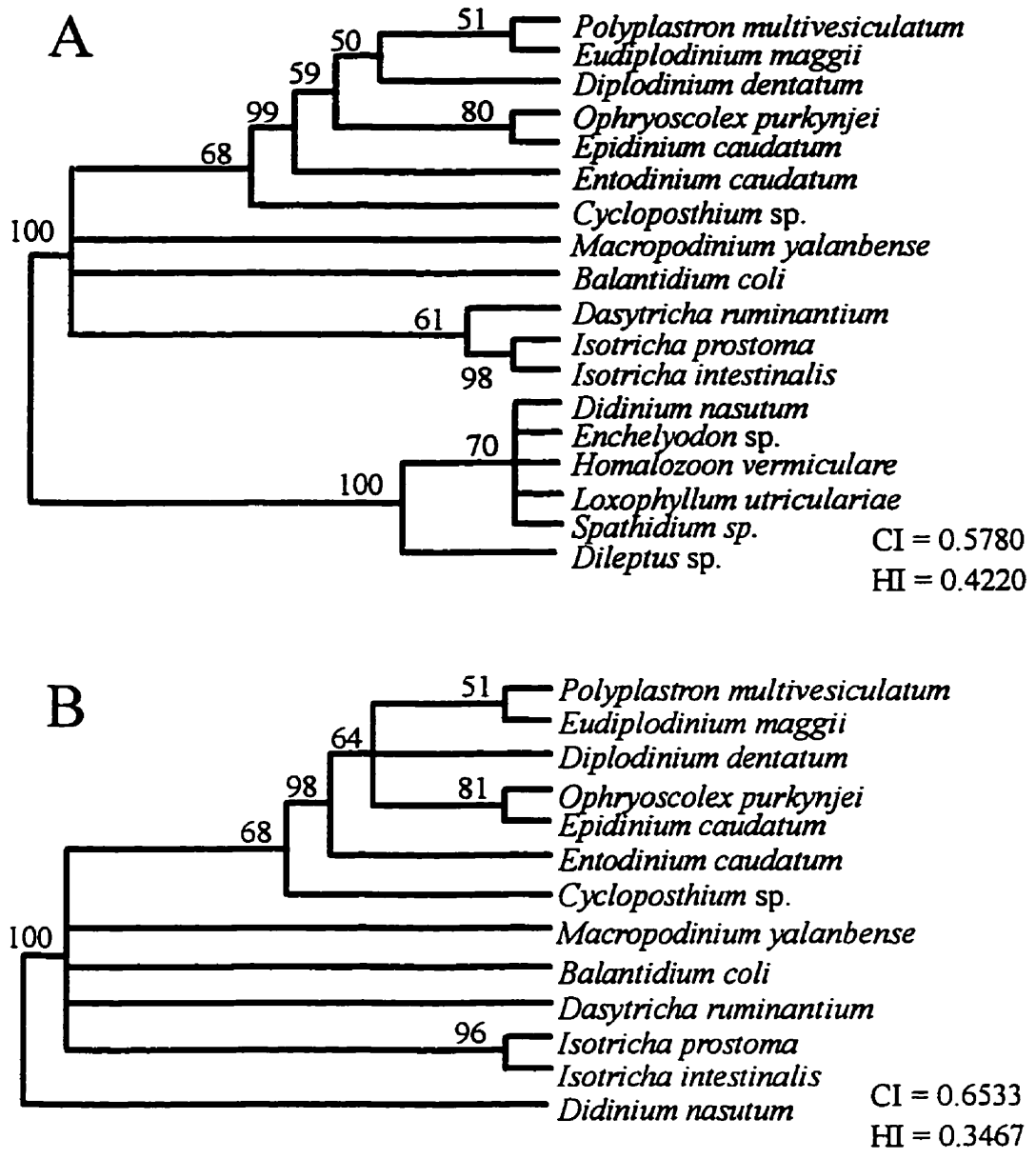
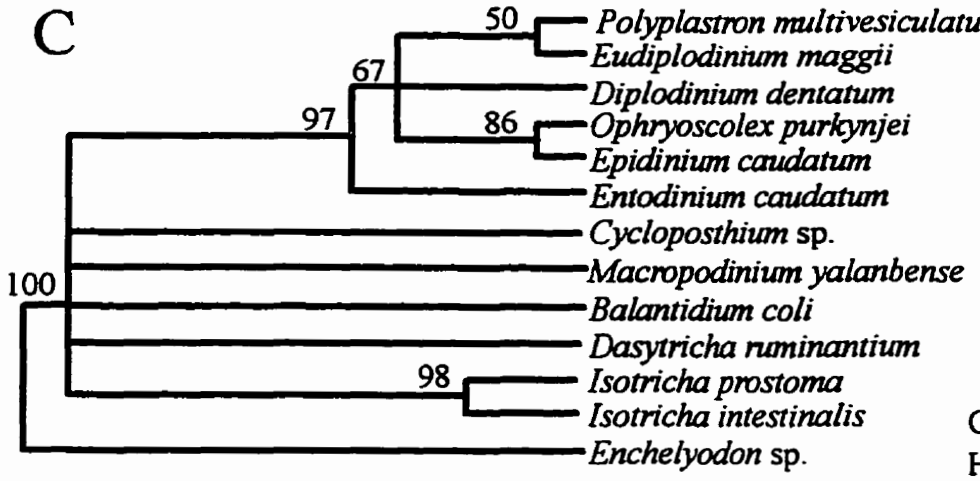


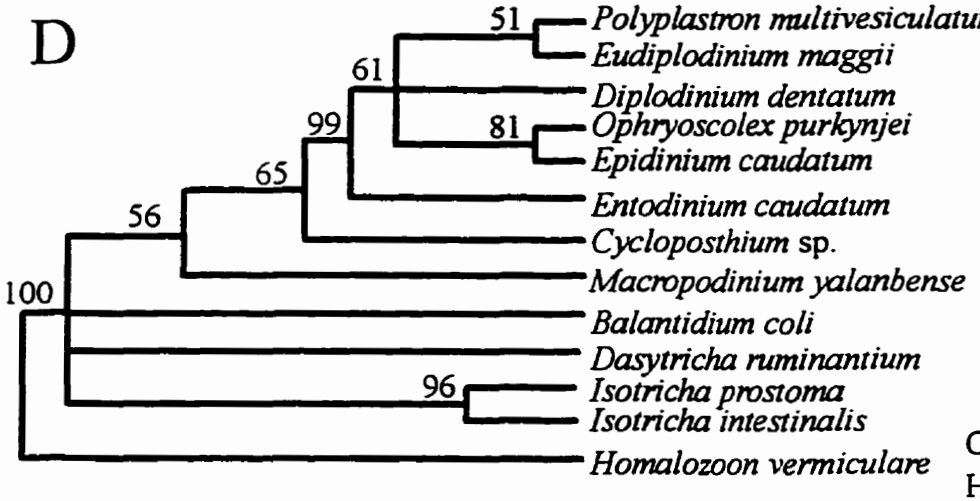
Fig. 2.32. Maximum parsimony trees of the trichostome ciliates inferred from complete SSrRNA gene sequences with a bootstrap resampling of the reduced data set. The numbers at the forks represent the percentage of times the group occurred out of 1,000 trees. Branches with less than 50% bootstrap support were collapsed. No significance is placed on the lengths of the branches connecting the species. In these five trees (continued on next page), either: **(A)** *Spathidium* or all the haptorians was used as the outgroup to the trichostomes; **(B)** *Didinium* was used singly as the outgroup to the trichostomes; **(C)** *Enchelyodon* was used singly to root the ingroup; **(D)** *Homalozoon* was used singly as the outgroup; or **(E)** either *Loxophyllum* or *Dileptus* was used to root the trichostomes.

C



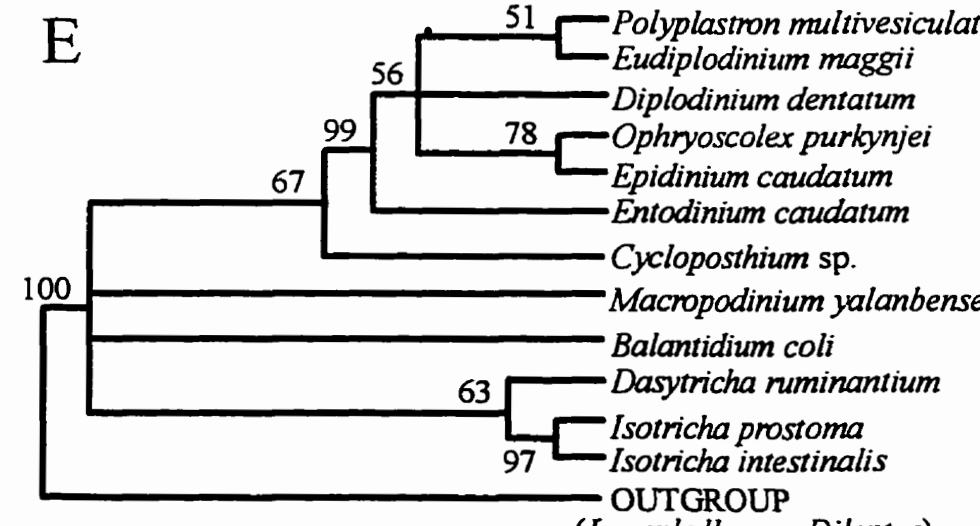
CI = 0.6569
HI = 0.3431

D



CI = 0.6591
HI = 0.3409

E



CI = 0.6711
HI = 0.3289

the two groups have the same characters.

In contrast, when each of the same six intramacronucleate lineages was used as the outgroup for the trichostomes, the same tree was produced (Figure 2.33) and the consistency and homoplasy indices, as well as the bootstrap values were very similar (see Table 2.12). Moreover, when different combinations of these six lineages were used as the outgroup (e.g. armophorids plus nassophoreans), or when the haptorians were the only taxa removed from the initial data set, the same tree topology was obtained (see Figure 2.33). The newly inferred tree for the trichostomes (Figure 2.33) depicted the vertebrate endosymbionts as a monophyletic group with *Macropodinium* as the earliest branching ciliate before a dichotomy including the entodiniomorphids on one branch and the vestibuliferids on the other branch. This would suggest that the entodiniomorphids are paraphyletic because *Macropodinium* does not group with either *Cycloposthium* or the ophryoscolecids. *Macropodinium* is morphologically dissimilar to other ciliates belonging to the Entodiniomorphida and it is possible that it was improperly placed within the Entodiniomorphida only because it shares a similar gastrointestinal habitat with other members of this group. As far as we know, *Macropodinium* only occurs in macropod marsupials (Dehority, 1996), which are more primitive than placental mammals. Thus, it is possible that *Macropodinium* could have diverged before the radiation of the endosymbionts of placental mammals. Although a probable scenario, sequence evidence is needed from more species of *Macropodinium* before concluding that this genus should be removed from the Entodiniomorphida.

The phylogenetic trees from the original data set and the newly inferred tree (c.f. Figures 2.24, 2.25, 2.33) grouped *Cycloposthium* with the ophryoscolecids to form a clade,

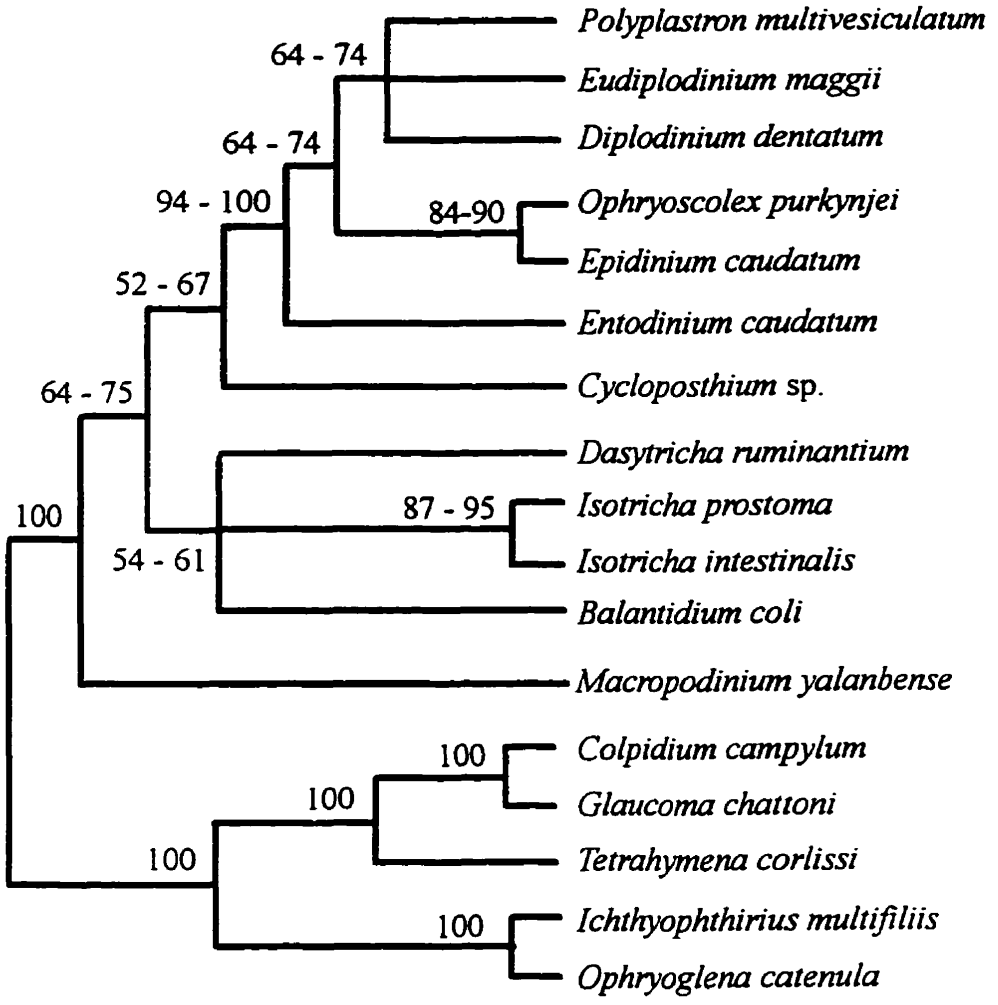


Fig. 2.33. A maximum parsimony tree of the trichostome ciliates inferred from complete SSrRNA gene sequences with a bootstrap resampling of the reduced data set. When representatives from either the armophorids, colpodeans, oligohymenophoreans, nassophoreans, prostomateans or spirotricheans were used as the outgroup, the same tree was produced. In the tree shown here, the oligohymenophoreans were used as the outgroup. The numbers at the forks represent the range (i.e. depending on the outgroup) of the percentage of times the group occurred out of 1,000 trees. Branches with less than 50% bootstrap support were collapsed. No significance is placed on the lengths of the branches connecting the species

Table 2.12. Consistency and homoplasy indices for different outgroup comparisons for the trichostome ciliates

Outgroup		Consistency Index	Homoplasy Index
Taxon	Rank		
Metopus contortus *M. palaeformis*	Order Armophorida	CI = 0.719	HI = 0.281
Colpoda	Class Colpodea	CI = 0.712	HI = 0.289
Furgasonia *Pseudomicrothorax*	Class Nassophorea	CI = 0.726	HI = 0.274
Colpidium *Glaucoma* *Ichthyophthirius* *Ophryoglena* *Paramecium* *Tetrahymena*	Class Oligohymenophorea	CI = 0.703	HI = 0.297
Coleps *Prorodon*	Class Prostomatea	CI = 0.726	HI = 0.274
Protocruzia *Oxytricha*	Class Spirotrichea	CI = 0.718	HI = 0.282

consistent with its placement within the order Entodiniomorphida. *Cycloposthium* inhabits herbivorous animals (i.e. hippopotamus, rhinoceros) that have diverged before the appearance of the ruminants and similarly, it is the earliest branching entodiniomorphid before a clade consisting of rumen ciliates (Figure 2.33). The entodiniomorphids are the sister group to the vestibuliferids (Wright and Lynn, 1997b) and although the vestibuliferids are monophyletic, the relationships within the group are still unresolved with *Isotricha* spp., *Balantidium*, and *Dasytricha* forming a trichotomy. This may in part be due to low species sampling of the vestibuliferid clade relative to these other two litostome clades (Smith, 1994). Moreover, the phylogenetic positions of *Diplodinium* and *Eudiplodinium* remain uncertain (c.f. Figures 2.24, 2.25, 2.30, 2.32, 2.33) (Wright and Lynn, 1997a, 1997b; Wright *et al.*, 1997).

The lack of phylogenetically informative sites for the haptorians very likely prevents the elucidation of their branching pattern. The unresolved polychotomy of the haptorians also appears to affect the topology of the ingroup (i.e. the trichostomes) (see Nixon and Carpenter, 1993). This suggests that the unresolved haptorians should not be used, as part of, or as the outgroup to the trichostomes. Further, the six congruent phylogenies inferred without the haptorians present in the dataset probably depict the true relationships within the trichostomes.

In light of these results, phylogeneticists should also consider the relationships within perspective outgroup taxa before designating them as the outgroup for phylogenetic studies.

CHAPTER THREE

Analysis Of Intraspecific Sequence Variation

Among Eight Isolates Of *Isotricha prostoma* From Two Continents

"We have two ears and one mouth, in order that we may listen more and talk less."

Zeno; Epictetus, year unknown.

INTRODUCTION

The identification of closely related organisms has always proven difficult when they display few morphological differences. The terms "sibling species" or "species complex" have been used to describe multiple members of a genus that cannot be differentiated by obvious morphological characters by light or electron microscopy (Borden *et al.*, 1977). There are two major reasons why it is important to identify morphologically similar species or strains. First from a practical perspective, it is important to identify pathogenic from non-pathogenic strains so that a correct diagnosis can be made leading to proper treatment. Second, from an evolutionary perspective, analysis of the detailed genetic structure of strains and sibling species may increase the understanding of the evolutionary mechanisms giving rise to this genetic differentiation.

The internally transcribed spacer regions 1 and 2 (ITS-1 and ITS-2) (see Figure 1.2) that separate nuclear ribosomal genes are considered to be quite variable and have been useful for identifying and discriminating interspecific and intraspecific genetic variation (White *et al.*, 1990; Allard and Honeycut, 1991; Hillis and Dixon, 1991; Schlötterer *et al.*, 1994). In fact, there are over 1,650 entries in GenBank (*pers. obs.*) and most of these were deposited within the last five years (*pers. obs.*). ITS regions have been used to identify virulent strains of bacteria (Gürtler, 1993; McLaughlin *et al.*, 1993), toxin-producing strains of the marine dinoflagellate *Alexandrium tamarense* (Adachi *et al.*, 1994; Scholin and Anderson, 1994), and pathogenic strains of *Eimeria*, a coccidian parasite of chickens (Barta *et al.*, 1998). Proper identification of these strains using ITS

sequence characters lead to rapid and correct diagnosis and appropriate treatment preventing deaths of humans and their domestic animals.

ITS regions have also been used to probe evolutionary relationships. Pleyte *et al.* (1992) used ITS sequences to infer phylogenetic relationships within the salmonid fish genus *Salvelinus*, and Schlötterer *et al.* (1994) used ITS sequences to identify and distinguish fruit flies belonging to the *Drosophila melanogaster* complex. Thus, the ITS spacer region is potentially useful for discovering patterns of evolution of rumen ciliates. Some genera like *Entodinium* have species complexes where species differentiation is limited because of the large number of similar forms and the lack of morphological characters. For example, the *Entodinium dubardi* species complex is made up of about 12 presumed species (*E. bimastus*, *E. bovis*, *E. caudatum*, *E. convexum*, *E. dubardi*, *E. exiguum*, *E. longinucleatum*, *E. nanellum*, *E. ovinum*, *E. ovoideum*, *E. parvum* and *E. simplex*), whose identification is difficult and sometimes arbitrary (Dehority, 1994). Although Dehority (1994) suggested using SSrRNA gene sequences from various *E. dubardi* types to differentiate these species, the more variable ITS domain may be more suitable.

Other species like *Isotricha prostoma* are prevalent in all domestic and wild ruminants, pseudoruminants, and non-ruminants world-wide, but are morphologically the same. *Isotricha*-like organisms have even been reported in at least five species of marsupials (e.g. quokkas, kangaroos, wallabies) (Obendorf, 1984; Dellow *et al.*, 1988) and in the hoatzin (Domínguez-Bello *et al.*, 1993), a unique foregut-fermenting bird of South America. Even though the distribution of *I. prostoma* is global, little information is

known about the extent of genetic divergence among isolates: (1) do populations of *I. prostoma* in the same host species on different continents differ? or (2) do populations of *I. prostoma* in different hosts on the same continent differ?

Of the rumen ciliates, *Isotricha prostoma* was the first choice to study the intraspecific variation because it is found in a variety of hosts, is universally distributed, is easy to recognize, is large enough to collect easily, and is typically numerous. In this study, eight isolates of *I. prostoma* from two continents, representing three countries, Australia, Canada, and the United States, were sequenced and examined to discover the extent of variation in their ITS-1 and ITS-2 regions, as well as their 5.8S LSrRNA. A single isolate of another vestibuliferid, *Balantidium coli*, was used as a reference species.

MATERIALS AND METHODS

Source Of Isolates.

Eight isolates of *I. prostoma* were collected from either fresh abattoir samples or from rumen fistulated cattle and sheep: two isolates were freshly obtained at the University of Guelph's abattoir (Department of Animal and Poultry Science) from two cows that came from a herd in Elora, Ontario; two isolates were freshly obtained at a large commercial abattoir in Guelph (Better Beef Limited, 781 York Rd., Guelph, ON., N1E 6N1) from cows from a herd in Ashton, Ontario (Note: Because of strict food and health regulations, these animals are not permitted to come in contact with other herds at the abattoir); three isolates were obtained from rumen-fistulated cattle at the Commonwealth States Industrial Research Organization (CSIRO) - Yalanbee Research Station near Perth,

Western Australia; and one isolate was obtained from a fistulated sheep at the Ohio Agricultural Research and Development Centre in Wooster, Ohio, USA, and was a gift of Dr. Burk Dehority. Genomic DNA from *Balantidium coli*, an endosymbiotic ciliate of vertebrate animals taken from the colon of a lowland gorilla (*Gorilla gorilla gorilla*), was a gift of Dr. C. Graham Clark (Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, London, England).

Isolation Of Fresh Isolates.

Rumen fluid samples were filtered through two layers of cheese cloth to remove plant and feed material, and put into either a separatory funnel or a beaker for 1 h at 39° C to sediment protozoa. In some cases, the more motile vestibuliferid ciliates (*Dasytricha ruminantium, I. prostoma, I. intestinalis*) aggregated together and formed on the surface of the rumen fluid white patches of cells that were easily removed using a hand-drawn Pasteur pipette. In most cases, the ciliates were passed through 100 μm and 70 μm Nitex meshes to remove the smaller entodiniomorphid species, such as *Entodinium*, *Diplodinium*, and *Eudiplodinium*. Most of the larger remaining cells were sorted under a dissecting microscope. Clean isolates of *I. prostoma* were obtained from Elora, Ontario, Wooster, Ohio, and from one of the two Ashton, Ontario samples.

However, some animals harboured both species of *Isotricha*, which made isolation of *I. prostoma* difficult as the only morphologically distinguishing trait is the position of the vestibulum. This trait is nearly impossible to detect if the cells are starved (i.e. internal structures are transparent) or if they have been fixed (i.e. the cytoplasm shrinks away from

the pellicle). In addition to containing *I. prostoma*, the second isolate from Ashton, Ontario and the three isolates from Australia contained *Isotricha intestinalis*, *Dasytricha ruminantium*, and at least three large entodiniomorphids, *Epidinium*, *Ophryoscolex*, and *Polyplastron*.

DNA Extraction, Amplification, and Sequencing.

A DNA extraction procedure using CTAB (cetyltrimethylammonium bromide) (Murray and Thompson, 1980; Reichardt and Rogers, 1994; Wilson, 1994) was used following the protocol of Wright *et al.* (1997) (see chapter 2). Because of the possible contamination by other ciliates, two species-specific forward primers were designed and designated IPRO600 (5'-GTTGGATTTCAAGGATTACTC-3') and IPRO1250 (5'-TCGTCCTCATATTATGGGGTA-3'). These new primers were based upon signature sequences within the SSrRNA gene (see chapter 2) and designed to amplify only *I. prostoma*. At least four regions of the SSrRNA gene were detected where oligonucleotide probes could be constructed for most of the rumen ciliates in my database. These four areas were designated as signature sequence regions 1, 2, 3, and 4 (see Appendix XVII).

Each of these species-specific forward primers was used in combination with Jerome primer C (Jerome and Lynn, 1996), which is located in the D2 region approximately 500 bp downstream from the 5' end of the 28S gene. Therefore, IPRO600 and IPRO1250 should amplify a fragment of about 1.9 kb and 1.3 kb respectively.

These new primers were rigorously tested under various PCR conditions (see

Appendix IX) for their specificity using genomic DNA from at least nine species of rumen ciliates and four species of free-living ciliates. These 13 species included the two other isotrichids *I. intestinalis* and *D. ruminantium*, and those species contaminating the *I. prostoma* samples. PCR reactions were set-up to amplify the templates of each ciliate, of different combinations of ciliates, with and without the presence of *I. prostoma*. No PCR product was obtained from the isotrichids, *I. intestinalis* and *D. ruminantium*, or from any of the other free-living or rumen ciliates. Only those samples containing *I. prostoma* produced a single PCR product of the expected size (Figure 3.1). Overlapping sequence of the 18S gene (IPRO600 produces a 950 bp overlap and IPRO1250 produces a 400 bp overlap) confirmed that the sequence was that of *I. prostoma*.

Templates containing DNA from a known number of *I. prostoma* cells were set-up and it was determined that a minimum of 50 cells was required for consistently reliable results. *Balantidium coli* was amplified using the 1055F forward primer (Elwood *et al.*, 1985) (see Table 2.3) with Jerome primer C (Jerome and Lynn, 1996).

A PTC-100™ thermal cycler (MJ Research, Inc., Watertown, MA) was used with the following stringent parameters: 30 sec denaturation at 94° C, 30 sec primer annealing at 60° C, and 90 sec primer extension at 72° C. On the 35th and last cycle, the primer extension was extended for 2.5 min. The resulting PCR product was run on a 1% agarose gel and the single band was visualized and immediately excised under long-wave ultraviolet light with minimal exposure to the DNA (< 60 sec) to avoid damage. The excised PCR fragment was purified using the GeneClean® kit (BIO/CAN, Mississauga,

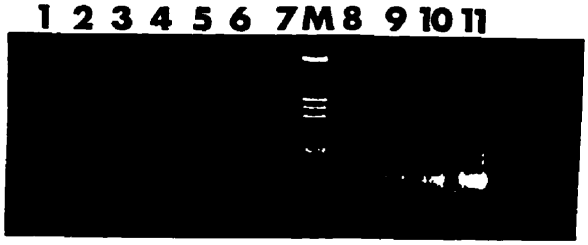


Fig. 3.1. Photograph of an 1% agarose gel showing the specificity of the specially designed PCR primers for *Isotricha prostoma* (see text for details). Lanes 1-7 contain negative controls and Lanes 8-11 contain positively amplified DNA from *Isotricha prostoma* isolates. Lane 1 contains a water control, Lane 2 contains *Paramecium*, Lane 3 contains *Entodinium*, Lane 4 contains *Epidinium*, Lane 5 contains *Ophryoscolex*, Lane 6 contains *Polyplastron*, Lane 7 contains *Isotricha intestinalis*, Lane M contains a *Hind*III/*Eco*Ri λDNA marker, Lanes 8 and 9 contain PCR amplified DNA from *I. prostoma* from AUSTRALIA, Lane 10 contains PCR amplified DNA from *I. prostoma* from Ashton, CANADA, and Lanes 11 contains PCR amplified DNA from *I. prostoma* from the UNITED STATES.

ON). The purified fragment was sequenced directly using an ABI Prism 377 Automated DNA Sequencer (Applied Biosystems Inc, Foster City, CA) using dye terminator and Taq FS with the 1400F primer (Elwood *et al.*, 1985) (see Table 2.3).

The ITS-1/5.8S/ITS-2 sequences from the eight *I. prostoma* isolates were aligned against each other, and against the ITS-1/5.8S/ITS-2 sequence from *B. coli* using ALIGN (Scientific and Educational Software).

RESULTS

The sequence length of the complete ITS-1/5.8S/ITS-2 region from each of the eight isolates of *I. prostoma* was 383 bp. Individually, the ITS-1, 5.8S, and ITS-2 sequences from all eight isolates of *I. prostoma* were as follows: 92 bp, 156 bp, and 135 bp (Figure 3.2). The sequence length of the complete ITS-1/5.8S/ITS-2 region from *B. coli* was 401 bp. Individually, the ITS-1, 5.8S, and ITS-2 sequences from *B. coli* were as follows: 106 bp, 154 bp, and 141 bp (Figure 3.3). These new ITS-1/5.8S/ITS-2 gene sequences have been deposited in the GenBank/EMBL databases under the accession numbers: AF045030 (*B. coli*) and AF045031 (*I. prostoma*).

No intraspecific variation was observed amongst the eight isolates of *I. prostoma* from Australia, Canada, or the United States (Figure 3.4). Moreover, there was no variation between the sheep isolate and those from cattle. Respectively, the G-C content for *I. prostoma* and *B. coli* was 21.7% and 23.8% for ITS-1 and 31.9% and 31.2% for ITS-2. Both ciliates had the same G-C content for the 5.8S LSrRNA gene at 41.6%. Overall, the G-C content of the entire ITS-1/5.8S/ITS-2 region was 33.4% for *I. prostoma*

ITS-1 Region

45 a 9 c 11 g 27 t

```
  1 tttactaaaa ctaaactaaa aactaactga ttattagaga gtaatctcta
 51 tttgaagtaa aagaaaacaa tataactgaa atggttgaat aa
```

5.8 LSrRNA Gene

47 a 31 c 34 g 44 t

```
  1 aatctaaatt ttcaacgatg gatgtcttgg ctcccatatc gatgaagaac
 51 gcagcaagaa tgcgatatgc agtgtgaatt gcagaaccac gaatcatcgg
101 attttctaac gttactgaca ctggtgaaga gccagtatac ttgtttcagt
151 gtcact
```

ITS-2 Region

51 a 20 c 23 g 41 t

```
  1 aaccaaacac ttaaacaaaa tgtatgagaa gttctcatat gaaattaatg
 51 ctctttgtag caatcacaga aatgtgaatc tataaggagg atttttattt
101 tgacctgaaa ttagtaagat gacccgctgg actta
```

Fig. 3.2. The complete ITS-1, 5.8S, ITS-2 sequences for *Isotricha prostoma.* Although the sequences are presented as three separate entities, ITS-1 is contiguous with the 5.8S LSrRNA gene, which in turn, is contiguous with the ITS-2 region. This 383 bp region separates the 18S and 28S rRNA genes.

ITS-1 Region

46 a 14 c 11 g 35 t

```
  1 ttaacaaaat ttgaactaat aactaactga acttgatagt gaaatttatt
 51 tcactatatt tgaactaata caagaaaacc ataaactact taattgggtg
101 aatatc
```

5.8S LSrRNA Gene

48 a 30 c 34 g 42 t

```
  1 aatctaaatt ttcaacgatg gatgtcttgg ctcccatatc gatgaagaac
 51 gcagcaagaa tgcgatatgc aatgtgaatt gcagaaccat gaatcatcgg
101 atattctaac gcaactggga ctggctaaac cagtatactt gtttcagtgt
151 gcgt
```

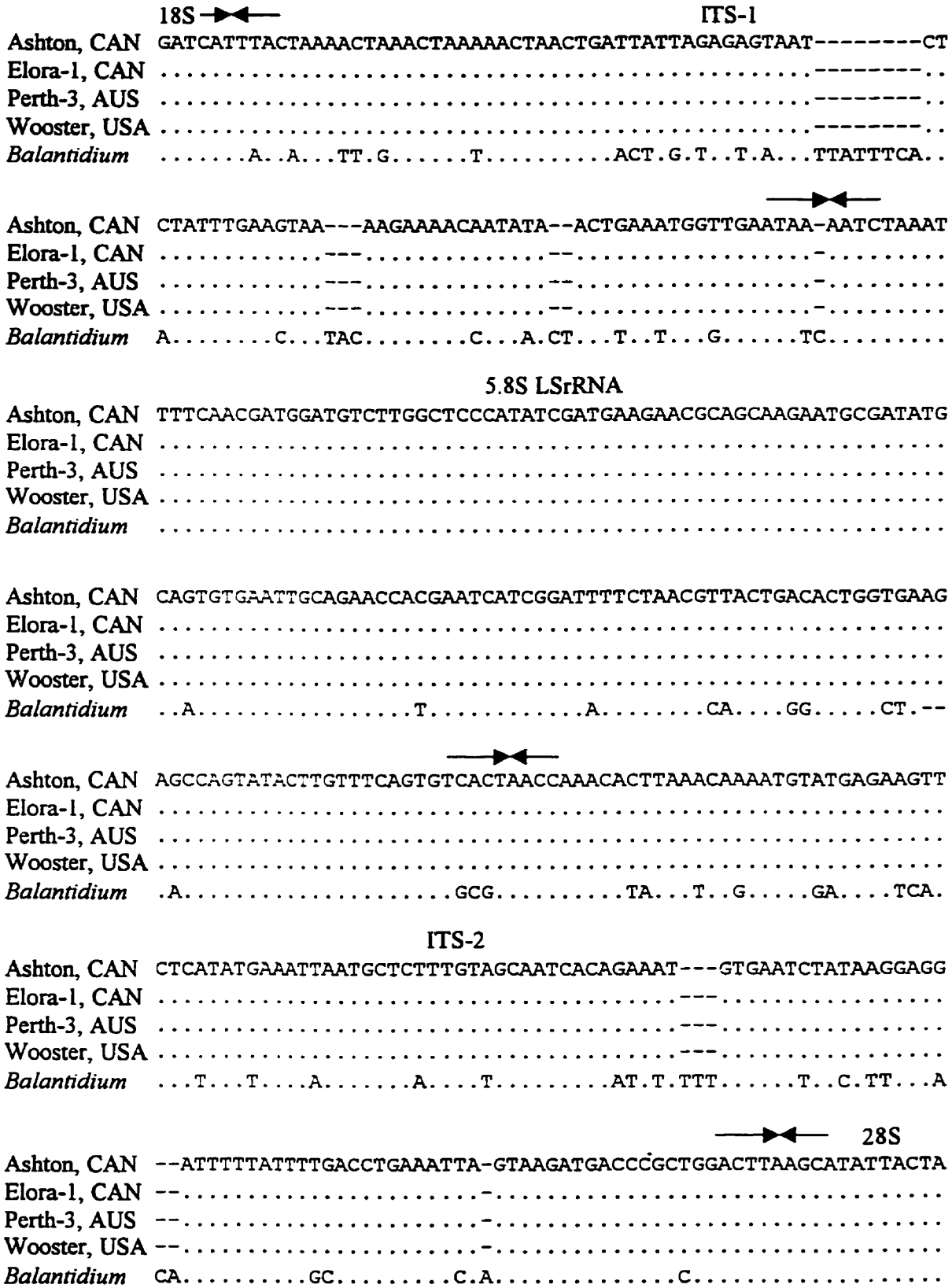
ITS-2 Region

51 a 24 c 20 g 46 t

```
  1 aaccaaacat ataatcagaa tgtgagagat catctcttat taaataaatg
 51 ctcattgttg caatcacaat atttttgtga atttacattg agacaatttt
101 tatttgcacc tgaaatcaag taagatgacc cgccggactt a
```

Fig. 3.3. The complete ITS-1, 5.8S, ITS-2 sequences for *Balantidium coli.* Although the sequences are presented as three separate entities, ITS-1 is contiguous with the 5.8S LSrRNA gene, which in turn, is contiguous with the ITS-2 region. This 401 bp region separates the 18S and 28S rRNA genes.

Fig. 3.4. Comparison of the ITS-1, 5.8S, and ITS-2 sequences from Canadian, American and Australian isolates of *Isotricha prostoma* with the isolate of *Balantidium coli*, which was obtained from an ape in London, England. The Wooster, USA isolate of *I. prostoma* was obtained from a sheep, while all other isolates of *I. prostoma* were obtained from cattle. As there were no sequence differences within the eight isolates of *I. prostoma* (see text for details), only one isolate from each site is shown.


18S
ITS-1
Ashton, CAN GATCATTTACTAAAACTAAACTAAAAACTAACTGATTATTAGAGAGTAAT--------CT
Elora-1, CAN .....--------..
Perth-3, AUS .....--------..
Wooster, USA .....--------..
Balantidium .....A..A...TT.G.....T.....ACT.G.T..T.A...TTATTTCA..
Ashton, CAN CTATTTGAAGTAA---AAGAAAACAATATA--ACTGAAATGGTTGAATAA-AATCTAAAT
Elora-1, CAN .....---.....--.....-.....
Perth-3, AUS .....---.....--.....-.....
Wooster, USA .....---.....--.....-.....
Balantidium A.....C...TAC.....C...A.CT...T..T...G.....TC.....
5.8S LSrRNA
Ashton, CAN TTTCAACGATGGATGTCTTGGCTCCCATATCGATGAAGAACGCAGCAAGAATGCGATATG
Elora-1, CAN .....
Perth-3, AUS .....
Wooster, USA .....
Balantidium .....
Ashton, CAN CAGTGTGAATTGCAGAACCACGAATCATCGGATTTTCTAACGTTACTGACACTGGTGAAG
Elora-1, CAN .....
Perth-3, AUS .....
Wooster, USA .....
Balantidium ..A.....T.....A.....CA....GG.....CT.--
Ashton, CAN AGCCAGTATACTTGTTTCAGTGTCACTAACCAAACACTTAAACAAAATGTATGAGAAGTT
Elora-1, CAN .....
Perth-3, AUS .....
Wooster, USA .....
Balantidium .A.....GCG.....TA...T..G.....GA....TCA.
ITS-2
Ashton, CAN CTCATATGAAATTAATGCTCTTTGTAGCAATCACAGAAAT---GTGAATCTATAAGGAGG
Elora-1, CAN .....---.....
Perth-3, AUS .....---.....
Wooster, USA .....---.....
Balantidium ...T...T....A.....A....T.....AT.T.TTT.....T..C.TT...A
28S
Ashton, CAN --ATTTTTATTTTGACCTGAAATTA-GTAAGATGACCCGCTGGACTTAAGCATATTACTA
Elora-1, CAN --.....-.....
Perth-3, AUS --.....-.....
Wooster, USA --.....-.....
Balantidium CA.....GC.....C.A.....C.....

and 33.2% for *B. coli*.

The sequence divergence between *I. prostoma* and *B. coli* was 26.6% for ITS-1, 9.6% for the 5.8S gene, and 19.3% for ITS-2. Overall, the sequence divergence between *I. prostoma* and *B. coli* for the complete ITS-1/5.8S/ITS-2 region was 16.2%.

DISCUSSION

The ITS regions have been useful for distinguishing and differentiating morphologically identical species and strains from a wide diversity of life, such as bacteria (Barry *et al.*, 1991; McLaughlin *et al.*, 1993; Matar *et al.*, 1993, Gürtler, 1993), apicomplexans (Cevallos *et al.*, 1993; Goggin, 1994; Barta *et al.*, 1998), dinoflagellates (Adachi *et al.*, 1994), trematodes (Anderson and Barker, 1993), fungi (Lee and Taylor, 1992), plants (Baldwin, 1992), dipterans (Wesson *et al.* 1992; Fritz *et al.*, 1994; Schlötterer *et al.*, 1994), and salmonid fish (Pleyte *et al.*, 1992).

Jerome and Lynn (1996) used a RFLP analysis of the 18S/ITS-1/5.8S/ITS-2 region as an alternative means to mating experiments and isoenzyme analysis for identifying and distinguishing sibling species within the *Tetrahymena pyriformis* complex. Different sibling species had different RFLP patterns. Although they did not rigorously assess intraspecific variation using the ITS regions, there was no variation in the RFLP pattern either between two strains of *T. thermophila* or among 18 isolates of *T. empidokyrea*. Moreover, six *Tetrahymena* isolates from the wild had identical RFLP patterns to one of the recognized patterns of a species in the *T. pyriformis* complex (Jerome and Lynn, 1996). Thus, intraspecific variation within the tetrahymenine ITS-1/5.8S/ITS-2 region

appeared to be quite low. Similarly, a lack of genetic diversity was detected using RFLP analysis of the ITS-1 region from several geographical isolates of the apicomplexan parasite, *Sarcocystis gigantea* (Jeffries *et al.*, 1996).

Diggles and Adlard (1997) examined the intraspecific variation within the ITS-1 from 16 isolates of the obligate marine fish ectoparasite, *Cryptocaryon irritans*, and discovered as much as 5.9% sequence divergence amongst the 13 isolates (seven wild and six laboratory raised) from Australia. Even if the laboratory-raised isolates were excluded from the analysis, there was still a 4.1% sequence divergence amongst the Australian isolates. In contrast, there was only one nucleotide difference in the 170 bp region amongst the single isolate from the United States and the two isolates from Israel. Diggles and Adlard (1997) also examined the ITS-1 region from another ciliate, *Ichthyophthirius multifiliis*, and determined that the sequence divergence between the isolates of *C. irritans* (class Prostomatea) and *I. multifiliis* (class Oligohymenophorea) ranged between 40.7 – 42.4%. In comparison, genetic distance between the two vestibuliferids (order Vestibuliferida), *I. prostoma* and *B. coli*, was only 26.6%.

In the present study, the eight isolates of *I. prostoma* from three countries, representing two continents, have remarkably conserved ITS-1, 5.8S, and ITS-2 regions. Moreover, there were no sequence differences between *I. prostoma* isolates from cattle and sheep hosts. This suggests that populations of *I. prostoma* on two continents are very recently diverged, consistent with human migration with their domestic animals to these continents in the 18th and 19th centuries. Although the 100% sequence conservation found in the eight isolates of *I. prostoma* contrasts with examples where these ITS regions are

highly variable, presumably because they are not under functional constraints (White *et al.*, 1990; Allard and Honeycut, 1991; Hillis and Dixon, 1991; Schlötterer *et al.*, 1994), other researchers have not found significant variation. For example, Hoste *et al.* (1993) found no differences in the ITS-2 region of the nematode, *Trichostrongylus colubriformis.* De Wit and Klatser (1994) detected no differences in the ITS spacer region of 75 isolates of *Mycobacterium leprae*, the causative agent of leprosy, from four continents. More recently, Homan *et al.* (1997) examined 20 isolates of the intracellular protozoan parasite, *Toxoplasma gondii,* and detected no differences in the ITS-1 and 5.8S sequences.

In the present study, the ITS-1 regions for *I. prostoma* (92 bp) and *B. coli* (106 bp) were much shorter than that for *C. irritans* (170 bp) and *I. multifiliis* (182 bp) (Diggles and Adlard, 1997). To account for the considerable variability in ITS-1 lengths between our two studies, a careful inspection of the 3′ end of their ITS-1 sequences with the 5′ end of the 5.8S sequences from my study revealed that Diggles and Adlard's (1997) published ITS-1 sequences contained 40 bp belonging to the 5′ end of the 5.8S gene. To support this conclusion, the last 80 bp of the ITS-1 sequences from *C. irritans* and *I. multifiliis* were put through GenBank's Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) to determine whether the sequences were isologous with the ITS-1 or 5.8S gene from other eukaryotes in the database. The results indicated that the 40 suspect nucleotides were >78% similar to the 5′ end of the 5.8S gene from at least 100 diverse eukaryotes ranging from *Caenorhabditis elegans* (a nematode) to *Chlamydomonas callosa* (a chlorophyte). The best match of similarity was 90% with the ciliate, *Tetrahymena thermophila.* Thus, it appears that the correct ITS-1 lengths for *C.*

irritans and *I. multifiliis* should be 130 and 142 bp respectively, very similar to the ITS-1 length of *T. thermophila* of 131 bp (Engberg and Nielsen, 1990) (see Table 3.1), but still longer than that of *I. prostoma* (92 bp) and *B. coli* (106 bp).

It has been suggested that the length and G-C content of the ITS-1 region tends to increase in "higher" eukaryotes (Takaiwa *et al.*, 1985; Torres *et al.*, 1990). For example, the ITS-1 in plants is 72% G-C and over 220 bp long, whereas the ITS-1 in humans and other mammals is 80% G-C and longer than 1,000 bp (see Table 3.1). Diggles and Adlard (1997) used this rationale to suggest that because the ITS-1 length (142 bp) and 28.5% G-C content of *I. multifiliis* is greater than that of *C. irritans* (130 bp; 20.6 – 22.4%), it must be more derived than *C. irritans*.

If longer ITS-1 and higher G-C content indicate more recently derived organisms (Takaiwa *et al.*, 1985; Torres *et al.*, 1990), then ciliates must be old as they have shorter sequences like *Tritrichomonas* and *Entamoeba* (Table 3.1). Although this supports the conclusion based on the SSrRNA molecular clock (see chapter 4) that the ciliates are a very old assemblage of crown-eukaryotes, this is likely to be too simplistic a perspective, like other trends related to genome characteristics (e.g. genome size). For example, it is widely accepted that ciliates are derived from dinoflagellate-like ancestors (Taylor, 1976). Therefore, I expect to see shorter ITS-1 sequences in dinoflagellates, like *Alexandrium tamarense* and *Prorocentrum micans*, and longer ITS-1 sequences in the ciliates, but the opposite occurs (see Table 3.1). The apicomplexans are also believed to be derived from dinoflagellate-like ancestors and while their ITS-1 sequences are longer than their dinoflagellate relatives (see Table 3.1), they are also longer than several other metazoans

Table 3.1. Comparisons of the different sequence lengths of ITS-1, 5.8S, and ITS-2.

Species	Taxonomic Group or Common Name	Accession #	Nucleotide Lengths				References
			ITS-1	5.8S	ITS-2	Total	
Homo sapiens	human	U13369	1094	156	1154	2402	Nazar *et al.*, 1976
Mus musculus	mouse	J00623	1000	156	1090	2246	Michot *et al.*, 1982
Xenopus laevis	frog	X02995	556	161	261	978	Boseley *et al.*, 1979
Neospora caninum	**apicomplexan**	L49389	419	157	323	899	Payne and Ellis, 1996
Lytechinus variegatus	sea urchin	X00350	368	157	437	962	Hindenach and Stafford, 1984
Cylicocyclus ashworthi	nematode	Y08586	366	152	314	832	Hung, *unpubl data*
Crithidia fasciculata	kinetoplastid	Y00055	365	170	415	950	Spencer *et al.*, 1987
Trypanosoma evansi	kinetoplastid	D89527	340	169	586	1095	Urakawa, *et al.*, *unpubl data*
Eimeria maxima	**apicomplexan**	AF027722	321	155	282	758	Barta *et al.*, 1998
Herdmania momus	urochodate	X53538	285	154	275	714	Degnan *et al.*, 1990
Trebouxia jamesii	green algae	Z68701	275	152	230	657	Bhattacharya *et al.*, 1996
Corycium dracomontanum	seed plants	AJ000125	246	161	245	654	Douzery *et al.*, *unpubl data*
Solanum lycopersicum	tomato	X52265	226	152	234	612	Kiss *et al.*, 1988
Trichoderma longibrachiatum	fungi	Z79627	222	157	168	547	Gueho *et al.*, *unpubl data*
Prorocentrum micans	**dinoflagellate**	M14649	210	153	194	557	Maroteaux *et al.*, 1985
Alexandrium tamarense	**dinoflagellate**	AB006992	170	159	183	512	Adachi *et al.*,1996
Ascomycetes sp.	fungi	Z54274	168	156	207	531	Rollo *et al.*, 1995
Tetrahymena thermophila	**ciliate**	X54512	**131**	153	178	462	Engberg and Nielsen, 1990
Entamoeba histolytica	amoeba	Y12249	122	150	124	396	Bhattacharya, *unpubl data*
Ochromonas danica	stramenopile	Y07976	118	156	47	321	van der Auwera and de Wachter, 1997
Balantidium coli	**ciliate**	AF045030	**106**	154	141	401	this study
Isotricha prostoma	**ciliate**	AF045031	**92**	156	135	383	this study
Tritrichomonas foetus	trichomonad	M81842	79	158	62	299	Chakrabarti *et al.*, 1992
Nannochloropsis salina	stramenopile	Y07974	69	162	40	271	van der Auwera and de Wachter, 1997
Naegleria sp.	amoeba	Y10197	33	173	99	305	de Jonckheere and Brown, 1997

(e.g. the sea urchin *Lytechinus variegatus*, the nematode, *Cylicocyclus ashworthi*, and the urochordate, *Herdmania momus*). Further, because the litostome ITS-1 sequences are shorter than that of other ciliates, I would expect to see the litostomes branching early near the base of the ciliate tree, but they do not.

Although no sequence divergence was observed among the eight isolates of *I. prostoma*, the ITS-1/5.8S/ITS-2 region may still be useful in other ciliate groups where species identification is uncertain. *Entodinium* is the smallest rumen ciliate and the most troublesome to classify into species because of the large number of similar forms. There are at least 120 presumed species of *Entodinium* and undoubtedly some, or most of these species are the same (Williams and Coleman, 1992) or are sibling species (e.g. the *E. dubardi* species-complex). Future testing of members belonging to these species-complexes, like the *E. dubardi* complex, may demonstrate the ITS-1/5.8S/ITS-2 region to be reliable for identifying and distinguishing species within *Entodinium* and increase our understanding of the evolutionary mechanisms that gave rise to this genetic differentiation.

CHAPTER FOUR

Maximum Ages Of Ciliate Lineages Estimated Using A Small Subunit rRNA Molecular Clock: Crown Eukaryotes Date Back To The Paleoproterozoic.

"An investment in knowledge
pays the best interest"

Benjamin Franklin, year unknown.

INTRODUCTION

Paleontologists have estimated the time at which major groups of multicellular eukaryotes appeared on earth by correlating index fossils with radioisotope data. However, there is much less known about the appearance of unicellular eukaryotes because fossilization does not preserve unicellular organisms well (Corliss, 1979; Sogin *et al.*, 1986c). This is especially true for the ciliated protozoa, a very diverse assemblage of unicellular eukaryotes (i.e. crown eukaryotes) that comprise the phylum Ciliophora Doflein, 1901. With over 8,000 species (Lynn and Corliss, 1991), their evolutionary history has been of interest to protozoologists, evolutionary biologists, and molecular geneticists because of their complex cytoarchitecture, diverse morphology, and dimorphic nuclei.

Because genetic divergence is roughly linearly correlated with divergence time (i.e. is clock-like), the use of genetic divergence data to estimate the origin of groups for which there is little or no fossil record evidence is a common application in molecular systematics (Hillis *et al.*, 1996). However, disagreement exists over using this approach: there are discrepancies between paleontological data and molecular divergence estimates (see Sarich and Wilson, 1967; Holmes, 1991); and rates of molecular divergence are known to be unequal over long periods of time and to vary between groups of organisms (i.e. humans and other higher primates; see Miyamoto and Goodman, 1990). Nonetheless, in the absence of a thorough fossil record, which is especially true for most protist groups (e.g. ciliates), and given that clock rate is more consistent within a group of related organisms, a molecular clock can provide useful insights into a group's evolution (Saunders and

Druehl, 1992, Doolittle *et al.*, 1996; Hillis *et al.*, 1996; Wray *et al.*,, 1996).

Ribosomal RNA sequences make the best molecular clocks because of their high degree of functional constancy, universal distribution, and their low levels of variability within related taxa (Woese, 1987). This permits the most distant relationships, up to 3,500 million years ago (Ma), to be measured (Woese, 1987, Hillis *et al.*,, 1996). Nuclear small subunit rRNA (SSrRNA) gene divergence estimates have already been determined for vertebrates (1% per 60 million years (My)) (see Saunders and Druehl, 1992), plants (1% per 25-My) (Saunders and Druehl, 1992), and prokaryotes (1% per 50-My) (Ochman and Wilson, 1987).

Although protozoa do not fossilize well, estimates of the maximum age of protozoa with symbiotic relationships with vertebrates may be obtained using the fossil record of their hosts. For example, the ciliated protozoan, *Ichthyophthirius*, is an obligate ectoparasite of freshwater teleosts (to my knowledge, there are no published accounts in the literature of *Ichthyophthirius* parasitizing non-teleost freshwater fishes, such as gars and sturgeons). *Ichthyophthirius*' closest relative, based upon morphological (Canella, 1964, Lynn *et al.*,, 1991) and molecular data (Wright and Lynn, 1995), is the free-living, but histophagous ciliate, *Ophryoglena*. Further, from the fossil record, the oldest known freshwater member of a living teleostean clade, such as Osteoglossomorpha or Elopomorpha, is undoubtedly (e.g. *Lycoptera*, a freshwater osteoglossomorph) from the very late Jurassic/early Cretaceous of China, approximately 145-Ma (Schaeffer and Patterson, 1984; Wilson and Williams, 1992; Patterson, 1993). From this information I can determine the rate of nucleotide substitution and calculate the **maximum** age of

divergence of the ciliates. This paper represents the first attempt to calibrate a SSrRNA molecular clock for the ciliated protozoa and to predict the time of divergence of ciliate lineages from pair-wise genetic distances.

MATERIALS AND METHODS

Sequence Acquisition and Analysis

The nucleotide sequences in this paper are available from the GenBank/EMBL databases under the following accession numbers: *Bresslaua* sp., *Bryometopus* sp., and *Bursaria* sp. (Lynn *et al.*, unpubl. data); *Climacostomum virens* X65152 (Hammerschmidt *et al.*,, 1996), *Coleps* sp. (Stechmann *et al.*,, 1998), *Colpidium campylum* X56532 (Greenwood *et al.*,, 1991a), *Colpoda inflata* M97908 (Greenwood *et al.*,, 1991b), *Cyclidium glaucoma* Z22879 (Embley *et al.*,, 1995), *Dasytricha ruminantium* U57769 and *Didinium nasutum* U57771 (Wright and Lynn, 1997b); *Diplodinium dentatum* U57764 (Wright and Lynn, 1997a); *Entodinium caudatum* U57765 and *Epidinium caudatum* U57763 (Wright *et al.*,, 1997), *Eudiplodinium maggii* U57766 (Wright and Lynn, 1997a); *Eufolliculina uhligi* U47620 (Hammerschmidt *et al.*,, 1996), *Furgasonia blochmanni* X65150 (Bernhard *et al.*,, 1995), *Glaucoma chattoni* X56533 (Greenwood *et al.*,, 1991a), *Homalozoon vermiculare* L26447 (Leipe *et al.*, 1994a), *Ichthyophthirius multifiliis* U17354 (Wright and Lynn, 1995), *Isotricha intestinalis* U57770 (Wright and Lynn, 1997b), *Labyrinthuloides minuta* L27634 (Leipe *et al.*, 1994b), *Loxodes striatus* U24248 (Hammerschmidt *et al.*, 1996), *Loxophyllum utriculariae* L26448 (Leipe *et al.*, 1994a), *Metopus contortus* Z29516 (Embley *et al.*, unpubl. data), *Metopus palaeformis*

M86385 (Embley *et al.*, 1992), *Obertrumia* sp. (Bernhard *et al.*, 1995), *Onychodromus quadricornutus* X53485 (Schlegel *et al.*, 1991); *Ophryoglena catenula* U17355 (Wright and Lynn, 1995), *Ophryoscolex purkynjei* U57768 (Wright and Lynn, 1997b), *Oxytricha granulifera*, X53486 (Schlegel *et al.*, 1991), *Paramecium tetraurelia* X03772 (Sogin and Elwood, 1986), *Platyophrya* sp. (Lynn *et al.*, unpubl. data), *Polyplastron multivesiculatum* U57767 (Wright *et al.*, 1997), *Prorocentrum micans* M14649 (Herzog and Maroteaux, 1986), *Prorodon teres* (Stechmann *et al.*, 1998), *Protocruzia* sp. X65153 (Hammerschmidt *et al.*, 1996), *Pseudomicrothorax dubius* X65151 (Bernhard *et al.*, 1995), *Pseudoplatyophrya* sp. (Lynn *et al.*, unpubl. data); *Sarcocystis muris* M64244, M34846 (Gajadhar *et al.*, 1991), *Spathidium* sp. Z22931 (Dyal *et al.*, 1995), *Stylonychia pustulata* M14600, X03947 (Elwood *et al.*, 1985), *Symbiodinium pilosum* X62650, S44661 (Sadler *et al.*, 1992), *Tetrahymena australis*, *T. borealis*, *T. canadensis*, and *T. capricornis* (Sogin *et al.*, 1986c), *T. corlissi* U17356 (Wright and Lynn, 1995), *T. empidokyrea* U36222 (Jerome *et al.*, 1996), *T. hegewischi*, *T. malaccensis*, *T. nanneyi*, *T. patula*, *T. pigmentosa*, and *T. pyriformis* (Sogin *et al.*, 1986c), *T. thermophila* (Spangler and Blackburn, 1985), *T. tropicalis* (Sogin *et al.*, 1986c), *Theileria buffeli* Z15106 (Allsopp *et al.*, 1994), *Tracheloraphis* sp. L31520 (Hirt *et al.*, 1995), and *Uronema marinum* Z22881 (Dyal *et al.*, unpubl. data).

Ciliate SSrRNA sequences were aligned against sequences from ciliates belonging to the same class and those from other classes using the Dedicated Comparative Sequence Editor (DCSE) program (de Rijk and de Wachter, 1993) and further refined by considering secondary structural features of the SSrRNA molecule. PHYLIP's (ver.

3.51C) (Felsenstein, 1993) DNADIST program was used to calculate the evolutionary distances (*d*) between pairs of nucleotide sequences using the Kimura (1980) two-parameter model.

The ciliates *Blepharisma*, *Discophrya*, *Euplotes*, *Opisthonecta*, *Plagiopyla*, *Trimyema*, and *Trithigmostoma* have a relatively fast evolving SSrRNA and were omitted from the analysis because their uncharacteristic divergence rates may over-estimate time of divergence (see Sogin *et al.*, 1986b; Saunders and Druehl, 1992; Philippe *et al.*, 1996) for the ciliates. The remaining genetic distances were used to estimate the divergence times of the major ciliate groups.

RESULTS

Molecular clock calibration

The genetic distance separating *Ichthyophthirius* and *Ophryoglena* is 1.8% (Tables 4.1 and 4.2). Depending upon minor changes in my SSrRNA sequence alignments, I have found that this genetic distance varies between 1.8 to 2.0%. Thus, the **maximum** divergence of the SSrRNA gene of *Ichthyophthirius* is approximately 1.8 to 2.0% over 145-My or 1% per 72 to 80-My (this is equivalent to a rate of nucleotide substitution per site, per year, per lineage of 1.25 to 1.4 x 10^{-8}).

Pair-wise genetic distances

Using the classification scheme of Small and Lynn (1985), the deepest divergence involving the postciliodesmatophorans (i.e. class Karyorelictea and class Heterotrichea)

Table 4.1. SSrRNA evolutionary distance data for ciliate representatives of major lineages

	Loxo..	*Trac..*	*Eufo..*	*Clim..*	*Prot..*	*Styl..*	*Oxyt..*	*Homa..*	*Loxo..*	*Didi..*	*Furg..*
Tracheloraphis	0.0903										
Eufolliculina	0.1611	0.1682									
Climacostomum	0.1714	0.1625	0.1067								
Protocruzia	0.2083	0.2015	0.1857	0.1919							
Stylonychia	0.2101	0.2094	0.1966	0.1981	0.1277						
Oxytricha	0.2143	0.2078	0.1910	0.1915	0.1245	0.0435					
Homalozoon	0.1935	0.1983	0.1866	0.1967	0.1522	0.1561	0.1527				
Loxophyllum	0.2221	0.2170	0.2054	0.2101	0.1673	0.1742	0.1688	0.0740			
Didinium	0.2058	0.2091	0.1941	0.1991	0.1596	0.1647	0.1561	0.0547	0.0778		
Furgasonia	0.1959	0.2033	0.1894	0.1866	0.1442	0.1511	0.1460	0.1632	0.1803	0.1633	
Obertrumia	0.1857	0.1959	0.1765	0.1836	0.1391	0.1411	0.1348	0.1575	0.1799	0.1559	0.0741
Pseudomicrothorax	0.1964	0.2012	0.1882	0.1970	0.1516	0.1649	0.1606	0.1810	0.1937	0.171 4	0.1279
Paramecium	0.2282	0.2236	0.2137	0.2152	0.1773	0.1773	0.1864	0.1759	0.1816	0.1686	0.1712
Uronema	0.2306	0.2339	0.2597	0.2480	0.1902	0.1978	0.2023	0.2101	0.2229	0.2110	0.1849
Cyclidium	0.2057	0.2089	0.2142	0.2232	0.1794	0.1743	0.1789	0.1846	0.1893	0.1753	0.1514
T. thermophila	0.2699	0.2725	0.2651	0.2719	0.2332	0.2353	0.2294	0.2248	0.2341	0.2226	0.2049
T. corlissi	0.2685	0.2711	0.2625	0.2706	0.2358	0.2317	0.2274	0.2250	0.2325	0.2217	0.2018
T. empidokyrea	0.2669	0.2695	0.2636	0.2746	0.2347	0.2276	0.2280	0.2239	0.2339	0.2221	0.2050
Ichthyophthirius	0.2561	0.2641	0.2645	0.2608	0.2166	0.2191	0.2257	0.2104	0.2314	0.2109	0.1969
Ophryoglena	0.2521	0.2558	0.2617	0.2609	0.2183	0.2246	0.2230	0.2118	0.2292	0.2096	0.1910
Colpoda	0.1952	0.1883	0.2059	0.1943	0.1466	0.1646	0.1589	0.1672	0.1721	0.1584	0.1202
Pseudoplatyophrya	0.2014	0.1962	0.2051	0.1986	0.1409	0.1671	0.1620	0.1650	0.1684	0.1525	0.1252
Platyophrya	0.1947	0.1931	0.1899	0.1948	0.1421	0.1567	0.1497	0.1622	0.1691	0.1592	0.1121
Prorodon	0.2037	0.2018	0.2039	0.1985	0.1403	0.1555	0.1498	0.1628	0.1670	0.1518	0.1200
Coleps	0.2119	0.2116	0.2111	0.2117	0.1586	0.1636	0.1632	0.1771	0.1874	0.1738	0.1475

The evolutionary distance for all pairs of aligned SSrRNA sequences were determined following Kimura two-parameter model. Table 4.1 is continued on next page.

Table 4.1. (Continued). SSrRNA evolutionary distance data for ciliate representatives of major lineages

	Ober..	*Pseu..*	*Para..*	*Uron..*	*Cycl..*	*T.the.*	*T.cor.*	*T.emp.*	*Ich...*	*Ophr..*	*Colp..*	*Pseud.*	*Plat..*	*Pror..*
Pseudomi....	0.1146													
Paramecium	0.1579	0.1733												
Uronema	0.1633	0.1843	0.1740											
Cyclidium	0.1455	0.1645	0.1610	0.1328										
T. thermo....	0.1944	0.2065	0.1862	0.1931	0.1913									
T. corlissi	0.1946	0.2059	0.1863	0.1903	0.1902	0.0168								
T. empido.....	0.1912	0.2063	0.1906	0.1945	0.1891	0.0209	0.0168							
Ichthyoph....	0.1936	0.1925	0.1880	0.1869	0.1888	0.0638	0.0651	0.0658						
Ophryoglena	0.1894	0.1873	0.1852	0.1928	0.1828	0.0610	0.0605	0.0612	**0.0181**					
Colpoda	0.1157	0.1424	0.1720	0.1724	0.1631	0.2065	0.2092	0.2053	0.1828	0.1887				
Pseudopla...	0.1133	0.1406	0.1709	0.1723	0.1660	0.2044	0.2085	0.2031	0.1831	0.1844	0.030			
Platyophrya	0.1046	0.1291	0.1602	0.1713	0.1526	0.1975	0.1994	0.1919	0.1781	0.1739	0.0933	0.0941		
Prorodon	0.1220	0.1262	0.1473	0.1491	0.1298	0.1799	0.1862	0.1860	0.1698	0.1715	0.1035	0.0980	0.1008	
Coleps	0.1397	0.1413	0.1697	0.1518	0.1495	0.2122	0.1966	0.2022	0.1766	0.1793	0.1310	0.1355	0.1276	0.0976

The evolutionary distance for all pairs of aligned SSrRNA sequences were determined following Kimura two-parameter model. .

Table 4.2. SSrRNA evolutionary distance data for the Class Oligohymenophorea

	Para..	*Uron..*	*Cycl..*	*T.the...*	*T.heg..*	*T.cap..*	*T.can..*	*T.bor..*	*T.aus..*	*T.tro..*
Uronema	0.1740									
Cyclidium	0.1610	0.1328								
T. thermophila	0.1862	0.1931	0.1913							
T. hegewischi	0.1900	0.1942	0.1871	0.0180						
T. capricornis	0.1916	0.1934	0.1902	0.0162	0.0040					
T. canadensis	0.1887	0.1901	0.1886	0.0098	0.0139	0.0121				
T. borealis	0.1887	0.1901	0.1886	0.0098	0.0139	0.0121	0.0000			
T. australis	0.1916	0.1918	0.1894	0.0168	0.0046	0.0029	0.0127	0.0127		
T. tropicalis	0.1879	0.1942	0.1916	0.0098	0.0162	0.0145	0.0046	0.0046	0.0150	
T. pyriformis	0.1863	0.1868	0.1853	0.0104	0.0144	0.0127	0.0029	0.0029	0.0121	0.0063
T. pigmentosa	0.1923	0.1926	0.1910	0.0162	0.0052	0.0011	0.013	0.0133	0.0029	0.0156
T. patula	0.1916	0.1934	0.1902	0.0168	0.0046	0.0006	0.0127	0.0127	0.0023	0.0150
T. nanneyi	0.1923	0.1926	0.1910	0.0162	0.0052	0.0011	0.0133	0.0133	0.0029	0.0156
T. malaccensis	0.1880	0.1957	0.1931	0.0023	0.0186	0.0168	0.0104	0.0104	0.0174	0.0104
T. hyperangularis	0.1923	0.1926	0.1910	0.0162	0.0052	0.0011	0.0133	0.0133	0.0029	0.0156
T. corlissi	0.1863	0.1903	0.1902	0.0168	0.0174	0.0156	0.0104	0.0104	0.0139	0.0104
T. empidokyrea	0.1906	0.1945	0.1891	0.0209	0.0133	0.0092	0.0145	0.0145	0.0110	0.0192
Colpidium	0.2006	0.2032	0.1966	0.0383	0.0328	0.0316	0.0353	0.0353	0.0305	0.0365
Glaucoma	0.2007	0.2053	0.1941	0.0393	0.0325	0.0313	0.0338	0.0338	0.0313	0.0357
Ichthyophthirius	0.1880	0.1869	0.1888	0.0638	0.0657	0.0651	0.0639	0.0639	0.0645	0.0633
Ophryoglena	0.1852	0.1928	0.1828	0.0610	0.0598	0.0592	0.0630	0.0630	0.0586	0.0611

The evolutionary distance for all pairs of aligned SSrRNA sequences were determined following Kimura two-parameter model. Table 4.2 is continued on the next page.

Table 4.2. (Continued). SSrRNA evolutionary distance data for the Class Oligohymenophorea

	T.pyri..	*T.pig..*	*T.pat..*	*T.nan..*	*T.mal..*	*T.hyp..*	*T.cor..*	*T.emp..*	*Colp...*	*Glau...*	*Ichth..*
T. pigmentosa	0.0127										
T. patula	0.0121	0.0006									
T. nanneyi	0.0127	**0.0000**	0.0006								
T. malaccensis	0.0115	0.0168	0.0174	0.0168							
T. hyperangularis	0.0127	0.0000	0.0006	**0.0000**	0.0168						
T. corlissi	0.0121	0.0168	0.0162	0.0168	0.0180	0.0168					
T. empidokyrea	0.0139	0.0092	0.0086	0.0092	0.0216	0.0092	0.0168				
Colpidium	0.0341	0.0316	0.0311	0.0316	0.0389	0.0316	0.0353	0.0329			
Glaucoma	0.0350	0.0325	0.0319	0.0325	0.0399	0.0325	0.0356	0.0308	0.0319		
Ichthyophthirius	0.0626	0.0664	0.0657	0.0664	0.0645	0.0664	0.0651	0.0658	0.0723	0.0703	
Ophryoglena	0.0617	0.0605	0.0598	0.0605	0.0617	0.0605	0.0605	0.0612	0.0701	0.0668	**0.0181**

The evolutionary distance for all pairs of aligned SSrRNA sequences were determined following Kimura 2-parameter model.

(Figure 4.1) is 27.3% between the karyorelictean *Tracheloraphis* and the oligohymenophorean *T. thermophila* (Table 4.1), and 27.5% between the heterotrich *Climacostomum*, and the oligohymenophorean *T. empidokyrea* (Table 4.1). The average of pair-wise comparisons between the heterotrichs and karyorelicteans is 16.6% (16.1 - 17.1%) (Table 4.3). Within the class Karyorelictea, the distance separating the protostomatid *Tracheloraphis* and the loxodid *Loxodes* is 9.0% (Table 4.3). Within class Heterotrichea, the distance between the heterotrichs *Eufolliculina* and *Climacostomum* is 10.7% (Table 4.3).

The deepest divergence involving the oligohymenophoreans (class Oligohymenophorea) (Figure 4.1) is 27.5% between the heterotrich *Climacostomum* and the hymenostome *T. empidokyrea* (Table 4.1). Within the hymenostomatians (Subclass Hymenostomatia), the deepest divergence is 20.5% between the hymenostome (order Hymenostomatida) *Glaucoma*, and the scuticociliate (order Scuticociliatida) *Uronema* (Table 4.2). The average of pair-wise genetic distance comparisons between the hymenostomes and the peniculid *Paramecium* is 19.0% (17.5 - 20.1%) (Table 4.2). Within the hymenostomatids, the average genetic divergence between the ophryoglenines (suborder Ophryoglenina), *Ophryoglena* and *Ichthyophthirius*, and the tetrahymenines (suborder Tetrahymenina), *Tetrahymena*, *Glaucoma*, and *Colpidium*, was 6.4% (5.9 to 7.2%) (Table 4.2). The average of pair-wise genetic distances between *Glaucoma* and *Colpidium* with the tetrahymenas (family Tetrahymenidae) is 3.4% (3.1 - 4.0%) (Table 4.2), whereas the distance between *Colpidium* (family Turaniellidae) and *Glaucoma* (family Glaucomidae) is 3.2% (Table 4.2). The deepest divergence within the

Fig. 4.1. A phylogenetic tree of the ciliates drawn to show the estimated time of divergence of the major ciliate lineages. The upper-limit of 1% divergence per 80 million years was used to draw the branches on the tree (see text for details).

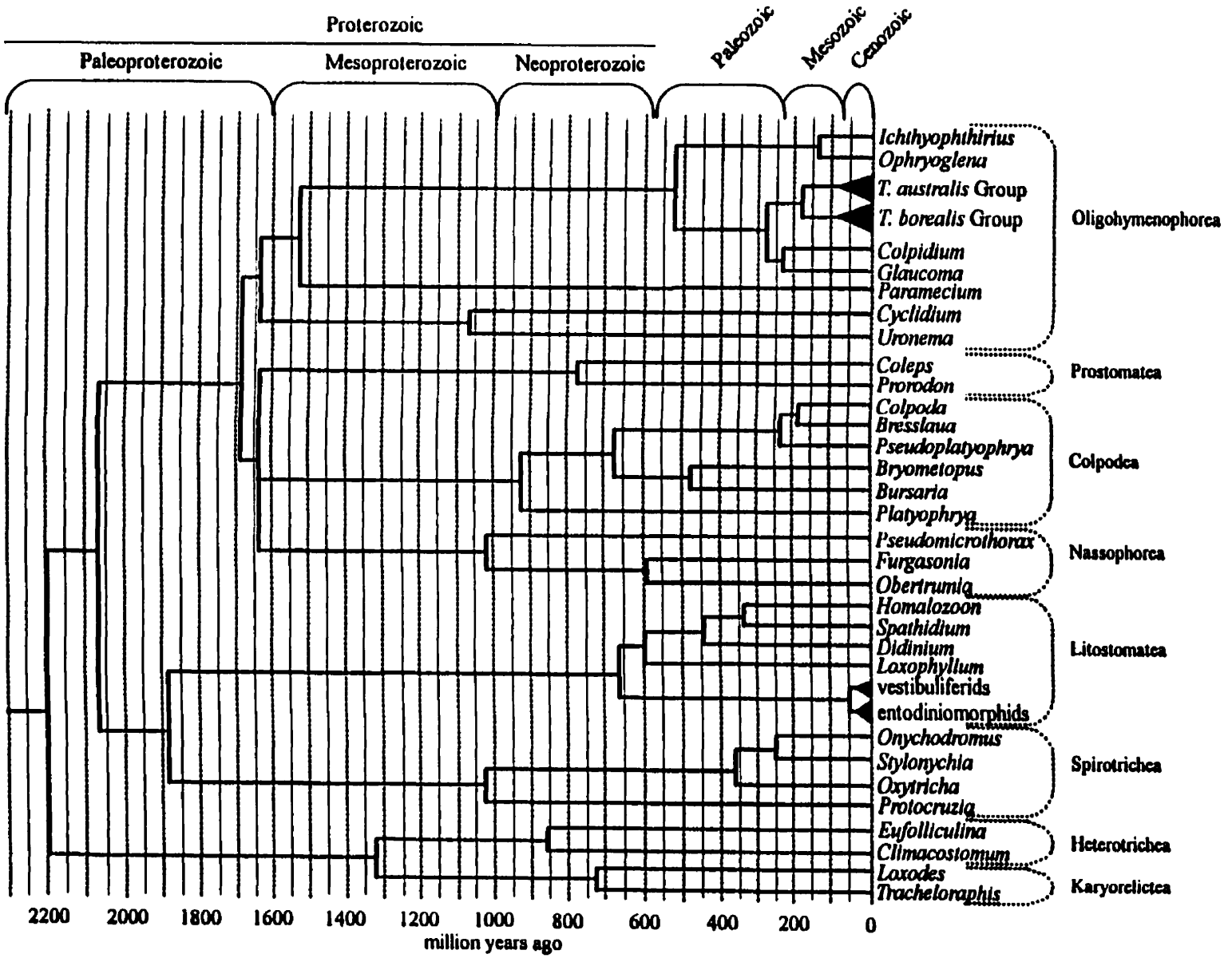

Proterozoic
Paleoproterozoic
Mesoproterozoic
Neoproterozoic
Paleozoic
Mesozoic
Cenozoic
Ichthyophthirius
Ophryoglena
T. australis Group
T. borealis Group
Colpidium
Glaucoma
Paramecium
Cyclidium
Uronema
Coleps
Prorodon
Colpoda
Bresslaua
Pseudoplatyophrya
Bryometopus
Bursaria
Platyophrya
Pseudomicrothorax
Furgasonia
Obertrumia
Homalozoon
Spathidium
Didinium
Loxophyllum
vestibuliferids
entodiniomorphids
Onychodromus
Stylonychia
Oxytricha
Protocruzia
Eufolliculina
Climacostomum
Loxodes
Tracheloraphis
Oligohymenophorea
Prostomatea
Colpodea
Nassophorea
Litostomatea
Spirotrichea
Heterotrichea
Karyorelictea
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
million years ago

Table 4.3. SSrRNA evolutionary distance data for the Classes Heterotrichea and Karyorelictea

	Loxodes	*Tracheloraphis*	*Eufolliculina*
Tracheloraphis	0.0903		
Eufolliculina	0.1611	0.1682	
Climacostomum	0.1714	0.1625	0.1067

The evolutionary distance for all pairs of aligned SSrRNA sequences were determined following Kimura 2-parameter model.

tetrahymenas was 2.2% between *Tetrahymena empidokyrea* and *T. malaccensis* (Table 4.2; Figure 4.1). There is no genetic distance (d = 0.00%) separating *T. borealis* and *T. canadensis*, or *T. hyperangularis, T. pigmentosa*, and *T. nanneyi* (Table 4.2).

The deepest divergence involving the litostomes (class Litostomatea) (Figure 4.1) is 23.4% between the haptorian *Loxophyllum* and the oligohymenophorean *T. thermophila* (Table 4.1). The average distance between the haptorians (Subclass Haptoria) and the trichostomes (Subclass Trichostomatia) is 7.2% (6.6 - 10.8%) (Table 4.4). Within the Subclass Haptoria (Figure 4.1), the average distance between the pleurostomatid (order Pleurostomatida) *Loxophyllum* and the haptorids (order Haptorida), *Homalozoon*, *Spathidium*, and *Didinium*, is 7.5% (Table 4.4). The average distance between *Didinium* and either *Homalozoon* and *Spathidium* is 5.5% (Table 4.4), while the distance between *Homalozoon* and *Spathidium* is 4.3% (Table 4.4; Figure 4.1). Within the Subclass Trichostomatia (Figure 4.2), the average distance between the vestibuliferids (order Vestibuliferida) and the entodiniomorphids (order Entodiniomorphida) is 6.5% (5.6 - 7.8%) (Table 4.4). Within the Entodiniomorphida, the average distance separating *Entodinium* (Subfamily Entodiniinae) from the other entodiniomorphds (Subfamilies Diplodiniinae and Ophryoscolecinae) is 4.7% (4.4 - 5.1%) (Table 4.4). The average distance between the Diplodiniinae and Ophryoscolecinae is 3.5% (3.2 - 4.0%) (Table 4.4), whereas, the distance between the two vestibuliferids *Dasytricha* and *Isotricha* is 5.2%. The most recent divergence within the rumen ciliates is 1.8% between *Diplodinium* and *Eudiplodinium* (Table 4.4).

Fig. 4.2. A phylogenetic tree of the rumen ciliates drawn to show the estimated time of divergence of the rumen ciliate lineages. The lower-limit of 1% divergence per 8 million years was used to draw the branches on the tree (see text for details).

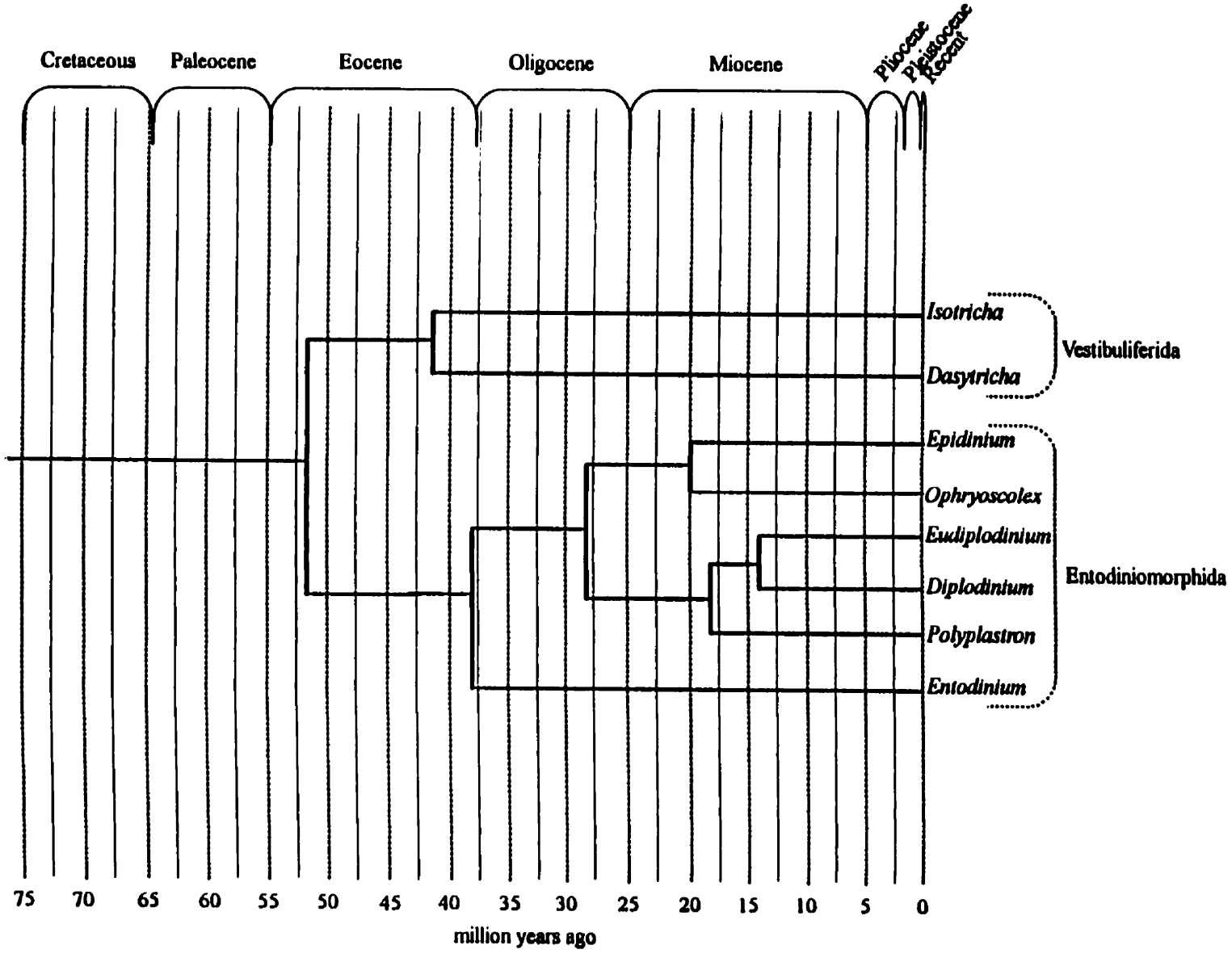

Cretaceous
Paleocene
Eocene
Oligocene
Miocene
Pliocene
Pleistocene
Recent
Isotricha
Dasytricha
Vestibuliferida
Epidinium
Ophryoscolex
Eudiplodinium
Diplodinium
Polyplastron
Entodinium
Entodiniomorphida
75 70 65 60 55 50 45 40 35 30 25 20 15 10 5 0
million years ago

Table 4.4. SSrRNA evolutionary distance data for the Class Litostomatea

	Homa..	*Loxo..*	*Spat..*	*Didi..*	*Isot..*	*Dasy..*	*Ento..*	*Epid..*	*Dipl..*	*Eudi..*	*Ophr..*
Loxophyllum	0.0740										
Spathidium	0.0427	0.0708									
Didinium	0.0547	0.0778	0.0531								
Isotricha	0.0807	0.1002	0.0662	0.0812							
Dasytricha	0.0743	0.1002	0.0667	0.0750	0.0517						
Entodinium	0.0961	0.1081	0.0785	0.0896	0.0784	0.0651					
Epidinium	0.0855	0.0986	0.0784	0.0883	0.0678	0.0644	0.0491				
Diplodium	0.0839	0.1020	0.0796	0.0866	0.0712	0.0570	0.0476	0.0326			
Eudiplodinium	0.0804	0.0985	0.0755	0.0818	0.0658	0.0557	0.0438	0.0320	0.0180		
Ophryoscolex	0.0869	0.0979	0.0729	0.0856	0.0632	0.0592	0.0439	0.0250	0.0346	0.0326	
Polyplastron	0.0804	0.1020	0.0783	0.0854	0.0720	0.0577	0.0510	0.0397	0.0217	0.0205	0.0384

The evolutionary distance for all pairs of aligned SSrRNA sequences were determined following Kimura two-parameter model.

The deepest divergence involving the spirotrichs (class Spirotrichea) (Figure 4.1) is 23.8% between *Protocruzia* and the oligohymenophorean *T. corlissi* (Table 4.1). The average of pair-wise distance comparisons between *Protocruzia* and the stichotrichs (order Stichotrichia) is 12.8% (12.5 - 13.1%) (Table 4.5). Within the stichotrichs, the deepest divergence is 4.7% between *Onchyodromus* and *Oxytricha*, whereas the most recent divergence is 3.1% between *Onchyodromus* and *Stylonychia* (Table 4.5).

The deepest divergence involving the prostomes (class Prostomatea) (Figure 4.1) is 21.2% between *Coleps* and the oligohymenophorean *T. thermophila* (Table 4.1), whereas, the distance between *Coleps* and *Prorodon* is 9.8%.

The deepest divergence involving the nassophoreans (class Nassophorea) (Figure 4.1) is 20.7% between *Pseudomicrothorax* and the oligohymenophorean *T. thermophila* (Table 4.1). Within the Nassophorea, the deepest divergence is 12.8% between *Pseudomicrothorax* and *Furgasonia*, whereas the closest divergence is 7.4% between *Furgasonia* and *Obertrumia* (Table 4.6; Figure 4.1).

The deepest divergence involving the colpodeans (class Colpodea) (Figure 4.1) is 20.9% between *Colpoda* and the oligohymenophorean *T. corlissi* (Table 4.1). The deepest divergence within the Colpodea is 11.6% between *Bursaria* and *Platyophyra*, whereas the closest divergence is 2.2% between *Bresslaua* and *Colpoda* (Table 4.7).

Table 4.5. SSrRNA evolutionary distance data for the Class Spirotrichea

	Protocruzia	*Stylonychia*	*Onychodromus*
Stylonychia	0.1277		
Onychodromus	0.1313	0.0309	
Oxytricha	0.1245	0.0435	0.0466

The evolutionary distance for all pairs of aligned SSrRNA sequences were determined following Kimura two-parameter model.

Table 4.6. SSrRNA evolutionary distance data for the Class Nassophorea

	Furgasonia	*Obertrumia*
Obertrumia	0.0741	
Pseudomicrothorax	0.1279	0.1146

The evolutionary distance for all pairs of aligned SSrRNA sequences were determined following Kimura two-parameter model.

Table 4.7. SSrRNA evolutionary distance data for the Colpodea

	Colp...	*Pseud..*	*Platy..*	*Bress..*	*Bryom..*
Pseudoplatyophrya	0.0301				
Platyophrya	0.0933	0.0941			
Bresslaua	0.0224	0.0317	0.0915		
Bryometopus	0.0797	0.0801	0.0951	0.0801	
Bursaria	0.0957	0.0956	0.1155	0.0983	0.0597

The evolutionary distance for all pairs of aligned SSrRNA sequences were determined following Kimura 2-parameter model.

DISCUSSION

There is no fossil record evidence of protozoan endo- or ectosymbionts. Therefore, I cannot determine the exact time (i.e. within the past 145-My) that the obligate freshwater fish ectoparasite, *Ichthyophthirius*, appeared on freshwater teleosts. However, since *Ichthyophthirius* is unlikely to be older than the appearance of freshwater fish, it is possible to calculate the maximum time and therefore divergence rate that *Ichthyophthirius* could have diverged from *Ophryoglena* (i.e. 1% per 72 to 80-My). As this is the first attempt to calibrate a SSrRNA molecular clock for the ciliates, it is important that as more independently-timed events are discovered, they be used to further refine my ciliate SSrRNA molecular clock calibration. With this in mind, the deepest divergence within the ciliates, 27.5% between the heterotrich *Climacostomum* and the oligohymenophorean *T. empidokyrea* (Table 4.1), would have occurred 1,980 to 2,200-Ma during the Paleoproterozoic (Table 4.8; Figure 4.1). This represents the maximum divergence time from a common ciliate-like ancestor. Although my time frame of 1,980 to 2,200-Ma doubles the divergence time speculated for the ciliated protozoa at 1,000-My (Schlegel and Eisler, 1996), this estimate is consistent with information that the oldest known fossil that appears to be protistan is approximately 1,800 to 1,900-My old from the Chuanlinggou Formation, China (Knoll, 1992). With fossil record evidence, it is important to note that the oldest known fossil group is seldom as old as the group itself (Wilson and Williams, 1992; Doolittle *et al.*, 1996). Beside this paleontological information, Knoll (1992) also mentioned biogeochemical evidence that eukaryotic cells were present during the Paleoproterozoic [e.g. steranes in bitumens from the 1,690-My

Table 4.8. Summary of maximum age of ciliate lineages based on a SSrRNA molecular clock calibrated to the origin of the freshwater teleost fish ectoparasite *Ichthyophthirius*[1]. This table is continued on the next page.

Deepest Divergence Involving			
Lineage	Lineage	Geological Time	Age (millions of years ago)
Subphylum Postciliodesmatophora			
Class Heterotrichea	*T. empidokyrea*	Paleoproterozoic	2200 - 1980
Climacostomum	*Eufolliculina*	Neoproterozoic	856 - 770
Class Karyorelictea	*T. thermophila*	Paleoproterozoic	2184 - 1966
Tracheloraphis	*Loxodes*	mid-Neoproterozoic	720 - 648
Subphylum Intramacronucleata[2]			
Class Oligohymenophorea	*Climacostomum*	Paleoproterozoic	2200 - 1980
Order Scuticociliatida	hymenostomatids	early-Mesoproterozoic	1640 - 1476
Order Hymenostomatida	*Paramecium*	Mesoproterozoic	1520 - 1368[3]
Suborder Tetrahymenina	Suborder Ophryoglenina	late-Cambrian/Ordovician	512 - 461[3]
Glaucoma/Colpidium	Family Tetrahymenidae	Permian	272 - 245[3]
T. australis group	*T. borealis* group	late-Jurassic	176 - 158
Class Spirotrichea	*T. corlissi*	late-Paleoproterozoic	1888 - 1699
Subclass Stichotrichia	*Protocruzia*	Mesoproterozoic/Neoproterozoic	1024 - 922[3]
Onychodromus	*Oxytricha*	Devonian/Carboniferous	376 - 338
Stylonychia	*Onychodromus*	Triassic	248 - 223

1. Genetic distance separating *Ichthyophthirius* from *Ophryoglena* is 1% per 72 - 80 My (see text for details).
2. The Classes Phyllopharyngea and Plagiopylea are not included in this Table as the only two representatives for these classes have a relatively fast evolving SSrRNA and were omitted from the analysis.
3. Based on mean percentage.

Table 4.8. (Continued). Summary of maximum age of ciliate lineages based on a SSrRNA molecular clock calibrated to the origin of the freshwater teleost fish ectoparasite *Ichthyophthirius*[1].

Deepest Divergence Involving			
Lineage	Lineage	Geological Time	Age (millions of years ago)
Subphylum Intramacronucleata[2]			
Class Litostomatea	*T. empidokyrea*	late-Paleoproterozoic	1872 - 1685
Subclass Haptoria	Subclass Trichostomatia[3]	late--Neoproterozoic	656 - 590
Loxophyllum	Order Haptorida	late-Neoproterozoic/Cambrian	600 - 540[4]
Didinium	*Homalozoon*	Silurian/Devonian	440 - 396
Homalozoon	*Spathidium*	Carboniferous	344 - 310
Class Prostomatea	*T. thermophila*	Paleoproterozoic/Mesoproterozoic	1696 - 1526
Coleps	*Prorodon*	Neoproterozoic	784 - 706
Class Nassophorea	*T. thermophila*	Paleoproterozoic/Mesoproterozoic	1656 - 1490
Pseudomicrothorax	*Furgasonia*	Neoproterozoic	1023 - 921
Furgasonia	*Obertrumia*	Neoproterozoic/Cambrian	592 - 533
Class Colpodea	*T. corlissi*	Paleoproterozoic/Mesoproterozoic	1672 - 1505
Bursaria	*Platyophyra*	Neoproterozoic	928 - 835
Bresslaua	*Colpoda*	Jurassic	176 - 158

1. Genetic distance separating *Ichthyophthirius* from *Ophryoglena* is 1% per 72 - 80 My (see text for details).
2. The Classes Phyllopharyngea and Plagiopylea are not included in this Table as the only two representatives for these classes have a relatively fast evolving SSrRNA and were omitted from the analysis.
3. The rumen ciliates are not included in this Table as they appear to have a different molecular clock rate (see Table 4.9).
4. Based on mean percentage.

old Barney Creek Formation in Australia (Hoffman and Chen, 1981)].

The class Oligohymenophorea de Puytorac *et al.*,, 1974 is the most speciose group of ciliates (Lynn and Corliss, 1991) and the group for which the most SSrRNA sequence data are available, especially for the genus *Tetrahymena*. According to my data, the tetrahymenas split into two distinct lineages, the *T. australis* group and the *T. borealis* group (Sogin *et al.*, 1986c; Wright and Lynn, 1995; Jerome *et al.*, 1996), during the late Jurassic Period 158-Ma (Figure 4.1). Within the two main groups, further radiations occurred 4 to 72-Ma (Table 4.2). Because genetic distance data in my analysis are limited to two decimal places, I cannot account for distances of less than 0.01% (i.e. within the last 720,000 to 800,000 years). Therefore, the most recent divergence (d = 0.00%) between *T. borealis* and *T. canadensis*, as well as the split amongst *T. hyperangularis*, *T. nanneyi*, and *T. pigmentosa*, must have taken place within the last 720,000 to 800,000 years. My relatively young age estimate of the genus *Tetrahymena* strongly contrasts with Nanney's (1984) suggestion that *Tetrahymena* was over 1,000-My old. Nanney's (1984) claim was based on the percentage of G-C variation, which he noted was a crude measure of divergence.

As indicated, the litostomes (class Litostomatea) date back to the late Paleoproterozoic some 1,685 to 1,872-Ma and divided into at least two major lineages, the haptorians (Subclass Haptoria) and the trichostomes (Subclass Trichostomatia). The haptorians consist of free-living organisms, whereas the trichostomes are primarily endosymbionts of vertebrates and are collectively known as the rumen ciliates. These obligate endosymbionts fall into two major lineages, the entodiniomorphids (order

Entodiniomorphida) and the vestibuliferids (order Vestibuliferida), and are found in the rumens of ungulates, which are well documented in the fossil record. Based on morphology (Dogiel, 1925, 1927, 1947; Lubinsky, 1957a, 1957b, 1957c) and molecular data (Wright *et al.*, 1997; Wright and Lynn, 1997a, 1997b), the genus *Entodinium* is the most primitive of the entodiniomorphids. Because *Entodinium* is found in camellids (e.g. camels, llamas, alpacas, vicunas, guanacos), which are pseudoruminants (Williams and Coleman, 1992), but not in non-ruminants (i.e. hippopotamus), *Entodinium* must have arisen after the non-ruminants diverged from the other Artiodactyla 50-Ma and before the separation of the pseudoruminants 40-Ma (Williams and Coleman, 1992). Based on this information, I now have a maximum and a minimum age (40 to 50-My) for the appearance of *Entodinium*. Within the Entodiniomorphida, the deepest and most recent divergences involving *Entodinium* is 5.1% (Table 4.4) with *Polyplastron* and 4.4% (Table 4.4) with either *Eudiplodinium* or *Ophryoscolex*. Thus, these changes in the SSrRNA gene of *Entodinium* occurred over the past 40 to 50-My or at a divergence rate of 1% per 8 to 11-My. In addition, the rumen ciliates, *Eudiplodinium* and *Epidinium*, are separated by a genetic distance of 3.2% (Table 4.4) and are only found in cervids and bovids (Williams and Coleman, 1992). Since cervids and bovids date back to 25-Ma (Williams and Coleman, 1992), then *Eudiplodinium* and *Epidinium* are diverging at a rate of 1% per 8-My (Table 4.9; Figure 4.2). This lower-limit of 1% divergence per 8-My is an order of magnitude faster than the divergence rate calibrated for *Ichthyophthirius* based on the appearance of freshwater fish.

Table 4.9. Summary of maximum age of the litostomes based on a SSrRNA molecular clock[1] calibrated to the origin the rumen ciliates, *Entodinium*, *Epidinium*, and *Eudiplodinium*.

Deepest Divergence Involving			
Lineage	Lineage	Geological Time	Age (millions of years ago)
Class Litostomatea			
Order Vestibuliferida	Order Entodiniomorphida	Cretaceous - Eocene	72 - 52[2]
Dasytricha	*Isotricha*	Paleocene - Eocene	58 - 42
Order Entodiniomorphida			
Subfamily Entodiniinae *(Entodinium)*	Subfamilies Ophryoscolecinae & Diplodiniinae	Eocene - Oligocene	52 - 38[2]
Subfamily Ophryoscolecinae	Subfamily Diplodiniinae	Oligocene	39 - 28[2]
Subfamily Ophryoscolecinae			
Epidinium	*Ophryoscolex*	Oligocene - Miocene	28 - 20
Subfamily Diplodiniinae			
Polyplastron	*Diplodinium*	Miocene	24 - 18
Diplodinium	*Eudiplodinium*	Miocene	20 - 14

1. Genetic distance is based on 1% divergence per 8 - 11 My (see text for details).
2. Based on mean genetic distance

If I use the calibrations based on the rumen endosymbionts of 1% divergence per 8 to 11-My, then the ciliates appeared some 220 to 303-Ma. This is undoubtedly a gross underestimation of the origin of ciliates, since there is fossil record evidence of a relatively derived group of ciliates, the tintinnids, dating back to the Ordovician (Tappan and Loeblich, 1968, 1973). Moreover, if I apply the 1% divergence per 72 to 80-My to the rumen ciliates, then the most recent divergence previously mentioned for *Entodinium* (Table 4.4) would have occurred between 318 to 352-Ma, well before the appearance of the ungulates. Neither of these predictions is reasonable, which leads to the conclusion that the rumen ciliates (endosymbionts) have a fast clock while ectosymbionts and free-living ciliates have a slower clock. A similar discovery was made by Moran and her collaborators (Moran *et al.*, 1995; Moran, 1996) when they compared divergence rates of ribosomal DNA for aphids and their endosymbiotic bacteria, *Buchnera* spp. They determined that substitution rates were twice as fast in *Buchnera* spp. as in related free-living bacterial lineages (Moran *et al.*, 1995; Moran, 1996).

The greatest variation of substitution rates within a group is found within the foraminifera, one of the most abundant and diverse marine protists. Pawlowski and his collaborators (de Vargas *et al.*, 1997; Pawlowski *et al.*, 1997) estimated that the rate of rDNA evolution in planktonic foraminifera was 50 - 100 times faster than in some benthic foraminifera. Pawlowski *et al.* (1997) are doubtful that differences of generation time and productivity are sufficiently large enough to account for the 100-fold increase in evolution rates and further suggested that an increased exposure of the planktonic foraminifera to solar UV radiation as another contributing factor. De Vargas *et al* (1997) suggested the

adaptation to a planktonic mode of life from a benthic one as another contributing factor to explain the elevated divergence rates in planktonic foraminifera.

Dykhuizen (1990) believed that elevated divergence rates, such as those in the rumen ciliates, endosymbiotic bacteria, and planktonic foraminifera, reflected periods of rapid evolution into new niches where survival is more important than competitive ability. He argued that there would be intense selection pressure on the region of the genome responsible for survival in the new niche and relaxed selection pressure on the rest of the genome. Therefore, researchers should expect to see rapid and unusual changes in the ribosomal sequence in lineages, like the foraminifera or endosymbionts (i.e. parasites), that had adapted to a new niche. For example, McCutchan and his collaborators (McCutchan *et al.*, 1988, 1995; Li *et al.*, 1997) indentified three highly derived functional 18S SSrRNA genes in the nuclear compartment of *Plasmodium*, the causative agent of malaria. Referred to as S-, A-, and O-types, the S-type gene is transcribed in the sporozoites within the liver, the A-type gene is transcribed in the merozoites in infected erythrocytes and in the gametocytes of the vertebrate and mosquito blood meal, while the O-type gene is transcribed in the oocysts within the mosquito. These multiple genes may be in response to different developmental stimuli as these parasites moved from poikilothermic invertebrate hosts (e.g. insects, O-type gene) to homeothermic vertebrate hosts (e.g. humans, A- and S-type genes) (Barta, *pers. comm.*).

Although the faster clock for the rumen ciliates can be explained by this intense selection on survivability as they invaded the rumen niche, it can also be explained by the relatively high ambient temperature (39° C) of the rumen environment. High temperatures

are known to decrease the efficiency of DNA repair mechanisms leading to higher mutation rates (Sancar and Sancar, 1988). Which explanation is more appropriate must await future experiments. Generation times have also been suggested to play an important role on the rates of molecular evolution (Martin and Palumbi, 1993). However, generation times are not considered to have contributed to the higher mutation rates of the rumen ciliates as their generation times at 39° C (*in situ* ≈ 12 - 24 h and *in vitro* ≈ 6 - 48 h) (Williams and Coleman, 1992) are consistent with generation times observed for a variety of free-living ciliates at 15 - 29° C (≈ 3.9 - 52.8 h) (Heinbokel, 1988; Suzuki and Taniguchi, 1993; Allali et al., 1994; Hansen, 1995; Perez-Uz; 1995, 1996; Montagnes, 1996; Montagnes *et al.*, 1996; Gransden, 1997).

Paleontologists refer to the Cenozoic Era as the Age of Mammals, but a valid argument could also be made for the Age of Birds, Insects, or Flowering plants (Davis *et al.*, 1990). The Mesozoic Era is known as the Age of Reptiles, the Silurian Period as the Age of Fishes, and the Paleozoic Era, the Age of Ancient Life (Davis *et al.*, 1990). With this in mind, the ciliates first emerged during the Proterozoic Era, and within 600-My of the ciliate-like ancestor diverging from the main eukaryotic line, a major radiation within the ciliates occurred during the Mesoproterozoic and Neoproterozoic periods leading to the well-established lineages recognized as classes today (Colpodea, Heterotrichea, Karyorelictea, Litostomatea, Nassophorea, Oligohymenophorea, Prostomatea, Spirotrichea) (Table 4.1). Within these major lineages, further radiations occurred 800 to 1,200-Ma. Although the SSrRNA molecular clock may only be a rough estimate of geological divergence time when applied to the ciliates, the time frames it suggests are

consistent with information from the paleontological record that a major radiation of single-celled eukaryotes occurred 1,000 to 1,200-Ma during the Neoproterozoic (Knoll, 1992). According to Knoll (1992), the diversity of Neoproterozoic eukaryotes surpassed that of any earlier era, which suggests that the Neoproterozoic radiation of ciliates as determined by my molecular clock is a real phenomenon and not an artifact of sampling. In light of this new information, the Proterozoic could be called the Age of Ciliated Protozoa, since these protists are typically the top heterotrophs in the microbial food web (Azam *et al.*, 1983, Stoecker and Sanders, 1985; Sherr and Sherr, 1987; Rassoulzadegan *et al.*, 1988).

Recently, Doolittle *et al.*, (1996) determined the divergence times of the major kingdoms using amino acid sequences from 57 different enzymes. They suggested that the prokaryotes and eukaryotes last shared a common ancestor about 2,000-Ma and that the major protist lineages split from the other eukaryotes about 1,230-Ma. In contrast, data from ribosomal RNA sequences suggest that the prokaryotic-eukaryotic split occurred 3,500-Ma (Woese, 1987). Moreover, I suggest a much later time of divergence for the ciliated protists.

Further, Wray *et al.*, (1996) calibrated the rates of molecular sequence divergence for seven independent data sets of macromolecules, including the SSrRNA. Their averaged molecular data suggest that the invertebrates diverged from the chordates about 1,000 to 1,200-Ma, twice as long ago as previously believed. If this information is considered to be correct, then it puts serious doubt into the classical belief that the crown eukaryotes (i.e. ciliates) evolved 1,000-Ma; putting them after the appearance of complex

metazoans. However, the results by Wray *et al.* (1996) have now been recently challenged by Ayala *et al.* (1998) who analyzed 18 protein-coding gene loci, including six of the seven genes studied by Wray *et al.* (1996). In contrast, Ayala *et al.*, (1998) report divergence data that coincides with the "Cambrian explosion" theory that the first coelomates appeared in the Neoproterozoic 544 to 700-Ma.

Nevertheless, despite the controversy of the appearance of the metazoa, paleontological information that the oldest known fossil that appears to be protistan is 1,800 to 1,900-My old (Knoll, 1992), coupled with biogeochemical evidence that eukaryotic cells were present 1,690-My (Hoffman and Chen, 1981), corroborates my molecular divergence estimates that the ciliates as crown eukaryotes are much older than previously speculated, appearing about 1,980 to 2,200-Ma in the Paleoproterozoic.

CHAPTER FIVE

General Discussion And Conclusions

Tout est pour le mieux dans le meilleux des mondes possibles.

Voltaire, year unknown.

GENERAL DISCUSSION AND CONCLUSIONS

This study has examined rDNA diversity in vertebrate endosymbiotic ciliates with a view towards examining the phylogenetic history of ciliates at large and rumen ciliates in particular. The 15 new sequences represent two subclasses, five orders, and 10 families of vertebrate endosymbionts and free-living predators within the class Litostomatea. In earlier chapters, these new sequences were compared against SSrRNA sequences from representatives from most of the major ciliate lineages to examine the phylogenetic relationships within the class Litostomatea and the phylum Ciliophora. The outcome of all trees in this dissertation were consistent with recent phylogenies inferred from LSrRNA (Baroin-Tourancheau *et al.*, 1992, 1995) and SSrRNA data (Hirt *et al.*, 1995; Hammerschmidt *et al.*, 1996; Stechmann *et al.*, 1998); the ciliates were a monophyletic group that divided into two major clades corresponding to the subphylum divisions within the phylum. The "first" major clade in the ciliate tree (i.e. the subphylum Postciliodesmatophora Gerassimova and Seravin, 1976 *sensu* Small and Lynn, 1985) was a dichotomy with *Loxodes* and *Trachelorapbis* (class Karyorelictea) on one branch, and *Climacostomum* and *Eufolliculina* (class Heterotrichea) on another branch. The other major clade corresponded to the subphylum Intramacronucleata Lynn, 1996 and contained the remaining ciliates, those that use intramacronuclear microtubules to divide their macronucleus. Within the Intramacronucleata the new litostome sequences always formed a monophyletic group consistent with their placement in the class Litostomatea. Six other major lineages that correspond to classes recognized by Small and Lynn (1985) and Lynn and Small (1998) were strongly supported by bootstrap data in both parsimony and distance-matrix analyses. Within the litostome clade the

free-living ciliates routinely grouped together on one branch consistent with their placement within the subclass Haptoria, while the vertebrate endosymbionts always assembled together on another branch consistent with their placement within the subclass Trichostomatia (Small and Lynn, 1985; Lynn and Small, 1998).

Although the haptorians were consistently, albeit weakly depicted as a monophyletic group, relationships within the clade could not be resolved. This is probably because there were not enough phylogenetically informative sites among the six genera of haptorians as only 85 sites were informative (i.e. 5.1% of the gene). Similarly, there were 127 phylogenetically informative sites for 13 species of *Tetrahymena* and the SSrRNA gene could not resolve some relationships among the tetrahymenas (Wright and Lynn, 1995). This suggests that there are not enough phylogenetically informative sites in the SSrRNA of closely related (i.e. the haptorians), or recently diverged species, like those in the genus *Tetrahymena*. In light of this information, another macromolecule that is less conserved than the SSrRNA gene, such as the ITS-1/5.8S/ITS-2 region, could be used to resolve relationships among these closely related taxa.

The vestibuliferids, *Dasytricha*, *I. Prostoma*, *I. intestinalis*, and *Balantidium*, consistently formed a monophyletic group on a branch within the trichostome clade and not within the haptorian clade. Even when the cladistic analysis by Lipscomb and Riordan (1992) was re-examined using their 46 morphological and ultrastructural characters (see Table 2.10) for 21 genera of litostomes, the vestibuliferids (*Balantidium*, *Isotricha*) always formed a monophyletic group outside of the haptorian clade (Figure 2.29). In light of this information, it is clearly incorrect to remove the vestibuliferids from the subclass Trichostomatia to the

subclass Haptoria as suggested by Leipe and Hausmann (1989) and Lipscomb and Riordan (1990, 1992). This supports the view of Small and Lynn (1985) and Lynn and Small (1998) that the vestibuliferids are the sister group to the entodiniomorphids within the subclass Trichostomatia. Further, the ophryoscolecids always formed a monophyletic group with *Entodinium* (i.e. subfamily Entodiniinae) as the earliest branching ciliate before a dichotomy containing *Epidinium* and *Ophryoscolex* on one branch (i.e. subfamily Ophryoscolecinae) and *Polyplastron*, *Diplodinium*, and *Eudiplodinium* (subfamily Diplodiniinae) on the other branch. These groupings corresponded to Lubinsky's (1957c) subfamilial division of the Ophryoscolecidae based upon morphological characters. Further, *Entodinium*'s basal position to the other ophryoscolecids also supported the idea that *Entodinium* was a representative of the ancestral ophryoscolecids (Crawley, 1923; Dogiel, 1925, 1947; Lubinsky, 1957b, 1957c).

Cycloposthium was the sister group to the ophryoscolecids consistent with its placement in the order Entodiniomorphida by Small and Lynn (1985). However, its putative entodiniomorphid relative *Macropodinium* did not group with the other entodiniomorphids making them paraphyletic. Perhaps *Macropodinium* was arbitrarily placed within the Entodiniomorphida because of its similar gastrointestinal habitat. In fact, *Macropodinium*'s basal position to the entodiniomorphids and vestibuliferids (i.e. two major groups of mostly eutherian mammalian endosymbionts) suggested perhaps the establishment of a new order for these unique marsupial endosymbionts. However, as previously stated, more sequence evidence from other species of *Macropodinium* is needed before such a decision is warranted. Furthermore, molecular sequence evidence from representatives of the other entodiniomorphid families, Ditoxidae Strelkow, 1939 (in horses), Polydiniellidae Corliss, 1960 (in elephants),

Rhinozetidae Van Hoven, 1988 (rhinoceros), Spirodiniidae Strelkow, 1939 (in horses), Telamodiniidae Latteur and Dufey, 1967 (in warthogs), and Troglodytellidae Corliss, 1979 (in apes) is needed to establish whether other endosymbionts may have been improperly placed within the Entodiniomorphida.

Although the SSrRNA gene corroborates the monophyly of the ciliates and supports the 10 classes of ciliates that were established based upon ultrastructural characters, it could not resolve the deep relationships among these major lineages. For example, the Nassophorea-Phyllopharyngea-Prostomatea-Colpodea-Oligohymenophorea clade, Spirotrichea clade, Armophorida clade, and Litostomatea clade are all on one branch, but not unambiuously resolved. As a result of this polychotomy, my analysis and other studies using SSrRNA (Leipe *et al.*, 1994a; Embley *et al.*, 1995; Hirt *et al.*, 1995; Hammerschmidt *et al.*, 1996; Wright and Lynn, 1997a, 1997b; Wright *et al.*, 1997; Stechman *et al.*, 1998) and LSrRNA (Baroin-Tourancheau *et al.*, 1992, 1995), could not unambiguously resolve the sister group to the litostomes. Molecular phylogenetic analyses, using other tree building algorithms and software, produced trees (not shown) that corroborated these results. If these organisms are a very old assemblage of protozoa, the accumulation over time of transitions, transversions, and back mutations would make it impossible to resolve these deep relationships.

The best way to find the most accurate phylogeny is to compare trees constructed from different macromolecules using various methods. In order to elucidate the deep branching pattern within the phylum Ciliophora another macromolecule should be used. Other macromolecules are being employed to corroborate SSrRNA based phylogenetic

relationships within various protistan groups and to test the deep relationships near the base of global eukaryotic trees. For example, chaperonin (cpn60) and heat shock protein (hsp70) sequences are being used to test deep relationships within the three amitochondriate lineages, the diplomonads (phylum Metamonada), trichomonads (phylum Parabasala), and microsporidians (phylum Microspora) (Clark and Roger, 1995; Bui *et al.*, 1996; Germot *et al.*, 1996, 1997; Horner *et al.*, 1996; Roger *et al.*, 1996, 1998; Hirt *et al.*, 1997; van der Giezen *et al.*, 1997). These macromolecules are proving to be quite useful in corroborating and resolving these deep branches. Perhaps macromolecules like cpn60 and hsp70 could be used to resolve the deep branches within the ciliates.

In chapter 3, rumen ciliate signature sequences (8-16 nucleotides that are unique to each species) from four regions of the SSrRNA gene (see Appendix XVII) were used to construct a species-specific oligonucleotide primer for *I. prostoma.* This new primer anneals approximately 400 bp upstream from the 3' end of the SSrRNA and was used with the Jerome C reverse primer to amplify a section of DNA that included the complete ITS-1,/5.8S/ITS-2 region of *I. prostoma*. This region is less conserved than the SSrRNA gene and has been used successfully to distinguish and differentiate morphologically identical species and strains from a wide diversity of life ranging from bacteria (Barry *et al.*, 1991; McLaughlin *et al.*, 1993; Matar *et al.*, 1993, Gürtler, 1993) to salmonid fish (Pleyte *et al.*, 1992). Because of *I. prostoma*'s global distribution and various ruminant and non-ruminant hosts, its ITS-1/5.8S/ITS-2 region was examined for intraspecific sequence variation. In contrast to other studies (Barry *et al.*, 1991; Pleyte *et al.*, 1992; Matar *et al.*, 1993; McLaughlin *et al.*, 1993; Gürtler, 1993), my analysis showed that there were no

differences in the ITS-1/5.8S/ITS-2 region among cattle and sheep isolates of *I. prostoma* from Australia, Canada, and the United States. Although these results indicated that the ITS-1/5.8S/ITS-2 region is 100% conserved among eight isolates from two continents, other researchers have reported identical results that support these findings (Hoste *et al.*, 1993, de Wit and Klatser, 1994; Homan *et al.*, 1997). The results from this study suggested that populations of *I. prostoma* on two continents are very recently diverged, consistent with human migration and colonization of these continents with their large domestic animals in the 18th and 19th centuries.

In chapter 4, my study was the first attempt to calibrate a SSrRNA molecular clock for the ciliated protozoa. The rate of nucleotide substitution was calibrated to be 1% divergence per 72 to 80 My and the origin of the ciliates was calculated to be much older than previously speculated, dating back 1,980 to 2,200 Ma. This represents the maximum divergence time from a common ciliate-like ancestor and doubles the divergence time speculated for the ciliated protozoa at 1,000 My (Schlegel and Eisler, 1996). Although Ayala *et al* (1998) disputed Wray *et al. 's* (1996) molecular divergence estimates that the invertebrates diverged from the chordates twice as long ago as previously believed (i.e. 1,000 - 1,200 Ma), paleontological information (Knoll, 1992), and biogeochemical evidence (Hoffman and Chen, 1981) corroborates my molecular divergence estimates that the ciliates, as crown eukaryotes, are much older than previously speculated.

Moreover, the rate of nucleotide substitution for rumen ciliates was almost a magnitude faster (1% per 8-11 My) than that for free-living ciliates. This faster clock might be explained by intense selection on survivability as they invaded the rumen

(Dykhuizen, 1990), or by the relatively high ambient temperature (39° C) of the rumen environment, as such high temperatures are known to decrease the efficiency of DNA repair mechanisms leading to higher mutation rates (Sancar and Sancar, 1988). Which explanation is more appropriate must await future experiment.

Living representatives of fossilized tintinnids (Tappan and Loeblich, 1968, 1973) could be used to further calibrate the ciliate SSrRNA molecular clock. In addition, more divergence data from other ciliate endosymbionts are needed to independently test my notion that endosymbionts (i.e. of vertebrate animals) have a faster SSrRNA molecular clock than the free-living ciliates. This approach to establish a chronometer for the ciliates, based on host fossil record information, may serve as an important model to researchers who may want to estimate the origin of other major lineages of protozoa (e.g. the apicomplexans, dinoflagellates, haemoflagellates) based on fossil record information of their hosts.

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"Talking and eloquence are not the same:
to speak and to speak well are two things.
A fool may talk,
but a wise man speaks."

Johnson, year unknown.

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APPENDICES

"We are none of us infallible,
not even the youngest among us."

William H. Thompson, 1810-1886.

APPENDIX I
GEL ELECTROPHORESIS BUFFERS AND REAGENTS

Reagents and Solutions

20X TAE buffer

96.8 g Tris base (Tris[hydroxymethyl]aminomethane) pH 8.0
57.1 ml Glacial acetic acid
40.0 ml 0.5M Na_2EDTA (Disodium ethylenediaminetetraacetic acid) pH 8.0

Make to 1 litre with dH_2O.

20X TBE buffer

108.0 g Tris base (Tris[hydroxymethyl]aminomethane) pH 8.0
55.0 g Boric acid
40.0 ml 0.5M Na_2EDTA (Disodium ethylenediaminetetraacetic acid) pH 8.0

Make to 1 litre with dH_2O.

Stop Solution

0.3 % Bromophenol blue
0.3 % Xylene Cyanol FF
10.0 mM EDTA (pH 7.5)
97.5% deionized formamide

Ethidium Bromide (extreme mutagen wear gloves at all times)

1. Add 20 µl Ethidium bromide (EtBr) to 250 ml of dH_2O in a designated EtBr stain tray. Once tray is contaminated with EtBr, it should be labelled and used only for EtBr staining. Gently slide the gel off the tray and into the stain for 2-5 min. and agitate frequently.

2. Rinse gel in dH_2O for 5 min. and visualize under UV light.

APPENDIX II
CULTURING RUMEN CILIATES USING MEDIUM *M*

Reagents and Solutions

Mineral Mix M
6.00 g NaCl
0.20 g $MgSO_4$
0.26 g $CaCl_2*2H_2O$
2.90 g KH_2PO_4
Dissolve in 1.0 litre of distilled H_2O.

Food Substrate
1.5 g ground wheat and 1.0 g dried orchard grass

Make up to 100 ml with dH_2O, gas with CO_2 until reduced, and store 2 ml aliquots in test tubes at –20° C.

Rumen Fluid Supernatant
Take 200 ml of rumen fluid and strain through cheesecloth. Centrifuge filtered fluid at 200 x g for 10 min and pour off supernatant.

1.5% Sodium Acetate Solution
Dissolve 3.95 g CH_3COON_4 in 250 ml H2O.

3% Cysteine HCl
Dissolve 1.5 g cysteine in 50 ml HCl.

6% $NaHCO_3$
Dissolve 15.0 g $NaHCO_3$ in 250 ml distilled H_2O.

Basal Medium M
150 ml Mineral mix M
30 ml Rumen fluid supernatant
15 ml 1.5% CH_3COON
78 ml Distilled H_2O

Protocol

1. Gas with 10% CO_2 and add 25 ml of 6% $NaHCO_3$.
2. Continue gassing until fairly well reduced and add 2.0 ml of cysteine HCl.
3. Continue gassing and check pH (adjust to pH 6.6).
4. Anaerobically transfer 10 ml into 30 ml test tubes and autoclave.

APPENDIX III
CULTURING RUMEN CILIATES USING MEDIUM *C*

SP-1 Mineral Mix

20.0 g K_2HPO_4 in 1.0 litre of distilled H_2O.

SP-2 Mineral Mix

16.0 g KH_2PO_4
4.0 g NaCl
0.212 g $CaCl_2*2H_20$
0.154 g $MgSO_4$

Dissolve in 1.0 litre of distilled H_2O.

Basal Medium C

90.0 ml Mineral Mix SP-1
90.0 ml Mineral Mix SP-2
30.0 ml Rumen Fluid Supernatant (see Appendix II)
71.5 ml Distilled H_2O
15.0 ml 1.5% CH_3COON_4 (see Appendix II)

Protocol

1. Gas with 10% CO_2 and add 1.5 ml of 6% $NaHCO_3$.
2. Continue gassing until fairly well reduced.
3. Add 2.0 ml of 3% cysteine HCl.
4. Continue gassing and check pH (adjust to pH 6.6).
5. Anaerobically transfer 10 ml into 30 ml test tubes and autoclave.

APPENDIX IV
ROUTINE Extraction Of Ciliate DNA

This protocol uses the non-ionic detergent sodium dodecyl sulfate (SDS) to complex proteins. Ideal for routine DNA extractions.

Protocol

1. Harvest cells in a 1.5-ml microfuge tube by centrifugation for 5 min. at 5,000 rpm.

2. Pour off culture media, or alcohol fixative, and resuspend pellet in 300 μl of STE buffer (see next page).

3. Centrifuge for 5 min. at 5,000 rpm and discard supernatant.

4. Resuspend pellet in 300 μl STE and add 0.05 vol of 20% SDS (1% SDS final conc)

5. Vortex well and store on ice for 2-5 min. (check lysed cells under a microscope).

6. Add an equal volume of buffer-saturated phenol and chloroform:IAA. Vortex to emulsify phases and store on ice for 2-5 min.

7. Centrifuge for 5 min. at 5,000 rpm. If the interface is very cloudy, repeat this step.

8. Transfer top phase to a sterile 1.5 ml microcentrifuge tube and add an equal volume of chloroform:IAA and vortex to emulsify liquids.

9. Separate phases by centrifugation for 1 minute at 7,000 rpm.

10. Transfer top phase to a sterile 1.5 ml microcentrifuge tube and add 0.1 volumes of 2 M $NaC_2H_3O_2$ and 2.0 volumes of 100% ethanol for at least 1.0 hr. at –20° C.

11. Centrifuge for 10 min. at 12,000 rpm and remove supernatant without dislodging pellet.

12. Wash pellet in 400 μl of 70% ethanol for 20 min. at –20° C.

13. Centrifuge for 2 min. at 12,000 rpm and carefully invert microcentrifuge tube to air dry for 15-30 min. on several clean kim wipes. Pellet can also be lyophilized in a speed vacuum for < 5 min.

14. Resuspend pellet in 50 μl of ddH_20. The pellet will contain both DNA and RNA and will be referred to as bulk nucleic acids (BNA).

Reagents and Solutions

STE Buffer	Final Concentration
2.5 ml 2 M Tris-base (pH 7.5)	10 mM
12.5 ml 4 M NaCl (sodium chloride)	100 mM
2.5 ml 200 mM EDTA (pH 7.5)	1 mM

Add 482.5 ml dH_2O to make 500 ml and AUTOCLAVE

APPENDIX V
CTAB EXTRACTION OF RUMEN CILIATE DNA

This protocol uses the non-ionic detergent cetyltrimethylammonium bromide (CTAB) to complex polysaccharides and residual proteins. Ideal for rumen protozoa, bacteria, and plants.

Protocol

1. Pellet cells in a 1.5-ml microcentrifuge tube by centrifugation at 6,000 *g* for 2 min.
2. Resuspended cells in 500 μl TE buffer (see next page).
3. Add 30 μl proteinase K of (20 mg/ml) and incubate for 1 h at 37° C.
4. Following incubation, add 140 μl of 5 M NaCl mix thoroughly.
5. Then add 80 μl of CTAB/NaCl (see next page), mix the lysate completely by inverting the microcentrifuge tube several times and incubate for 10 min at 65° C.
6. Add an equal volume of chloroform, containing isoamyl alcohol (24:1) to the lysate and mix well to extract CTAB from the solution.
7. Centrifuge at 7,000 *g* for 5 min and remove the aqueous phase to a new microfuge tube.
8. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), mix, and centrifuge at 7,000 *g* for 5 min.
9. Transfer the aqueous phase to a new microfuge tube with an equal volume of chloroform/isoamyl alcohol, mix, and centrifuge at 7,000 *g* for 2 min.
10. Remove the aqueous phase and precipitate the DNA with 0.6 volumes of isopropanol at room temp for 5 min.
11. Collect DNA by centrifugation at 14,000 *g* for 10 min and wash the nucleic acid pellet with 70% ice-cold ethanol.
12. Air dry the pellet and resuspend in 50 ml of dH_2O.

Reagents and Solutions

CTAB

2% (w/v) of CTAB
100 mM Tris-Cl pH 8.0
20 mM EDTA pH 8.0,
1.4 M NaCl

TE Buffer	Final Concentration
1.0 ml 1. M Tris-base pH 8.0	10 mM
1.0 ml 0.1 M EDTA pH 8.0	1 mM

Add 98.0 ml ddH_2O to make 100 ml total volume.

APPENDIX VI
EXTRACTION OF CILIATE DNA FROM FORMALIN-FIXED CELLS

This protocol primarily uses proteinase K, long incubations, and heat (48 °C) to digest cells that have been fixed with either formalin or ethanol.

Protocol

1. Pellet fixed-cells in a 1.5-ml microfuge tube by centrifugation at 14,000 rpm for 2 min.

2. Resuspended cells in 1 ml of dH_20 and repeat steps 1 and 2.

3. Resuspended cells in 570 μl of dH_20 and store at 4° C for 24 h.

4. Add 30 μl proteinase K of (20 mg/ml) and incubate for 1 h at 37° C, then repeat step1

5. Resuspend cells in 500 μl of digestion buffer (see next page) and add proteinase K to a final concentration of 0.1-10 mg/ml (proteinase K concentration depends on the duration of cell fixation – longer the fixation, use more proteinase K.).

6. Incubate at 48° C for 2 to 5 days (this will depend on the number and size of cells and the length of fixation). **NOTE**: It is the slower released higher molecular weight DNA that is desired. After 24 h, a second aliquot of proteinase K can be added.

7. Centrifuge lysate at 14,000 rpm and transfer aqueous layer to a new 1.5 ml microfuge tube.

8. Incubate sample containing DNA/RNA at 95° C for 7 min to inactivate proteinase K.

9. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), mix, and centrifuge at 10,000 rpm for 5 min.

10. Transfer top phase to a sterile 1.5 ml tube and add 0.1 vol of 2 M $NaC_2H_3O_2$.

11. Add 2.0 volumes of 100% ethanol and store for at least 1.0 hr. at -20° C.

12. Spin for 10 min. at 14,000 rpm and remove supernatant without dislodging pellet.

13. Wash pellet in 400 μl of 70% ethanol for 20 min. at -20° C.

14. Centrifuge for 10 min. at 14,000 rpm and carefully invert microcentrifuge tube to air dry for 15 min. on several clean kim wipes. Pellet can also be lyophilized in a speed vacuum for < 5 min. Resuspend pellet in 50 ml of dH_2O.

Reagents and Solutions

Digestion Buffer For Fixed Cells

50 mM Tris pH 7.5
1 mM EDTA
0.5 % Tween 20
0.1–10 mg/ml Proteinase K

APPENDIX VII
QUANTIFYING NUCLEIC ACIDS

1. Remove 10 μl of BNA for spectrophotometer use and add to 990 μl TE buffer (1:100 dilution) in a clean cuvette. Measure against a 1 ml blank of TE buffer.

2. Scan the samples to determine the absorbency at A_{260} and A_{280} nm to calculate the quality and quantity of yield.

A_{260}/A_{280} gives a good estimate of nucleic acid quality
(A value circa 2.0 is a clean yield).

In addition, one $O.D_{260}$ unit = 50 μg of BNA.

$$\frac{(O.D_{260} \text{ units} * \text{dilution rate} * 50\ \mu g\ BNA)}{1{,}000} = BNA/\mu l$$

3. To ascertain the total amount of BNA present, multiply by the total volume of BNA harvested.

4. Depending on the amount of BNA harvested, DNA can be diluted to a working concentration of 50 ng/μl for PCR applications. The remaining BNA can be can be stored in 0.1 volumes of 2 M $NaC_2H_3O_2$ and 2 volumes of 100% ethanol at –20° C for future use.

APPENDIX VIII
CALCULATING PRIMER CONCENTRATIONS

Amount of primer needed to equal 10 pmol

primer length	ng of primer equals to 10 pmol.
15 mer	50 ng
16 mer	53 ng
17 mer	56 ng
18 mer	59 ng
19 mer	63 ng
20 mer	66 ng
24 mer	80 ng

Note: 10 μM/ml = 10 pmols/μl.

How to calculate amount of primer in pmols from nanograms.

- For example, we have 236.0 ug/ml of primer 690F and we want to have a working concentration of 2 pmol/μl for sequencing.
- First, we convert 236.0 ug/ml to nanograms by multiplying by 1,000 to get 236,000 ng/ml).
- Then we determine the ng of primer required to equal 10 pmol from the table above and divide this into the concentration of the primer in ng.
- 690F is 18 mer **(see Table 2.3)** which is 59 ng/10 pmol. Thus, (236,000 ng/ml) / (59 ng/10 pmol) equals 40,000 pmols/ml.
- Next, convert to pmols/μl by dividing by 1,000 to get 40.0 pmols/μl
- Then, dilute 20× with dH_2O to have a final concentration of 2 pmol//μl

How to calculate amount of primer in pmols from nanomoles.

- For example, we have 55 nmoles of a new primer and we want have a working concentration of 10 μM for PCR
- First we multiply 55 nmoles by 10 to determine the amount of dH_2O needed to have a concentration of 100 μM.
- Then, we dilute 10× with dH_2O to have a final concentration of 10 μM.

APPENDIX IX
POLYMERASE CHAIN REACTION (PCR)

PCR Amplification Protocol

1. Use the following DNA conc. as templates: 50 ng, 100 ng, 200 ng, and 500 ng.

2. Combine template with the following in a 0.6 ml ultra-thin microcentrifuge tube:

   10 µl 10X PCR Buffer (final conc. = 1X)
   10 µl 5' Medlin (1988) PCR primer (final conc. = 1 µM)
   10 µl 3' Medlin (1988) PCR primer (final conc. = 1 µM)
   10 µl dNTP mix (final conc = 250 µM)
   4 µl 50 M $MgCl_2$ buffer (final conc = 2 mM).

3. Add autoclaved or 0.1 filtered sterilized H_2O until the final volume of the PCR reaction mixture is 100 µl and mix well.

4. Dilute *Taq* DNA polymerase to 1 unit/µl with 1X PCR buffer, mix well, and add 1 µl of *Taq* to each reaction.

5. Centrifuge the reactions for 5 seconds at 14,000 rpm to collect material from the side of the microfuge tubes.

6. Place in a Thermal Cycler for 34 cycles under the following PCR parameters:

Standard Parameters		**Stringent Parameters**	
Denaturation:	94° C for 30 seconds.	Denaturation:	94° C for 30 seconds
Annealing:	55° C for 30 seconds.	Annealing:	65° C for 30 seconds
Extension:	72° C for 90 seconds.	Extension:	72° C for 90 seconds

The completion of the denaturation, annealing, and extension parameters constitutes 1 cycle.

7. After the 34th cycle, an extra cycle with a 3:00 min extension is used to ensure elongation of newly synthesized strands. The samples are then held at 4° C.

8. Check 10 µl aliquots from each PCR reaction by agarose gel electrophoresis.

9. The remaining successful PCR reaction (≈ 90 µl) can be purified for subsequent analyses.

APPENDIX X
PREPARATION OF COMPETENT CELLS

Protocol.

1. Inoculate 10 ml of LB broth (see Appendix XI) from a glycerol stock (or single colony) of *E. coli* XL1-Blue (or other strain), agitate culture, and grow overnight at 37° C.

2. Inoculate 500 ml of LB broth with 5 ml of the overnight culture, agitate flask and incubate at 37° C. Occasionally, aseptically remove 1 ml and scan using spectrophotometry at A_{600}. Grow cells until the culture reaches a density between 0.4 - 0.8 at $O.D_{600}$ (0.5 is best).

3. Centrifuge cells at 5,000 rpm for 5 min. in a 250 ml centrifuge tube. Resuspend the large pellet in 100 ml of cold 100 mM $MgCl_2$ and divide slush into two 50 ml centrifuge tubes.

4. Centrifuge at 5,000 rpm for 5 min, then wash pellets in cold 0.1 M $MgCl_2$. Cells are very sensitive at this stage so do not vortex (gently tap the side of the tube).

5. Centrifuge at 5,000 rpm for 5 min and resuspend each pellet in 7 ml of cold 0.1 M $MgCl_2$ and incubate on ice for 1 hr. to overnight. **Note:** Overnight works better for *E. coli* XL1-Blue and 1 hr. for *E. coli* DH5αF'.

6. Add 3 ml of autoclaved 50% glycerol in 50 mM $CaCl_2$ to each tube and gently mix.

7. Aliquot 400 μl into pre-chilled microcentrifuge tubes and drop into liquid nitrogen and store at -80° C. (cells will remain competent and keep for at least a year, if not thawed and frozen repeatedly.

APPENDIX XI
CLONING PCR PRODUCTS INTO PLASMID VECTORS USING PHARMACIA'S SURECLONE® LIGATION KIT

This protocol was modified from the Pharmacia Biotech SureClone® Ligation (cat # 27-9300-01) procedure.

Protocol.

1. Set-up the blunting/kinasing reaction by adding the following reagents to a microcentrifuge tube:

   300-400 ng of SSrRNA gene (1-16 μl)
   1 μl Klenow fragment
   2 μl 10X blunting/kinase buffer
   1 μl Polynucleotide kinase
   up to a final volume of 20 μl with sterile dH_2O

2. Mix the reagents gently, centrifuge at 10,000 rpm for 3 seconds, and incubate in a water bath at 37° C for 30 min.

3. Add 20 μl of phenol/chloroform, vortex for 5 sec, then centrifuge for 1 min at 10,000 rpm before collecting the upper aqueous layer.

4. Add 500 μl of resuspended Sephacryl® S-200 resin to a MicroSpin Column (with bottom closure snapped off) and place inside of a 1.5 ml microcentrifuge tube with its lid removed (snap lid can be cut with a pair of scissors).

5. Centrifuge for at full speed for 30 sec, discard the effluent, and place the MicroSpin Column containing the Sephacryl® resin into a new microcentrifuge tube with its lid removed.

6. Slowly apply the aqueous material from the blunting/kinase procedure to the centre of the resin and centrifuge again at full speed for 30 sec.

7. Discard the MicroSpin Column and save the effluent from the 1.5 ml microfuge tube.

8. Use this effluent to ligate into a dephosphorylated and blunt-ended pUC18 vector by adding the following components:

5 µl of effluent
1 µl of 50 ng of dephosphorylated vector
10 µl of 2X ligation buffer
1 µl of DTT solution
1 µl of T4 DNA ligase
2 µl of sterile dH_2O

9. Mix gently, centrifuge for 5 sec, and incubate at 16° C for 2 h.

10. Add 20 µl of this ligated material to 200 µl of *E. coli* XL1-blue competent cells and incubate on ice for 20 min.

11. Heat shock cells for 45 seconds at 42° C, then return quickly to ice for 5 min.

12. Add 1 ml LB broth and incubate at 37° C for 60 min.

13. Spread 200 µl, 400 µl, and 700 µl of transformation mixture onto 1.2% LB-Ampicillin-Xgal-IPTG (LBAXI) (see next page for recipe) agar plates, invert, and incubate at 37° C for 14-16 h.

14. Remove the plates with the transformed colonies and store at 4° C for several h. to increase the intensity of blue negative colonies (24-48 h is best).

Reagents and Solutions

10X Ampicillin Solution
Dissolve 100 mg of Ampicillin into 100 ml of LB broth. No need to autoclave as this is 10X recommended strength. Keep frozen at –20° C.

Xgal/IPTG (5:1) Solution
Dissolve 100 mg of Xgal (5-bromo-4-chloro-3-indolyl galactopyranoside) with 5 ml of dimethylformamide (20 mg/ml).

Dissolve 200 mg of IPTG (Isopropyl thio-β-D-galactoside) with 1.0 ml autoclave ddH_2O (200 mg/ml).

Mix 5 ml of Xgal solution with 1 ml of IPTG and store at -20 ^{o}C.

LB Broth
Heat 300 ml of ddH_2O and when hot add the following:

4.0 g NaCl
2.0 g Yeast Extract
4.0 g Bacto-Tryptone

Bring to 400 ml with ddH_2O and aliquot 9 ml into 40 test tubes. Autoclave and store at 4° C.

1.2% LBAXI Agar
Heat 500 ml of ddH_2O and when hot add the following:

10.0 g NaCl
5.0 g Yeast Extract
10.0 g Bacto-Tryptone
12.0 g Bacto-Agar

Bring to 1 litre with ddH_2O and autoclave. When cool enough to touch against wrist, add 100 mg Ampicillin (Sigma) and 1 ml of Xgal:IPTG (5:1) solution. Swirl the contents and pour into (25-35) petri dishes.

APPENDIX XII
CLONE CONFIRMATION PROTOCOL USING PLASMID CRACKING BUFFER

Protocol.

1. Use the blunt end of an autoclaved tooth pick to gently touch the centre of a single white colony and make three streaks 1-2 cm long onto a new 1.2% LBAXI agar plate. Repeat step using a new tooth pick for each white colony, then incubate at 37° C for 12-16 h.

2. Remove LBAXI agar plates and check triple streaks for blue colouration. Disregard blue steaks and number only those sets of triple streaks which are white.

3. For each group of white strikes, use the blunt end of an autoclaved toothpick to collect cells from one of the three streaks.

4. Stick toothpick and cells into 15 µl of dH_2O in a 0.6 ml microfuge tube. Twist tooth pick in the dH_2O, to dislodge bacterial cells and discard the tooth pick into a biohazardous waste autoclave bag. **Note**: water should turn cloudy.

5. Add 15 µl of 2X Plasmid Cracking buffer (see next page for recipe) and vortex. **Note**: mixture should turn clear.

6. Add 30 µl of dH_2O to the 1X Plasmid Cracking buffer and run 30 µl on a 0.75% agarose gel to check for plasmids containing the SSrRNA insert. **Note**: pUC18 is 2.7 kb and the litostome SSrRNA is ≈1.6 kb. Therefore, the recombinant plasmid should be 4.3 kb. **Note**: supercoiled DNA will migrate like a 2.2 kb fragment.

7. Aseptically remove one of the two remaining triple streaks from each positive clone, using an inoculation loop, and inoculate test tubes containing 9 ml of LB broth and 1 ml of 10X ampicillin solution.

8. Agitate tubes overnight on a platform shaker in an incubator at 37° C for 16-20 h.

Reagents and Solutions

2X Plasmid Cracking Buffer

0.5 ml 10N NaOH
1.0 ml 0.5 M Na_2EDTA pH 8.0
2.5 ml 20% SDS
5.0 ml Glycerol
41.0 ml dH_2O

APPENDIX XIII
PLASMID DNA PURIFICATION USING SPEED PREP AND GENE CLEAN®

This modified protocol of the gene clean procedure provides a quick and easy way to obtain ultrapure DNA (plasmid or PCR product) for automated DNA sequencing.

Protocol

1. Grow 10 ml of culture containing positive transformed colonies overnight at 37° C.

2. Aliquot overnight culture into three 1.5 ml microfuge tubes and collect cells by centrifugation at full speed for 30 sec.

3. Discard broth and again add overnight culture to the same three tubes Repeat step 2.

4. Resuspend pellets in the last remaining bit (≈10 µl) of LB broth by vortexing, and add 200 µl of speed prep mix to the cells.

5. Vortex for 5 sec, then add 200 µl of phenol/chloroform and vortex for another 5 sec..

6. Centrifuge for 2 min at full speed, then remove upper phase to a new microfuge tube and add 400 µl of ice-cold 100% ethanol

7. Mix by vortexing, centrifuge for 5 min at full speed, then discard supernatant and rinse pellet with 1 ml of 70% ethanol.

8. Centrifuge for 5 min at full speed, air dry the pellet for 10 min, and then dissolve the pellet in 20 µl of dH_2O.

9. Divide the product into two samples and run on an agarose gel for 1 hour. **Note**: PCR products can also be run on a gel and processed in the same way using this modified Gene Clean® protocol listed below:

10. Briefly stain with ethidium bromide and visualise the gel under long-wave UV light. Excise correct size DNA band from gel using a razor blade. **Note**: To minimise damage to DNA, it is important to use long-wave UV for as short a time as possible, i.e. 2 min).

11. Using forceps, place up to three gel slices into a 1.5 ml microfuge tube, add three times (≈ 700 µl) the volume of NaI solution, and incubate for 6 min in a 55° C water bath (inverting every 2 min). **Note**: If the gel slice has not completely dissolved, return to the water bath for another 5 min.

12. Resuspend Glassmilk® vigorously until completely suspended, then add 7 µl of Glassmilk® to the NaI solution.

13. Place on ice for 10 min and mix by inverting the sample every 1-2 min. Then centrifuge sample at full speed for only 5 sec and remove the NaI solution.

14. Centrifuge again at full speed for 5 sec and remove the remaining NaI solution with a small bore pipette.

15. Resuspend the pellet in 700 µl of NEW WASH solution by pipetting back and forth while digging into the pellet with the pipette tip and centrifuge at full speed for 5 sec. Repeat this step **three** times.

16. After the third wash, centrifuge the sample again at full speed for 5 sec and remove the remaining NEW WASH solution with a small bore pipette.

17. Completely dissolve the pellet with 15 µl of dH_2O and incubate at 55° C for 3 min.

18. Centrifuge at full speed for 30 sec and carefully collect the clear supernatant containing the eluted DNA and place in a new microfuge tube. DNA is now ultraclean and ready for sequencing.

Reagents and Solutions

Speed Prep Mix	Final Concentration
2.5 ml of 1 M Tris-HCl pH 8.0	50.0 mM
2.0 ml of 4% Triton-X 100	
25.0 ml of 5 M LiCl	2.5 M
6.3 ml of 0.5 M EDTA	62.3 mM
14.3 ml of ddH_2O	

APPENDIX XIV
PLASMID DNA PURIFICATION USING PEG8000

Protocol (modified from Lee and Rasheed, 1990).

1. Fill six 1.5 ml microcentrifuge tubes from a 10 ml overnight culture of confirmed positive cells and harvest cells by centrifugation at 5,000 rpm for 2 min.

2. Discard supernatant and resuspend cells in 200 μl of Solution (Soln) #1 (see next pg for recipe). Hold at room temp. for 5 min.

3. Add 400 μl of Soln. #2 (recipe next page), vortex, and incubate on ice for 10 min.

4. Add 300 μl of Soln. #3 (recipe next page), vortex, and incubate on ice for 10 min.

5. Centrifuge tubes at 14,000 rpm for 10 min. Transfer supernatant to a new 1.5 ml microcentrifuge tube and add 0.6 volumes of isopropanol.

6. Incubate at room temp. for 5 min, then centrifuge for 10 min. at 14,000 rpm.

7. Discard the supernatant, invert the tubes, and air dry the pellet until dry.

8. Resuspend the pellet in 100 μl of dH_2O, then add 50 μl of Solution #3.

9. Mix by inverting the tubes and incubate on ice for 5 min.

10. Centrifuge mixture at 14,000 rpm for 10 min. and transfer supernatant to a 1.5 ml tube.

11. Add 400 μl of 95% ethanol and precipitate on ice for 10 min.

12. Centrifuge mixture at 14,000 rpm for 10 min., discard supernatant, and air dry pellet for 15-20 min.

13. Dissolve pellet in 32 μl of dH_2O and add 8 μl of 4 M NaCl and 40 μl of autoclaved 13% PEG8000.

14. After a thorough mixing, incubate sample on ice for 20 min and then pellet plasmid DNA by centrifugation for 15 min at 14,000.

15. Wash pellet in 70% ethanol, centrifuge again, and air dry the pellet for 20 min before dissolving in 20 μl dH_2O. Check product on an agarose gel.

Reagents and Solutions

Solution #1	Final Concentrations
0.45 g $C_6H_{12}O_6$ (Glucose)	50 mM
1.25 ml 1 M Tris-Base (pH 8.0)	25 mM
5.00 ml 0.1 M EDTA (pH 8.0)	10 mM

Make up to 50 ml by adding ddH_20 and autoclave

Solution #2 (Lysis solution):	Final Concentration
2.5 ml 20% SDS	1%
5.0 ml 2N NaOH	0.2 N

Add NaOH last and make up to 50 ml by adding ddH_20 and autoclave

SDS may precipitate when cold and turn solution white. If this happens heat solution until solution becomes clear.

Solution #3 (7.5 M $NH_4C_2H_3O_2$) pH 7.6

dissolve 57.81 g of $NH_4C_2H_3O_2$ into 50 ml dH_20, pH to 7.6, and add dH_20 until the final volume is 100 ml and autoclave.

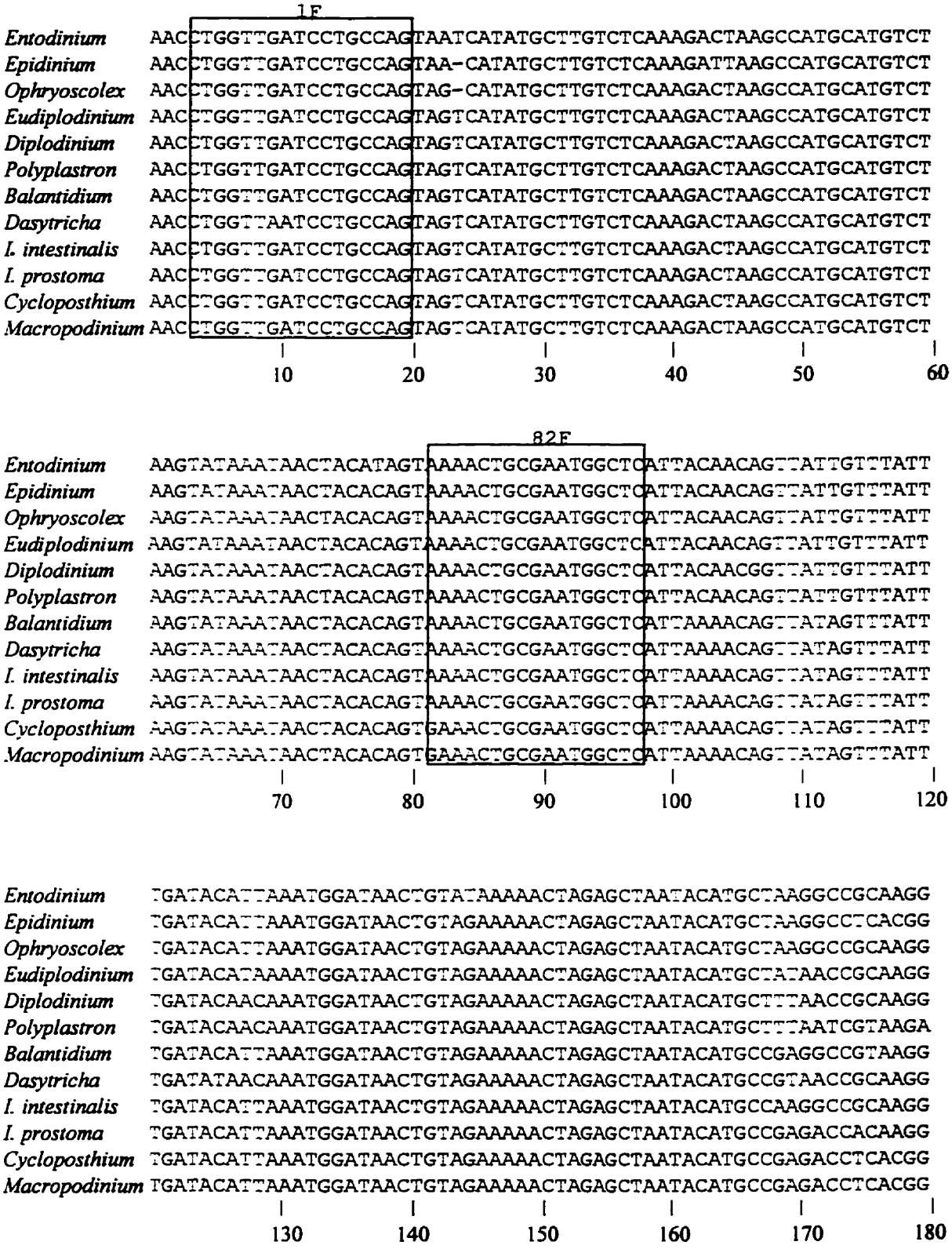
APPENDIX XV
SSrRNA ALIGNMENT POSITIONS (DCSE NUMBERING) FOR PHYLOGENETIC ANALYSES WITHIN THE PHYLUM CILIOPHORA

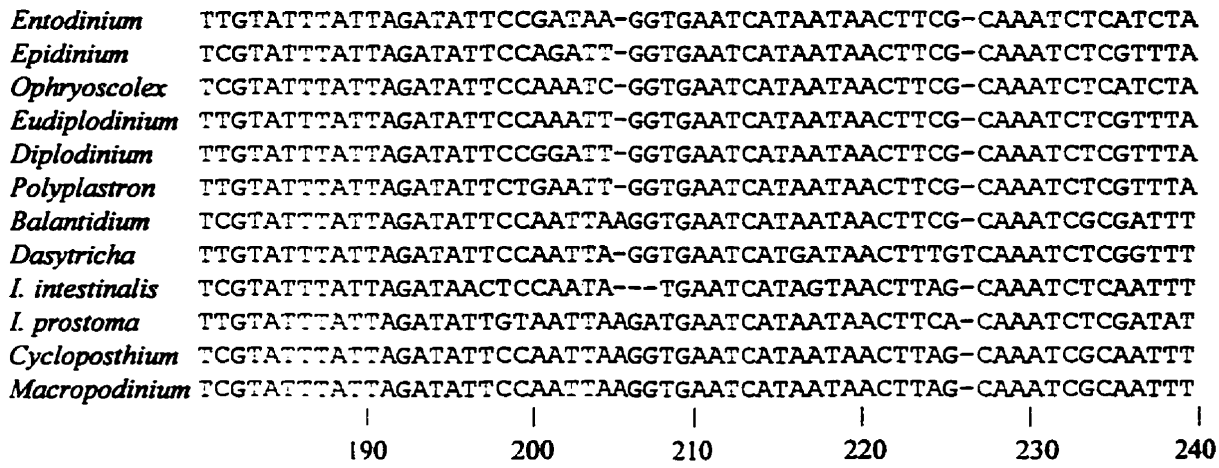
107-139	909-929	1851-1856
141-179	931-945	1858-1867
181-189	950-958	1870-1874
194-247	1004-1009	1876-1885
273-285	1059-1071	1887-1908
287-301	1079-1092	1910-1945
303-321	1098-1106	1947-1993
323-325	1150-1171	1995-2022
328-338	1173-1178	2024-2050
363-366	1181-1187	2056-2073
386-425	1195-1213	2075-2113
442-445	1215-1240	2115-2117
467-496	1250-1253	2119-2152
499-513	1262-1265	2305-2325
522-524	1267-1298	2327-2342
532-546	1311-1314	2345-2351
549-552	1318-1336	2355-2358
554-564	1343-1359	2360-2400
567-604	1469-1486	2402-2444
607-719	1499-1520	2508-2556
725-797	1522-1572	2558-2590
800-803	1576-1592	2592-2644
807-811	1594-1602	2646-2702
813-817	1604-1651	2704-2723
819-864	1653-1676	2737-2741
866-891	1678-1688	2766-2814
893-897	1759-1767	2816-2830
900-906	1840-1849	2832-2857

APPENDIX XVI
SSrRNA ALIGNMENT POSITIONS (DCSE NUMBERING) FOR PHYLOGENETIC ANALYSES WITHIN THE CLASS LITOSTOMATEA

107-138	622-641	1195-1203	1910-1945
141-178	643-683	1205-1213	1947-1993
181-189	685-719	1215-1240	1995-2022
194-195	725-758	1250-1253	2024-2050
197-201	763-765	1262-1264	2056-2073
203-212	767-769	1267-1298	2075-2113
214-244	771-773	1310-1314	2115-2117
273-285	776-797	1318-1322	2119-2152
287-293	800-803	1327-1336	2305-2325
295-301	807-811	1346-1350	2327-2337
303-325	813-817	1353-1359	2339-2341
328-338	819-837	1469-1486	2345-2351
363-365	839-840	1499-1520	2355-2358
386-393	842-859	1522-1572	2360-2400
395-414	861-864	1576-1592	2402-2444
416-425	866-891	1594-1602	2511-2536
472-496	893-897	1604-1651	2538-2543
499-502	900-906	1653-1676	2547-2556
504-511	909-929	1678-1687	2558-2590
532-534	931-945	1759-1766	2592-2644
536-539	950-957	1840-1849	2646-2702
541-542	1004-1008	1851-1856	2705-2717
544-546	1060-1063	1858-1867	2738-2741
549-552	1065-1071	1870-1874	2770-2792
554-564	1084-1092	1876-1885	2794-2814
567-604	1098-1103	1887-1900	2816-2830
607-620	1150-1161	1902-1908	2832-2857

APPENDIX XVII
SSrRNA ALIGNMENT OF VERTEBRATE CILIATE ENDOSYMBIONTS

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Dasytricha TTGTATTTATTAGATATTCCAATTA-GGTGAATCATGATAACTTTGTCAAATCTCGGTTT
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I. prostoma TTGTATTTATTAGATATTGTAATTAAGATGAATCATAATAACTTCA-CAAATCTCGATAT
Cycloposthium TCGTATTTATTAGATATTCCAATTAAGGTGAATCATAATAACTTAG-CAAATCGCAATTT
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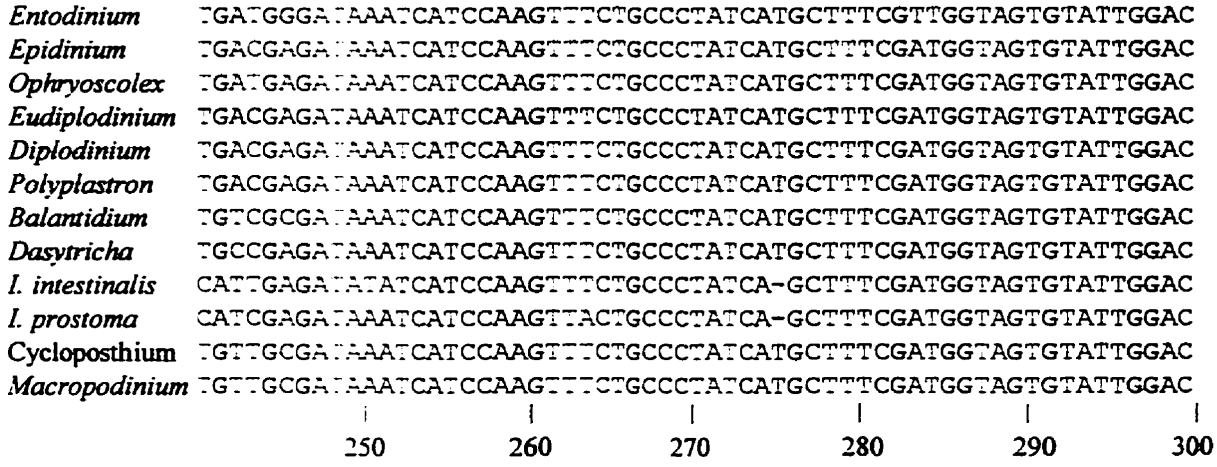
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Dasytricha TGCCGAGATAAATCATCCAAGTTTCTGCCCTATCATGCTTTCGATGGTAGTGTATTGGAC
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I. prostoma CATCGAGATAAATCATCCAAGTTACTGCCCTATCA-GCTTTCGATGGTAGTGTATTGGAC
Cycloposthium TGTTGCGATAAATCATCCAAGTTTCTGCCCTATCATGCTTTCGATGGTAGTGTATTGGAC
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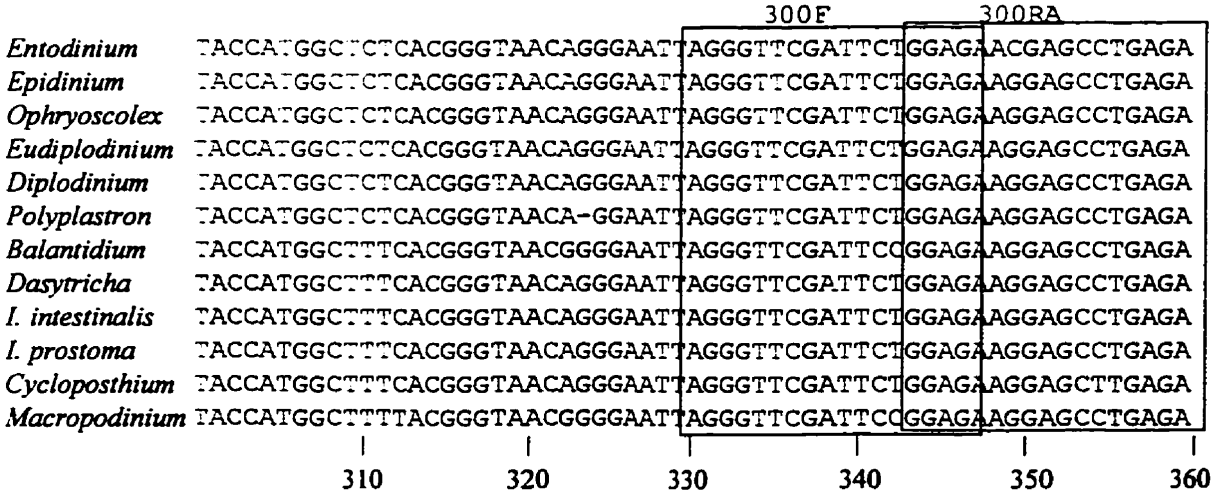
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300RA
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Ophryoscolex TACCATGGCTCTCACGGGTAACAGGGAATTAGGGTTCGATTCTGGAGAAGGAGCCTGAGA
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Balantidium TACCATGGCTTTCACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAAGGAGCCTGAGA
Dasytricha TACCATGGCTTTCACGGGTAACAGGGAATTAGGGTTCGATTCTGGAGAAGGAGCCTGAGA
I. intestinalis TACCATGGCTTTCACGGGTAACAGGGAATTAGGGTTCGATTCTGGAGAAGGAGCCTGAGA
I. prostoma TACCATGGCTTTCACGGGTAACAGGGAATTAGGGTTCGATTCTGGAGAAGGAGCCTGAGA
Cycloposthium TACCATGGCTTTCACGGGTAACAGGGAATTAGGGTTCGATTCTGGAGAAGGAGCTTGAGA
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310
320
330
340
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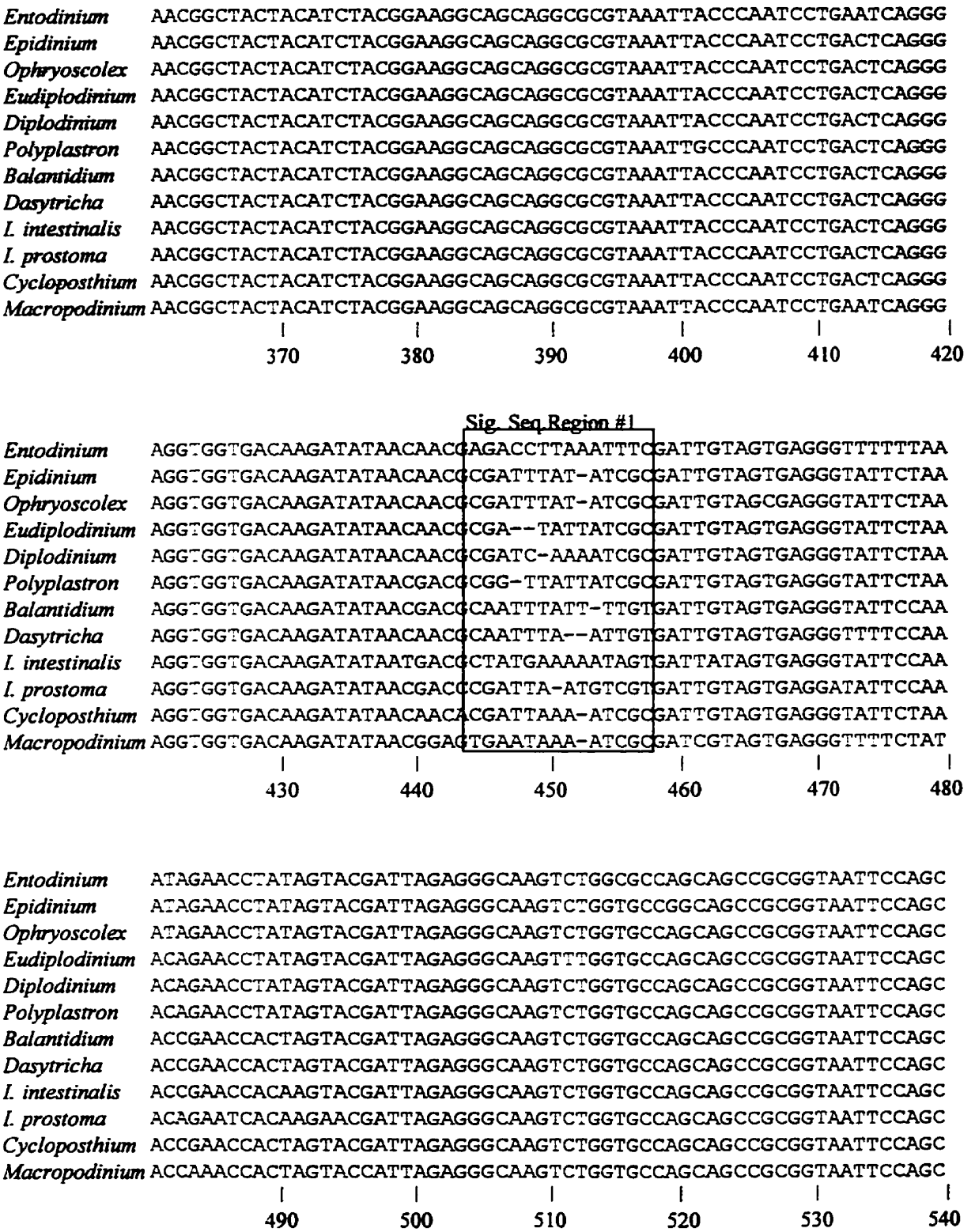
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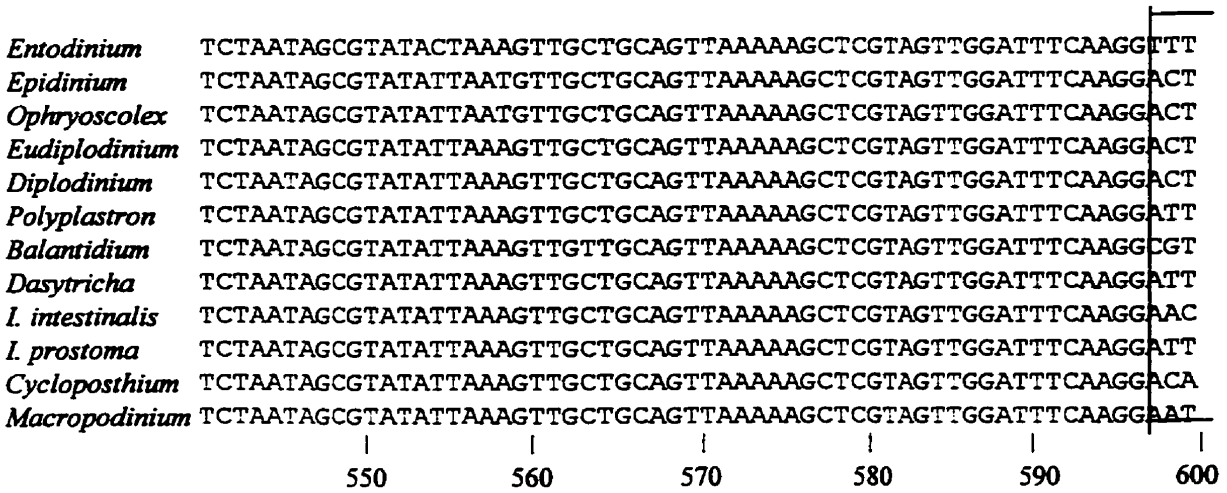
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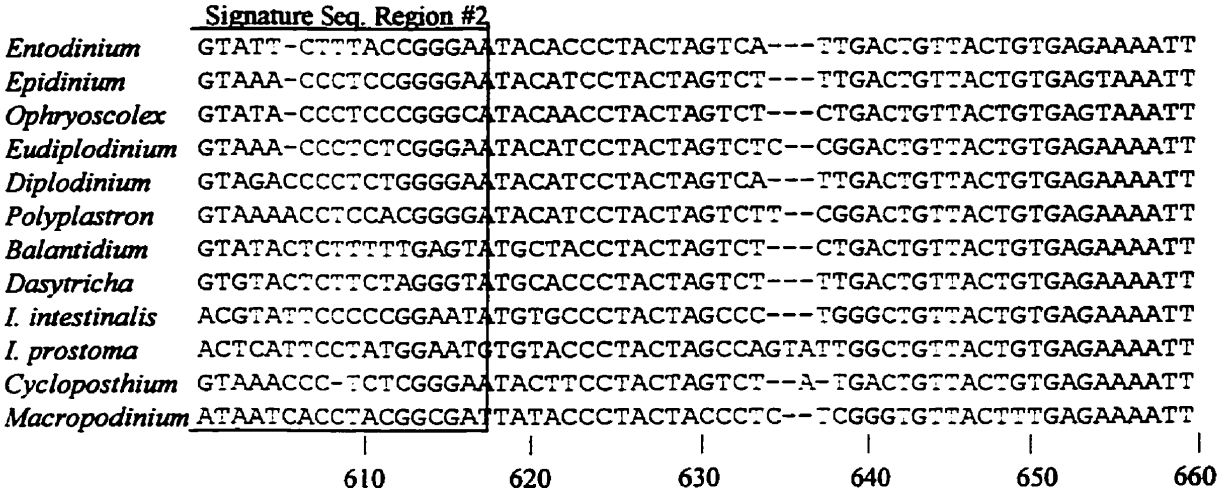
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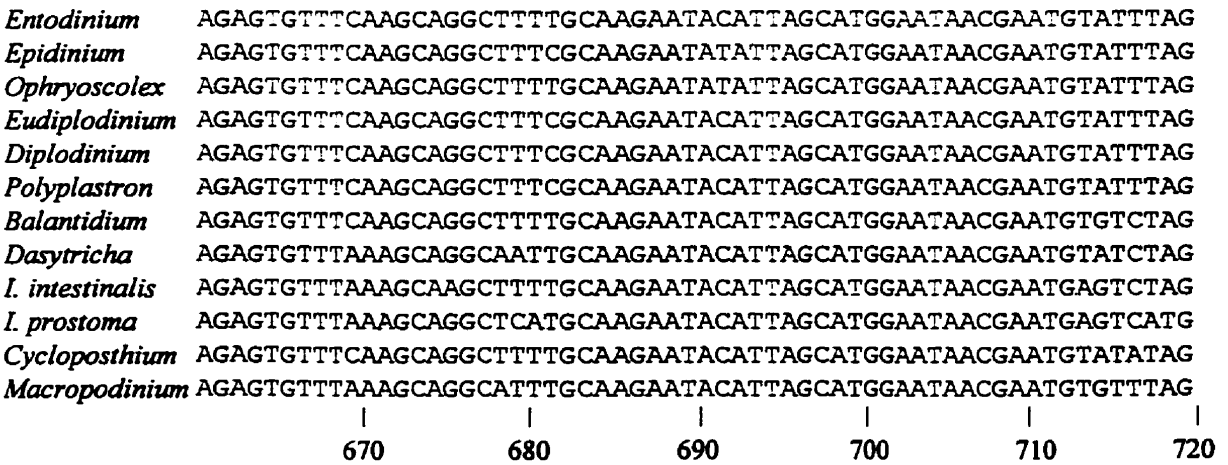
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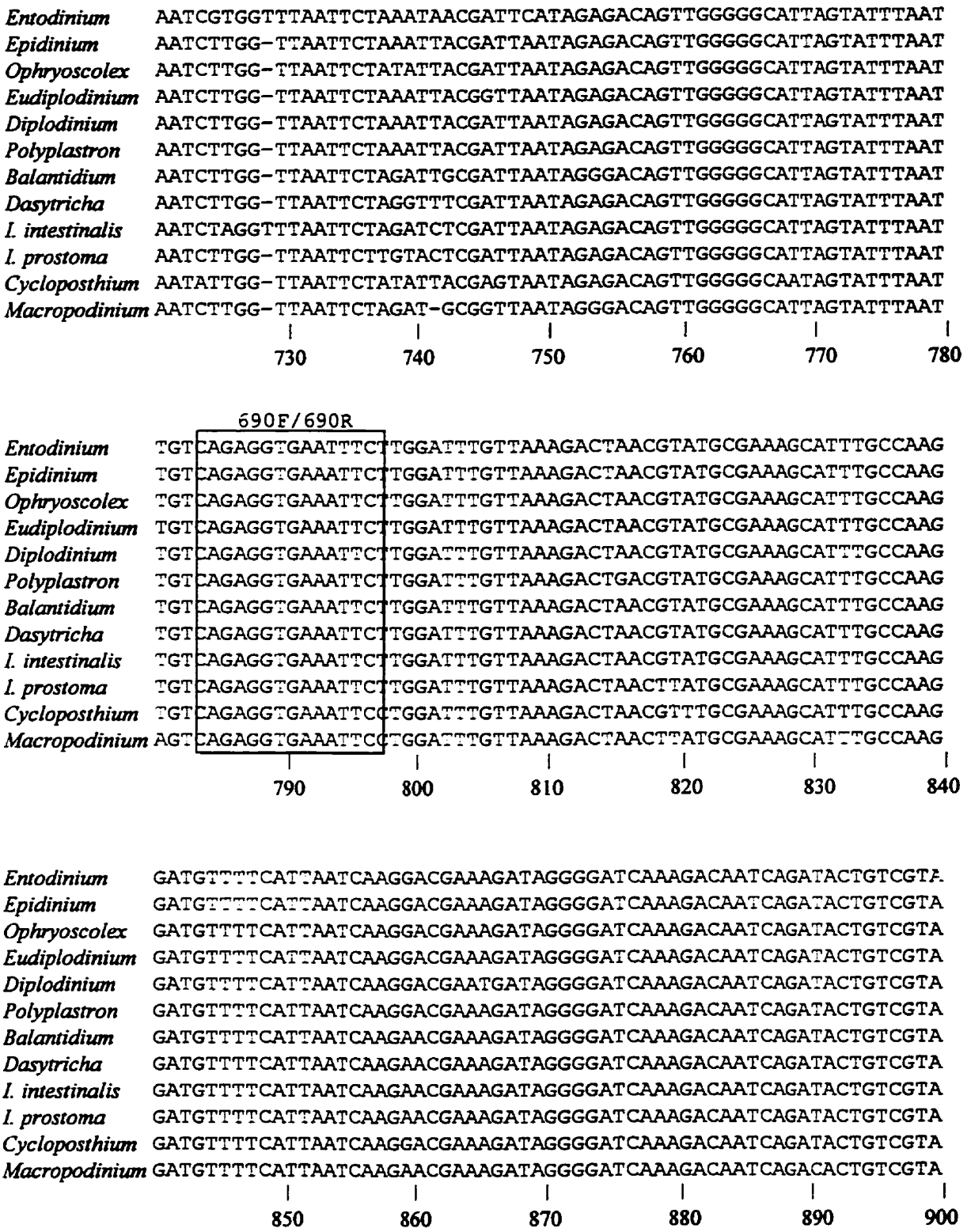
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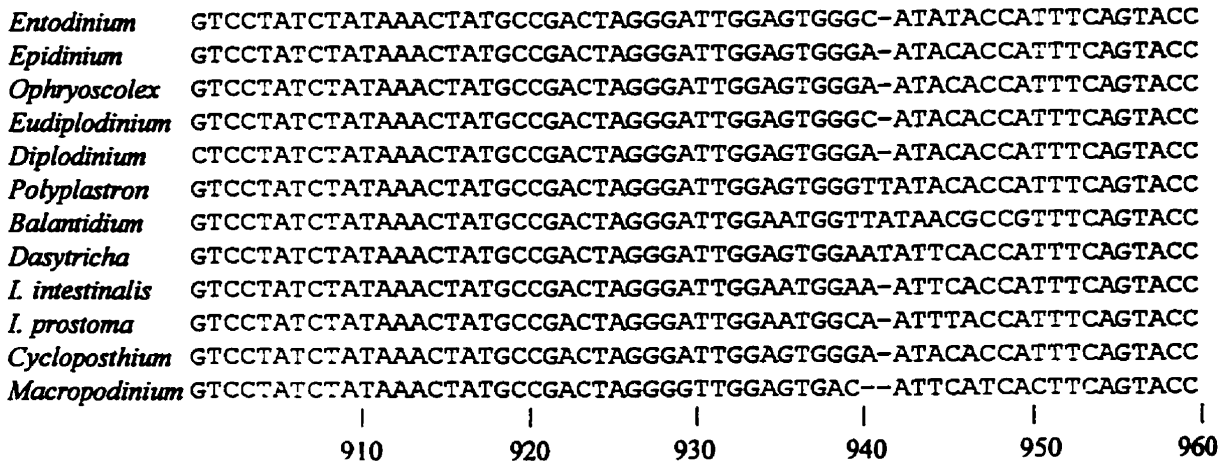
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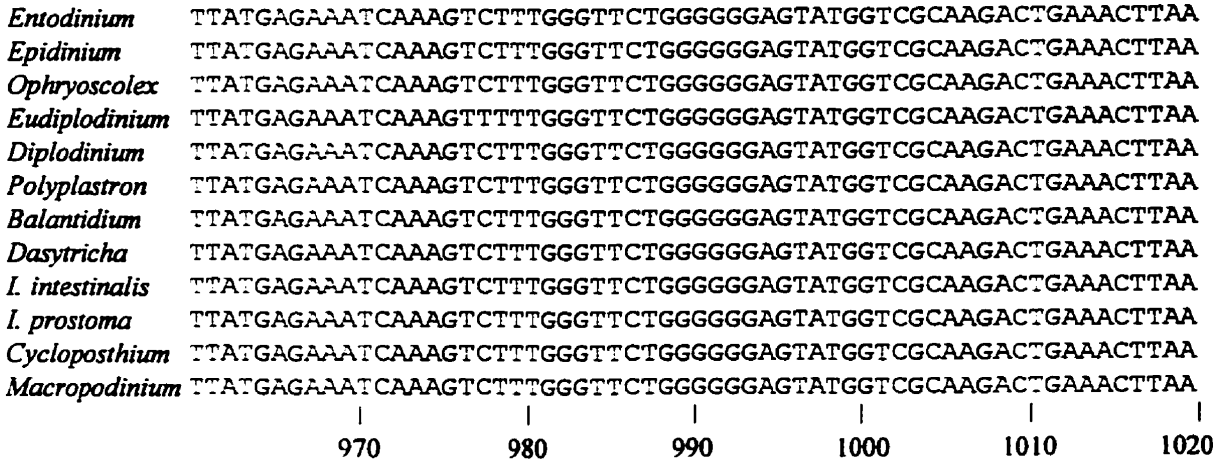
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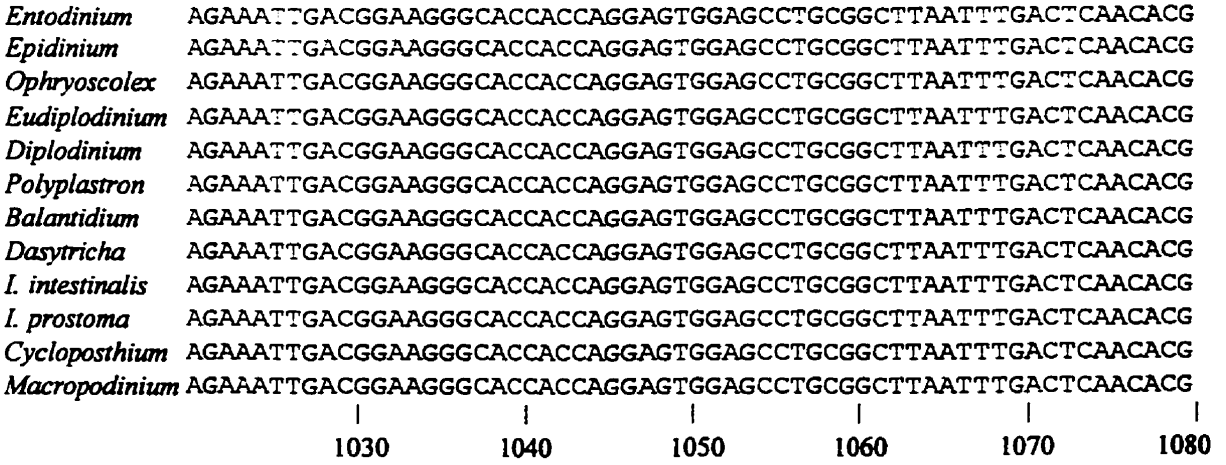
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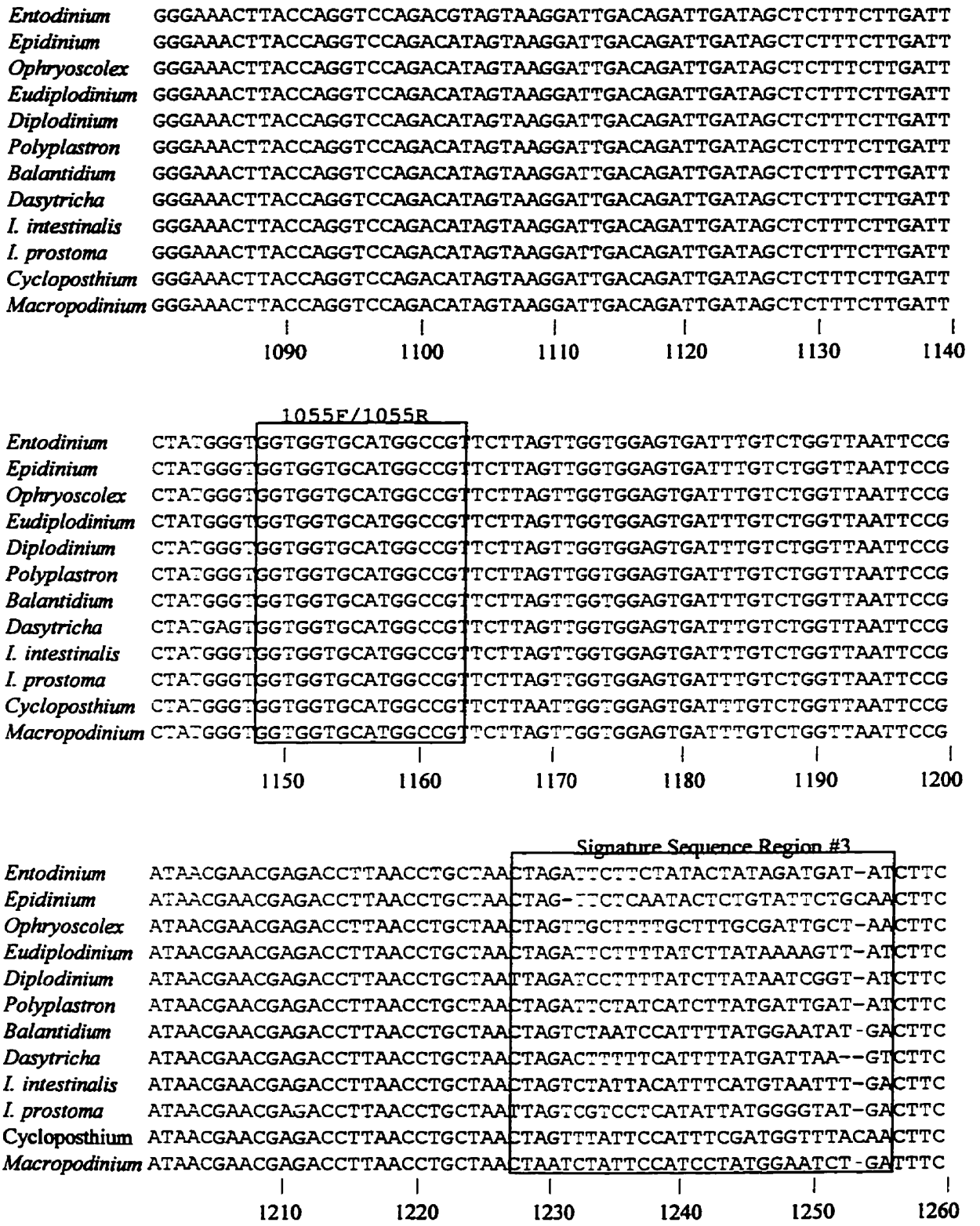
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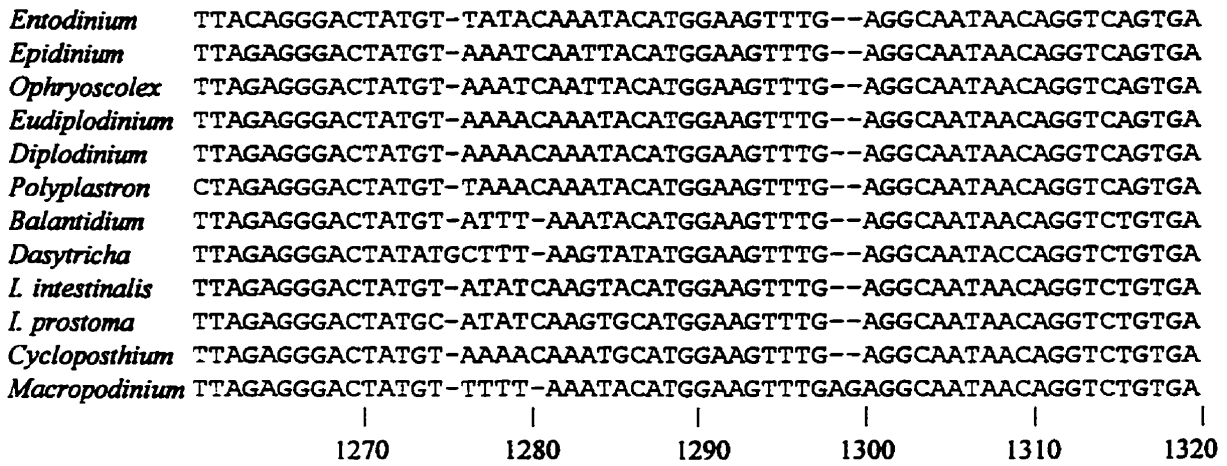
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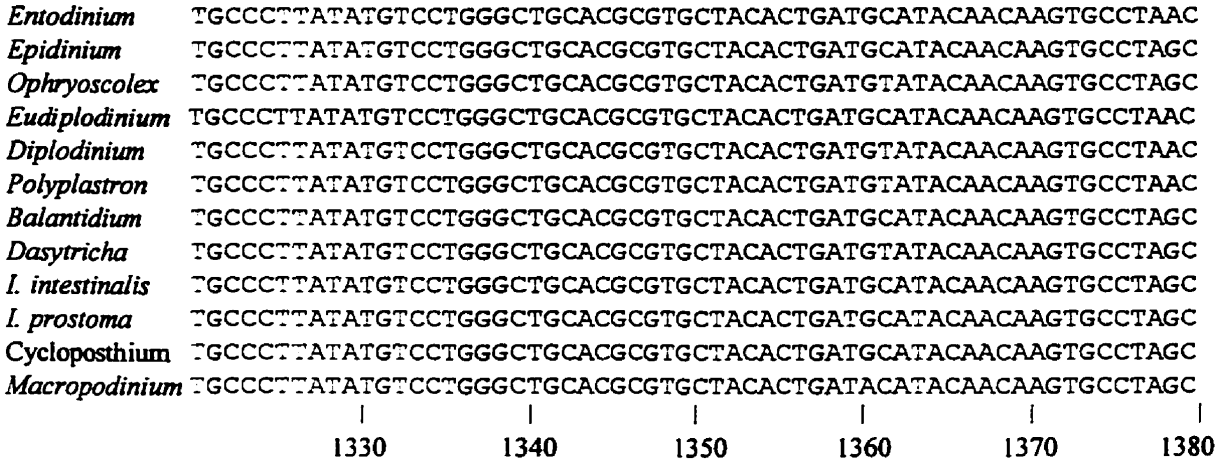
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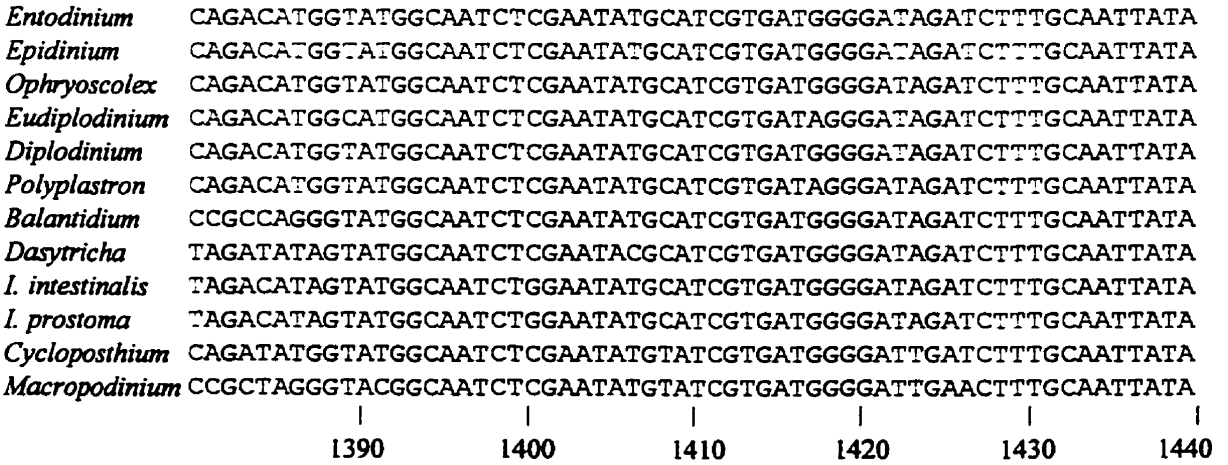
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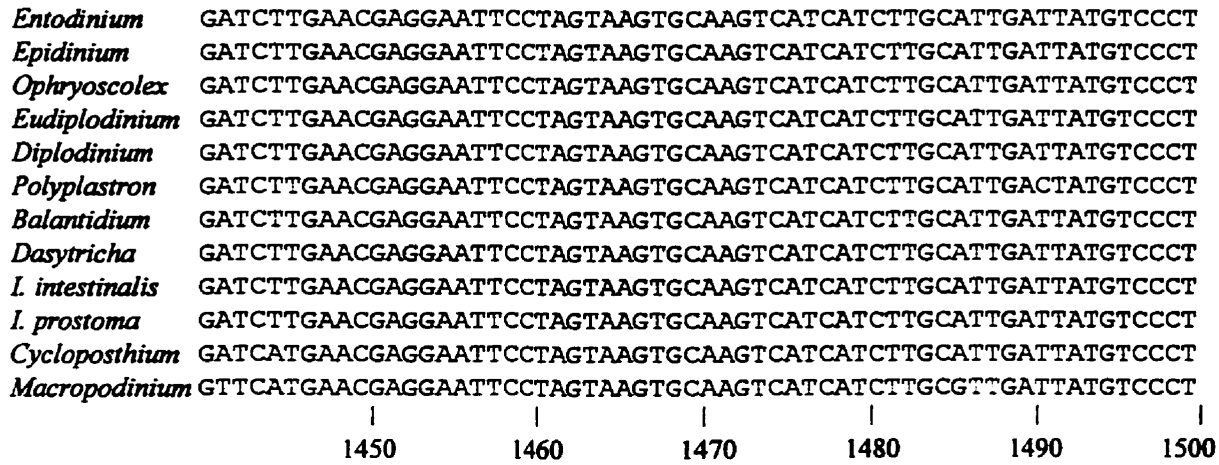
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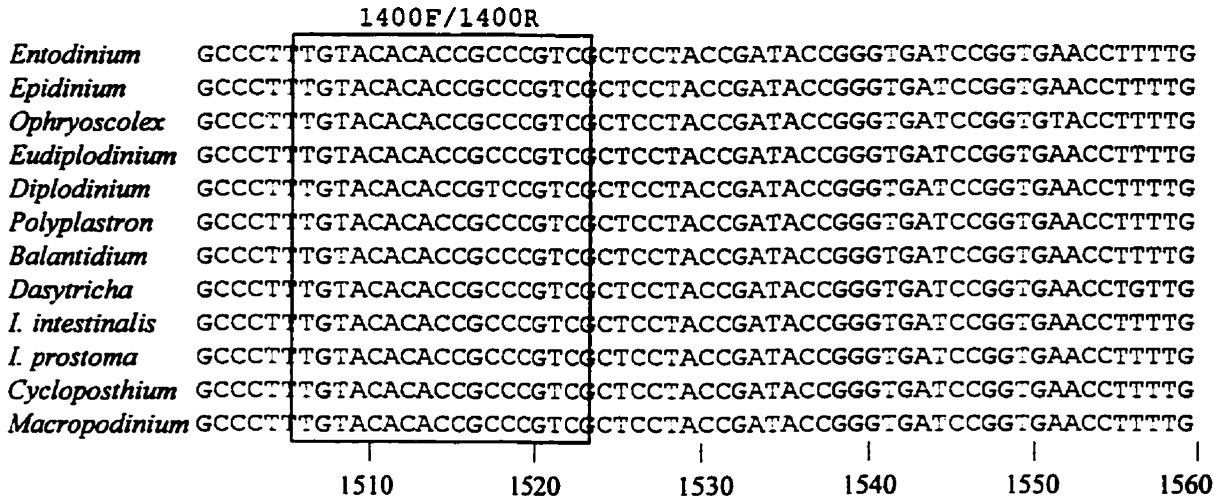
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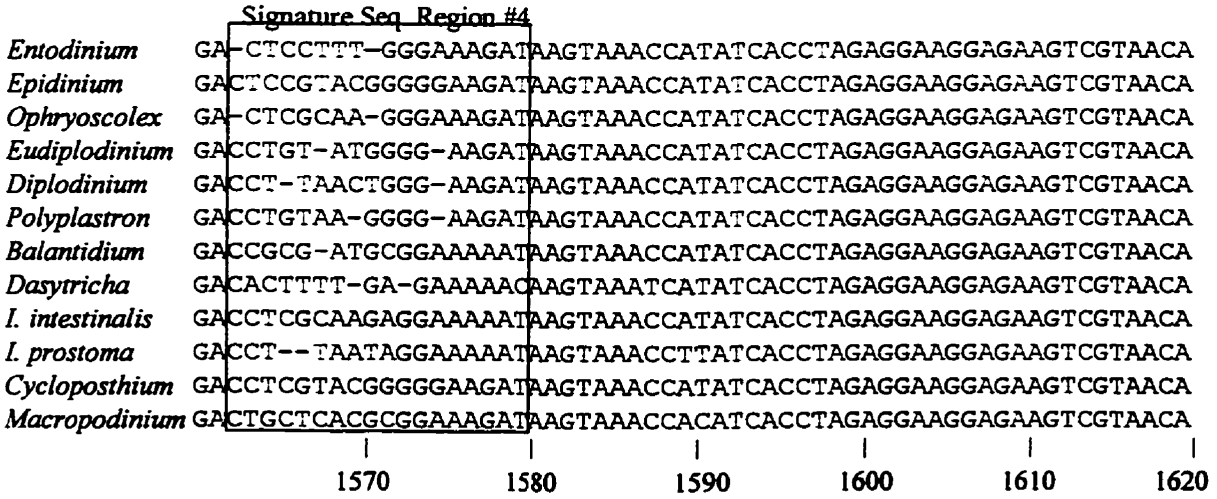
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I. prostoma GACCT--TAATAGGAAAAATAAGTAAACCTTATCACCTAGAGGAAGGAGAAGTCGTAACA
Cycloposthium GACCTCGTACGGGGGAAGATAAGTAAACCATATCACCTAGAGGAAGGAGAAGTCGTAACA
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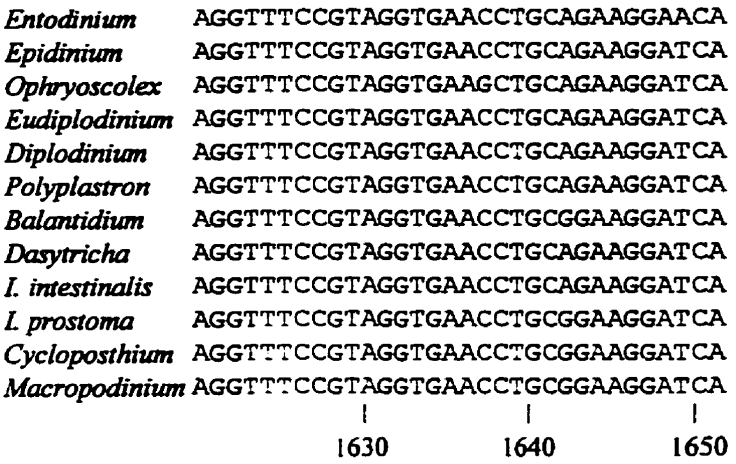
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Cycloposthium AGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
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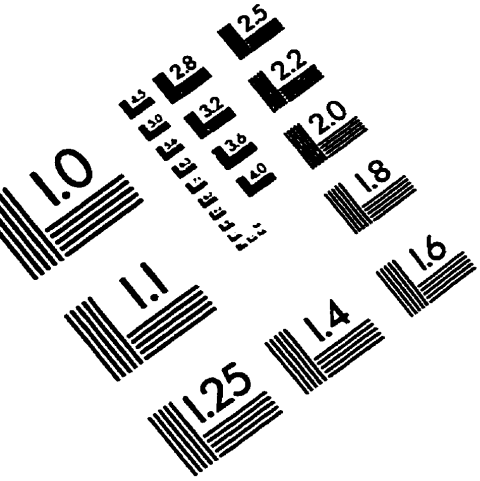
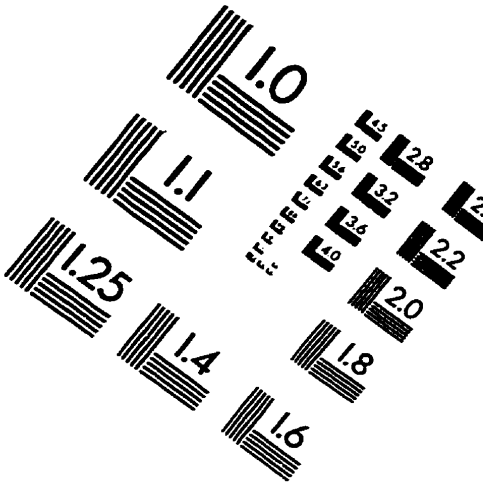
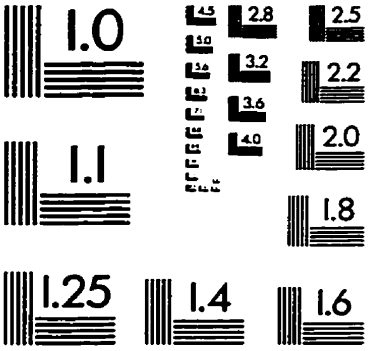
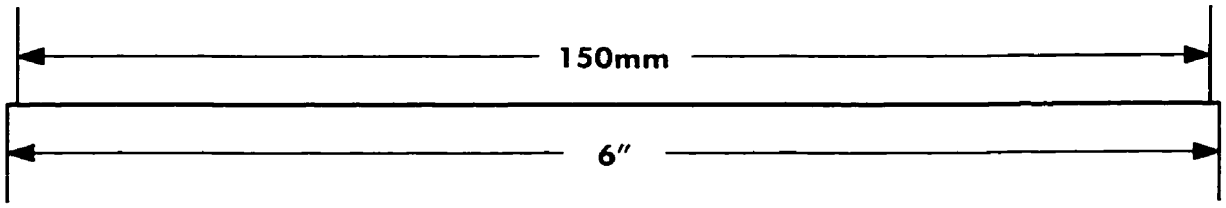
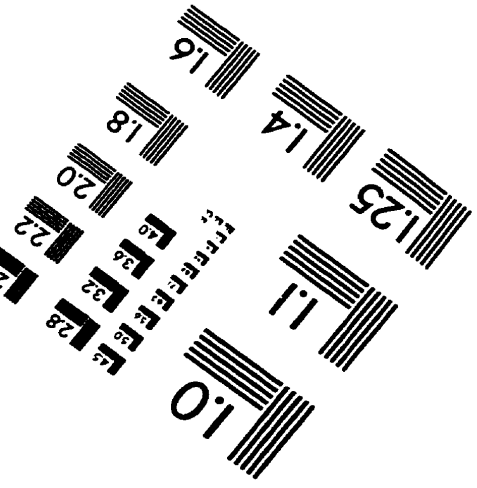
IMAGE EVALUATION
TEST TARGET (QA-3)

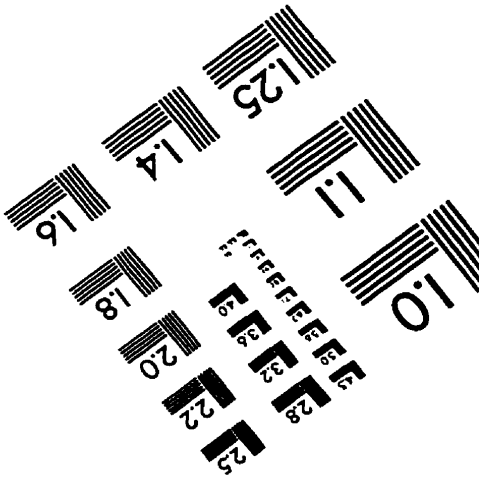












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