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Thraustochytrids are chromists, not Fungi: 18s rRNA signatures of Heterokonta

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SUMMARY

Thraustochytrids are a neglected group of non-photosynthetic marine protists characterized by the presence of a sagenogenetosome, an ectoplasmic net and a cell wall composed of non-cellulosic scales. Though originally classified as fungi, they are ultrastructurally closest to the labyrinthulids, which have often been treated as protozoa, but are now usually considered to be more closely related to the heterokont algae.

In order to clarify their controversial taxonomic position and evolutionary relationships, we have cloned and sequenced the 18s rRNA genes of the thraustochytrids *Thraustochytrium kinnei* and *Ulkenia profunda*. Phylogenetic analysis by several methods confirms earlier suggestions based on ultrastructure that thraustochytrids constitute a deeply divergent branch of the phylum Heterokonta, which is currently classified in the kingdom Chromista. The phylum Heterokonta also includes numerous chromophyte algae (e.g. brown algae and diatoms) as well as the oomycetes. Heterokont 18s rRNA sequences can be distinguished from those of all other eukaryotes by having an AU not a UA base pair at the very base of helix 47 in the V9 region. We also tabulate signature sequences that can be used to characterize 18s rRNA sequences from each of the major heterokont taxa.

1. INTRODUCTION

Thraustochytrids are common marine heterotrophic protists that feed non-phagotrophically as saprobes, or more rarely as parasites (Porter 1990). Their evolutionary relationships (Chamberlain & Moss 1988) and taxonomy (Gaertner 1972, 1977; Karling 1981; Bahnweg & Jäckle 1986) are still poorly understood. *Thraustochytrium*, originally described as a fungus of the order Chytridiales (Sparrow 1936) because of its superficial resemblance to chytrids, was later transferred to the oomycete order Saprolegniales on account of its biciliated heterokont zoospores (Sparrow 1943, 1960; Dick 1973; Goldstein 1973; Gaertner 1977). Following the description of additional genera (now about eight in all), thraustochytrids were segregated into their own order, Thraustochytriales (Sparrow 1973; emended by Alderman *et al.* 1974). Electron microscopy showed, however, that they were quite different from all fungi; instead they more closely resemble the labyrinthulids in having an ectoplasmic net generated by a unique organelle, the sagenogenetosome (Perkins 1972, 1973) or sagenogen (Olive 1975). This, coupled with the fact that their cell walls were found to be composed, not of cellulose fibres, but of non-cellulosic scales (Darley *et al.* 1973), led to their removal from the Oomycetes (Porter 1974) and their being grouped instead with the labyrinthulids in the class Labyrinthulea (Olive 1975), which Olive removed from the Fungi and placed instead in the kingdom Protista. Although Olive did not consider Labyrinthulea to be specifically related to Mycetozoa and plasmodiophorids, he grouped the three taxa (each as a subphylum) within a phylum Gymnomyxa. This clearly polyphyletic assemblage has not been accepted by subsequent authors, since their ultrastructure indicates that Labyrinthulea are less closely related to these two protozoan taxa than to the heterokont and haptophyte algae (Porter 1974; Perkins 1976; Moss 1985, 1986; Chamberlain & Moss 1988).

For this reason Labyrinthulea, Oomycetes and hyphochytrids have all been grouped together with the heterokont algae in the division (phylum) Heterokonta within the relatively new kingdom Chromista (Cavalier-Smith 1981; but the names Labyrinthulea and Heterokonta were not validated under the Botanical Code until 1986), which is fundamentally distinct from the classical kingdoms Fungi, Plantae and Protozoa (Cavalier-Smith 1986, 1987, 1989, 1993*a*,*b*): the other chromist phyla (divisions) are the Haptophyta, Cryptista, and Chlorarachniophyta (Cavalier-Smith 1993*a*). Initially Labyrinthulea were included in the same heterokont subphylum as the oomycetes and hypho-

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Table 1. *Classification of the phylum (division) Heterokonta*

- Phylum HETEROKONTA Cavalier-Smith, 1986
  - Subphylum 1. BICOECIA Cavalier-Smith, 1989
    - Class 1. Bicoecea orthogr. emend. pro Bicosoecea Cavalier-Smith, 1986
  - Subphylum 2. LABYRINTHISTA Cavalier-Smith, 1986 stat. nov., 1989
    - Class 1. Labyrinthulea Olive ex Cavalier-Smith, 1986
      - Subclass 1. Thraustochytridae Cavalier-Smith, 1989
      - Subclass 2. Labyrinthulidae Cavalier-Smith, 1989
  - Subphylum 3. OCHRISTA Cavalier-Smith, 1986
    - Infraphylum 1. Raphidoista Cavalier-Smith, 1986 emend. stat. nov. 1993
      - Superclass 1. Raphidomonadia Cavalier-Smith, 1993
        - Class 1. Raphidomonadea Chadefaud ex Silva, 1980 (syn. Chloromonadea)
          - Subclass 1. Raphidochloridae Cavalier-Smith, 1993
          - Subclass 2. Raphidochrysidae Cavalier-Smith, 1993
      - Superclass 2. Dictyochia Haeckel, 1894 stat. nov. emend. Cavalier-Smith, 1993
        - Class 1. Pedinellea Cavalier-Smith, 1986
        - Class 2. Silcoflagellatea Borgert, 1891 stat. nov. Cavalier-Smith, 1993
        - Class 3. Pelagophycea Andersen & Saunders, 1993
    - Infraphylum 2. Chrysista Cavalier-Smith, 1986 stat. nov. 1993
      - Class 1. Chrysophycea Pascher ex Hibberd, 1976
        - Subclass 1. Chrysomonadidae Saville Kent, 1881 stat. nov. Cavalier-Smith, 1993
        - Subclass 2. Synuridae stat. nov. [=class Synurea Cavalier-Smith, 1986 (syn. Synurophyceae Andersen, 1987)]
        - Subclass 3. Sarcinochrysidae Cavalier-Smith, 1993
        - Subclass 4. Chrysomeridae Cavalier-Smith, 1993
      - Class 2. Flavoretea Cavalier-Smith, 1993 (*Reticulosphaera*)
      - Class 3. Oikomonadea Cavalier-Smith, 1993
      - Class 4. Xanthophycea Allorge ex Fritsch, 1935 (syn. Tribophyceae Hibberd)
        - Subclass 1. Rhizochloridae Cavalier-Smith, 1993
        - Subclass 2. Tribophycidae Cavalier-Smith, 1993
      - Class 5. Phaeophycea Kjellman, 1891 (syn. Melanophyceae Rabenhorst, 1863, Fucophyceae Warming, 1884) orthogr. emend.
        - Subclass 1. Phaeophycidae Cavalier-Smith, 1986
        - Subclass 2. Fucophycidae Cavalier-Smith, 1986
    - Infraphylum 3. Eustigmista Cavalier-Smith, 1993
      - Class 1. Eustigmatophycea Hibberd and Leedale, 1971
    - Infraphylum 4. Diatomea Agardh, 1824 stat. nov. Cavalier-Smith, 1993
      - Class 1. Centricea Schütt, 1896 stat. nov. orthog. emend. (syn. Coscinodiscophyceae Round & Crawford, 1990)
        - Subclass 1. Eucentricidae Cavalier-Smith, 1993
        - Subclass 2. Corethrophycidae Round and Crawford, 1990
        - Subclass 3. Rhizosoleniophycidae Round and Crawford, 1990
      - Class 2. Pennatea Schütt, 1896 stat. nov. orthog. emend. (syn. Fragilariophycidae Round, 1990)
        - Subclass 1. Araphidae Hustedt, 1930 stat. nov. Cavalier-Smith, 1993
        - Subclass 2. Raphoidae Cavalier-Smith, 1993
  - Subphylum 4. PSEUDOFUNGI Cavalier-Smith, 1986 emend. 1989
    - Class 1. Pythiistea Cavalier-Smith, 1986 stat. nov. 1989
      - Subclass 1. Oomycetidae Winter in Rabenhorst, 1879 stat. nov. Cavalier-Smith, 1989
      - Subclass 2. Hyphochytridae orthogr. emend. Sparrow ex Dick, 1983 stat. nov. Cavalier-Smith, 1989

chytrids (Cavalier-Smith 1981), but more recently (Cavalier-Smith 1989) these groups have been segregated into two subdivisions (subphyla): Pseudofungi, comprising the oomycetes and hyphochytrids, and Labyrinthista containing the thraustochytrids (subclass Thraustochytridae) and labyrinthulids (subclass Labyrinthulidae). Together with the subdivisions (subphyla) Ochrista (several classes of heterokont algae, e.g. brown algae, diatoms and chrysophytes) and Bicoecia (bicosoecids) they constitute the botanical division (phylum) Heterokonta (see table 1). Though some mycologists have accepted the inclusion of labyrinthists in the Heterokonta within the kingdom Chromista (Barr 1992), many continue to treat thraustochytrids as fungi (Ulken 1990; Porter & Lingle 1992) or else as an isolated protist or protoctist phylum, Labyrinthulomycota (Porter 1990), a name not valid under the Botanical Code. This makes it important to obtain molecular sequence data to test more thoroughly their transfer from the kingdom Fungi (Cavalier-Smith 1987; Bruns *et al.* 1991) to the kingdom Chromista. Sequences of 5s and 5.8s rRNA have already demonstrated that thraustochytrids are indeed far removed from both Fungi and Mycetozoa (Walker 1985). However, because of their shortness these sequences lack the resolution to show positively the true affinities of thraustochytrids.

Therefore we have sequenced the 18s rRNA genes from two different thraustochytrid genera. Our phylogenetic trees show conclusively that thraustochytrids are indeed very deeply divergent from oomycetes, but confirm that oomycetes and heterokont algae (Ochrista) together are their closest known relatives. They also confirm that thraustochytrids are not specifically related to the true Fungi, and are even less so to the Mycetozoa. We also do an analysis of the 18s rRNA signature sequences for the phylum Heterokonta as a whole, and for the major taxa within it.

2. MATERIALS AND METHODS

Cells of *Thraustochytrium kinnei* Gaertner and *Ulkenia profunda* Gaertner (strains 1694d and N3077a, respectively, from the culture collection of the Alfred Wegener Institute für Polar und Meerschforschung, Germany) were provided by S.T. Moss. After lysis by vortexing in phenol (Medlin *et al.* 1988), DNA was extracted, and the 18s rRNA genes amplified by the polymerase chain reaction, cloned in both orientations into M13 phage, and sequenced on both strands with use of 12 conserved primers as described by Bhattacharya *et al.* (1990). The Genbank accession numbers are: *T. kinnei* L34668; *U. profunda* L34054.

The sequences were aligned manually with about 220 eukaryotic and 50 bacterial small ribosomal RNA genes by using the Genetic Data Environment software: the other sequences were obtained from Genbank or the EMBL database, except for 14 unpublished ones (*Axinella*, *Parazoanthus*, *Chilomonas*, *Prymnesium*, *Pavlova* from our own laboratory, *Chlorarachnion* and *Goniomonas* from G. McFadden and *Porphyra* from M.A. Ragan). Phylogenetic trees were calculated by using Phylip v. 3.5 (Felsenstein 1992) and by fastDNAml (Olsen *et al.* 1994). After running preliminary neighbour joining (Saitou & Nei 1987) and DNAPARS trees for the whole data set a representative subset of 87 sequences from species branching higher up the tree than Euglenozoa was selected for more detailed analysis.

3. RESULTS

From the neighbour-joining (NJ) distance tree (figure 1) it is clear that the two thraustochytrid genera are about as divergent from each other as are different phyla of bilateral animals. Yet they form a single clade well separated from all other eukaryotes. This NJ distance tree groups the thraustochytrids with the other heterokonts (bootstrap value 82%). The Fitch distance tree (figure 2) places them in exactly the same position at the base of the heterokonts. However the Fitch tree does differ in five ways from the NJ trees: (1) it groups choanoflagellates with fungi rather than radiate animals; (2) it groups the whole animal kingdom as a single clade, not two; (3) it groups the eustigmatophyte *Nannochloropsis* with chrysophytes, not with the xanthophyte–brown algal clade; (4) it groups Haptophyta with the Heterokonta, *Chlorarachnion* and Alveolata, not with green plants; (5) it separates the nucleomorphs into two clades, grouping one with red and one with green algae.

Inspection of the full alignment for over 220 eukaryotes revealed only two signature sequences that are universally present in all heterokonts including thraustochytrids but are absent from all other eukaryotes i.e. a change from a T to an A at position 3813 and from an A to a T at position 3986 of the alignment of Neefs *et al.* (1990). These two changes imply a change from a UA to an AU in the last base pair at the very base of helix 47 in the V9 region in the secondary structure model of Neefs *et al.* (1990). This suggests that there is a stringent requirement for either an AU or a UA base pair at this position; the uniqueness of the change is strong evidence for the monophyly of Heterokonta. There are about six other signature sequences shared by all Heterokonta, which differ from the vast majority of all other eukaryotes, but all of these are shared with one other group.

The distance trees show the thraustochytrid clade as a long branch (longer for the *Thraustochytrium* than for the *Ulkenia* lineage) compared with other heterokonts. This means that the 18s rRNA of thraustochytrids has evolved substantially faster than for other Heterokonta.

Perhaps that is why the parsimony tree (figure 3) does not group the thraustochytrids with the heterokonts but instead with another chromist phylum, the Chlorarachniophyta (which in the distance trees is a sister group of the Heterokonta); it is well known that parsimony methods are especially prone to an artefact in which two long branches group together incorrectly (Felsenstein 1978). The low bootstrap value (46%) of this placement of the thraustochytrids on the parsimony tree also suggests that in this respect it is less likely to be correct than the distance tree. None the less, because of this conflict between the parsimony and distance trees, we have also run a maximum likelihood tree (figure 4), since on simulations with unequal branch lengths maximum likelihood has proved much better able than parsimony (and somewhat better than neighbour joining) to reconstruct the correct tree topology (Nei 1991). Because maximum likelihood calculations of 87 species would have taken many weeks, even with use of nearly all the cpu time on our Sparc 2 work station, we reduced the number of genes to 48. The resulting tree clearly groups thraustochytrids with the other Heterokonta. Thus the distance and maximum likelihood trees, together with the signature sequence analysis, strongly support the classification of the thraustochytrids in the phylum Heterokonta. The discordant results with the parsimony tree can with confidence be attributed to the 'long branches attract' treeing artefact (Felsenstein 1978). A grouping of thraustochytrids with *Chlorarachnion* would not be supported by ultrastructure.

As an additional check on this point we also ran a more restricted parsimony tree including all the chromist nuclear and nucleomorph sequences, but having only *Haplosporidium* as outgroup. In this tree Heterokonta were holophyletic (*sensu* Ashlock 1971), with thraustochytrids at the base of the clade exactly as in the distance and fastDNAml trees, suggesting that their true position was perturbed by the non-chromist outgroups included on the parsimony tree in figure 3.

To see how sensitive the branching order within Heterokonta is to such perturbations by distant outgroups, we selected only the chromobiote

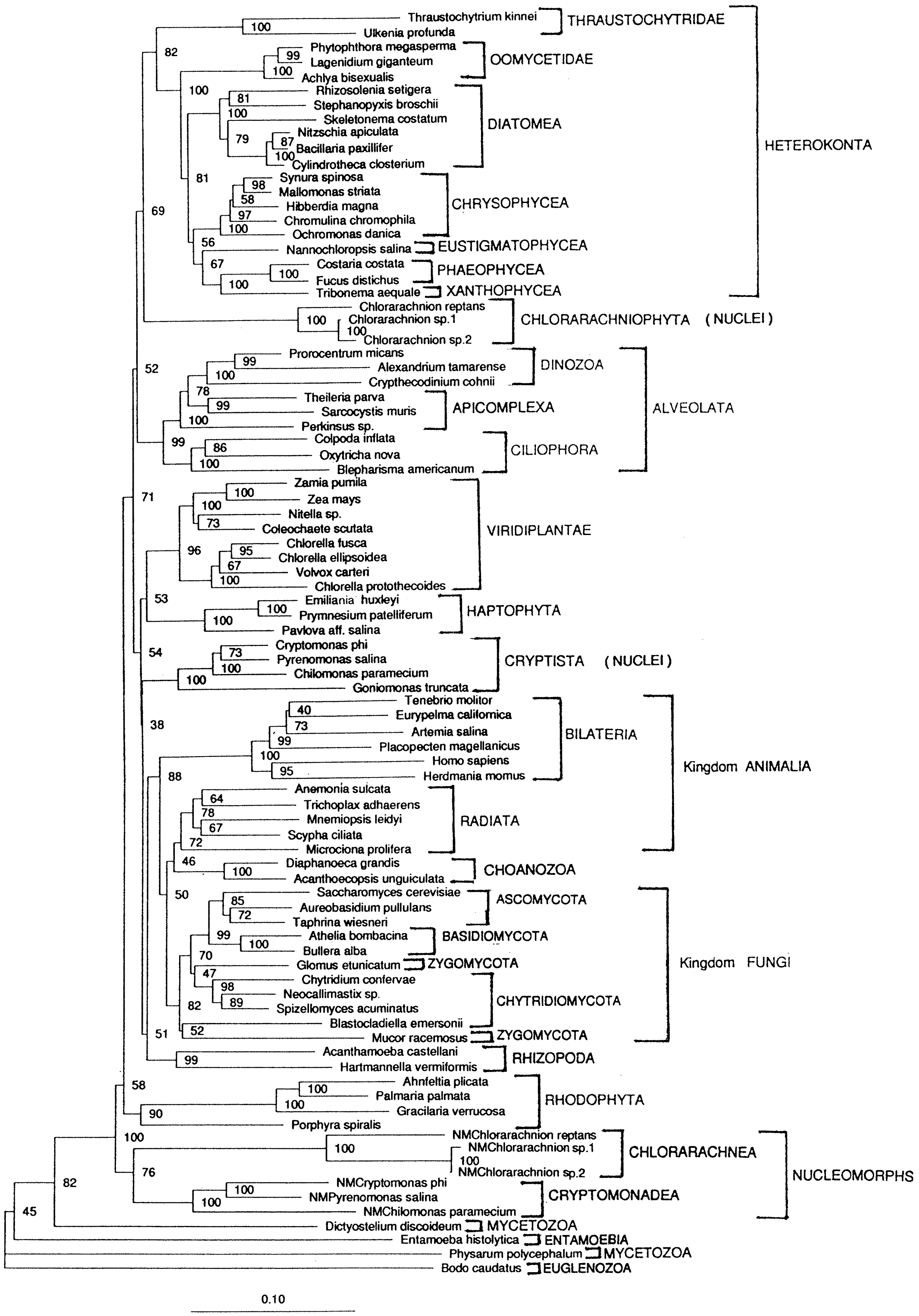

Figure 1. Neighbour joining distance tree for the 18s rRNA sequences of thraustochytrids (*Ulkenia* and *Thraustochytrium*) and 85 other eukaryotic sequences. Idiosyncratic insertions unique to one major group were masked and excluded from the phylogenetic analysis, which is based on 2179 (out of a total of 3110) nucleotide positions in the alignment. The distance matrix was calculated with use of the Jukes–Cantor correction and the neighbour joining tree was produced with the jumble option. The tree was rooted on the euglenozoan *Bodo caudatus* and includes representatives of all major taxa branching above Euglenozoa in the 18s rRNA tree (Cavalier-Smith 1993*a*). The bootstrap values for 100 replicates are shown at the nodes.

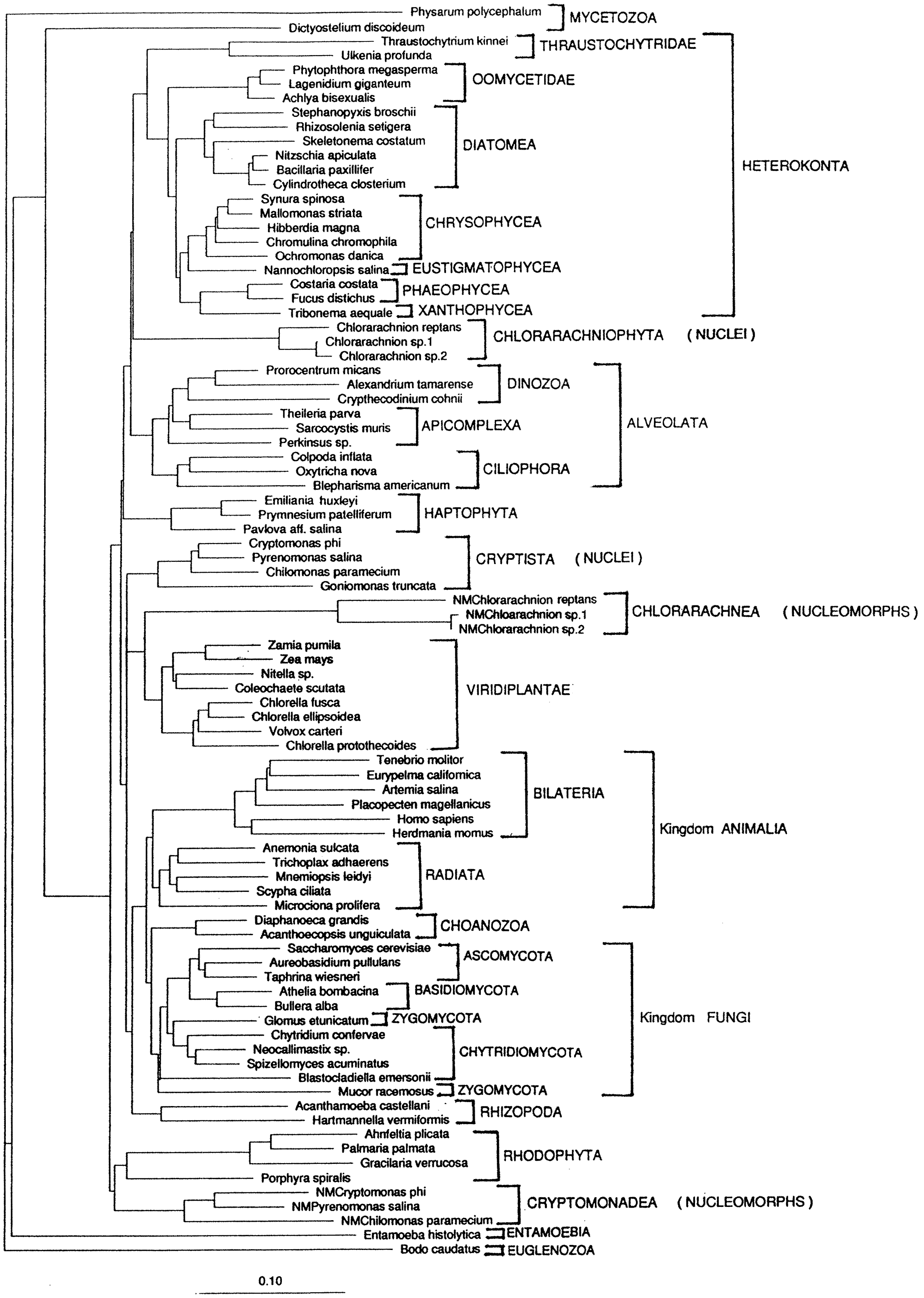

Figure 2. Fitch distance tree calculated from the same distance matrix as figure 1. Three jumblings gave the same tree.

sequences, i.e. the heterokont sequences plus those of the Haptophyta, which are classified as the sister group of the Heterokonta (Cavalier-Smith 1986; 1993*a*) with the infrakingdom Chromobiota, and therefore may be the closest outgroup, and then calculated trees by all four methods using this totally unmasked 1939 position and 26 species alignment. This alignment had three additional heterokont algae not shown in figures 1–4, including the recently discovered *Pelagomonas* (class Pelagophycea: Andersen

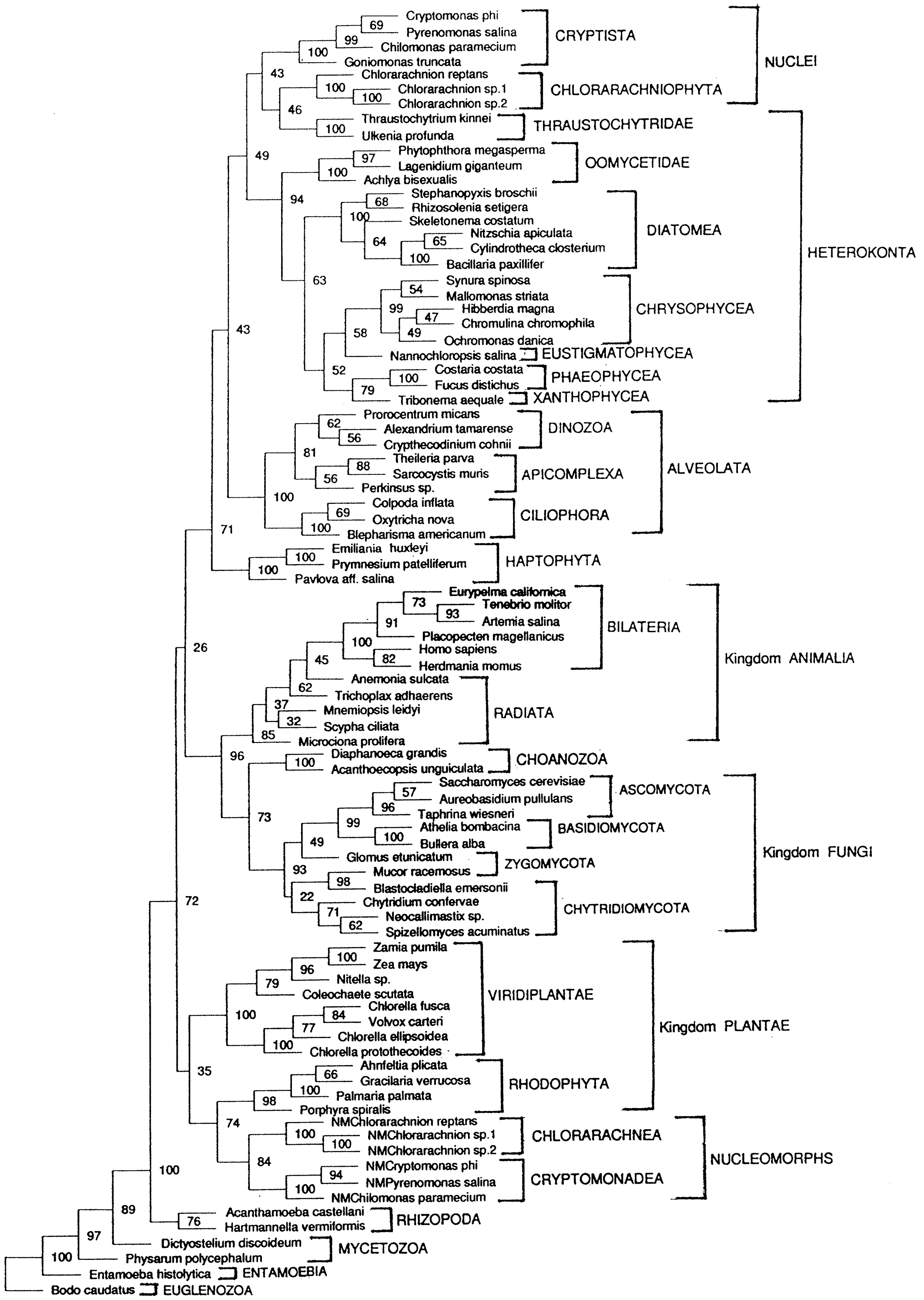

Figure 3. DNA parsimony tree for 18s rRNA from the same species and 2179 alignment positions as in figure 1. Bootstrap values for 100 replicates are shown at the nodes.

et al. 1993). With both distance methods the thraustochytrids and oomycetes formed a single clade (but with only weak bootstrap support) which was the sister group of the Ochrista. Parsimony also grouped thraustochytrids and oomycetes together as a clade, but within the Ochrista (sister group to diatoms). FastDNAml also placed oomycetes within the Ochrista as a sister group to the diatoms, but the thraustochytrids were at the base of the heterokonts as in figures 1, 3 and 4. In all these chromobiote trees Diatomea and Chrysophycea were holophyletic and Phaeophycea were grouped with Xanthophycea, as in

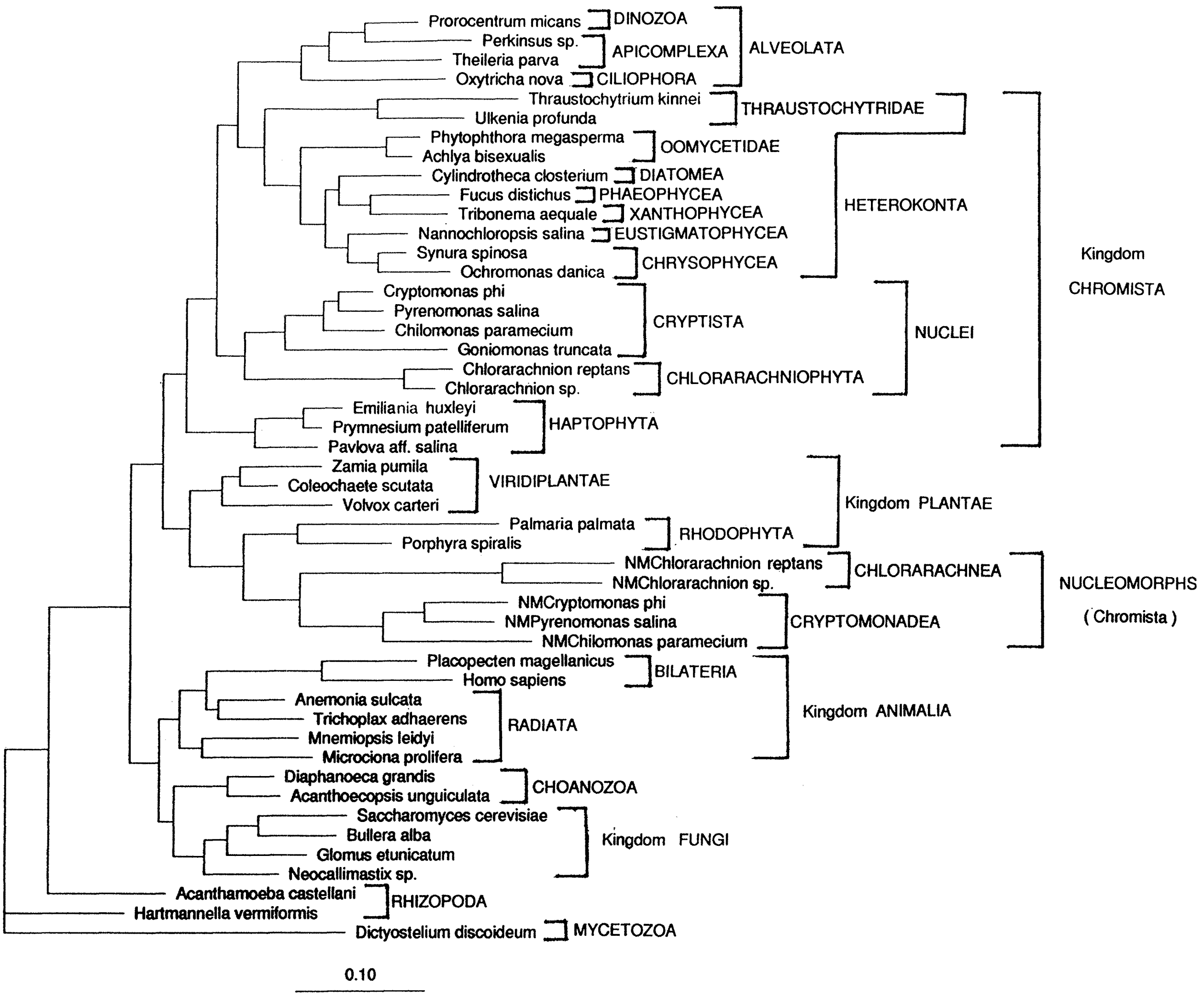

Figure 4. Maximum likelihood tree for 18s rRNA from the thraustochytrids *Ulkenia* and *Thraustochytrium* and 46 representative sequences from other eukaryote species that branch above the mycetozoan *Dictyostelium*. There were 2088 alignment positions included in the analysis by fastDNAml version 1.0.6 which was done with the quick add option and global rearrangements allowing crossing of three branches. Three different jumblings gave the same tree, as did a fourth run allowing crossing of five branches. All the branch lengths are significantly positive ($p < 0.01$).

figures 1–4, but the branching order of these three clades varied with the method used, as did the positions of Eustigmatea and Pelagophycea. The distinctiveness of Thraustochytridae and Oomycetes is also shown by signature analysis of the major heterokont taxa (table 2). Clearly, the branching order within Heterokonta (and even that within the class Chrysophycea) can be perturbed by using different sets of non-heterokont species.

4. DISCUSSION

None of our trees provides any support for the classification of thraustochytrids with the fungi (Sparrow 1936). Although we do not yet have sequences for *Labyrinthula*, the ultrastructural resemblances between thraustochytrids and labyrinthulids (sagenogenetosome and ectoplasmic net) leave little doubt that they are correctly classified together in the class Labyrinthulea (Olive 1975; Cavalier-Smith 1986; see table 1). The rRNA trees shown also do not support the treatment of thraustochytrids as oomycetes (Sparrow 1943; Dick 1973; Gaertner 1977). Instead, they support the separation of oomycetes and thraustochytrids into separate subphyla, Pseudofungi and Labyrinthista respectively (Cavalier-Smith 1989). Some caution is necessary here since the higher evolutionary rate of the thraustochytrids will tend to place them a little too low even on the distance and maximum likelihood trees. Moreover three of the four chromobiote trees (not shown), in which only Haptophyta were outgroup, did group the thraustochytrids (very distantly) with the oomycetes. However, this position had only very weak bootstrap support, and a study of the detailed patterns of substitution in different parts of the 18s rRNA molecule tends to support the conclusion from figures 1–4 and the fastDNAml chromobiote tree that oomycetes are more closely related to the ochrist algae than they are to the thraustochytrids, and that thraustochytrids diverged from other heterokonts very early in heterokont evolution.

This early divergence of the non-photosynthetic thraustochytrids from the lineage leading to the photosynthetic Ochrista, however, is not good

Table 2. *Signature sequences for major heterokont taxa*

(The numbering follows Neefs *et al.* (1990), even though we made our alignment entirely independently of theirs and that of Bhattacharya *et al.* (1992). In addition to those shown there are several signatures uniquely shared by Phaeophycea and Xanthophycea, which supports the proposal (Cavalier-Smith 1986) that they are sister groups and suggests that they should be grouped into a single superclass.)

	sequences in			
alignment position	the taxon shown below	other heterokonts	other chromists	other metakaryotes
Thraustochytridae				
134	G	A	A	A (very rarely G or —)
358–359	GTTTT	C/A/T/ – C	variable	variable
388	A	T	T or A (rarely C)	variable
390	T	A/G (rarely C)	A/T (rarely G)	G or T (rarely C)
404–405	TT	CC	CC	CC or CT
410	C	T	T	T (rarely C, G or A)
413	A	T	T (rarely C)	T (rarely C or A)
421	A	G	G (very rarely A)	G (very rarely A or T)
857	T	C	C	C (T only in *Dictyostelium*)
862	G	C	C	C (rarely G or T)
865	A	G	G (A only in *Goniomonas*)	G (rarely A)
875	G	A	A or G	A or G
877	C	G	G	G (rarely C, A or T)
909	G	C or T	variable	variable
915	G	C or T	variable	variable
954	C	A, G or T	variable	variable
960–967	A/C TCTTT G/C	T/G GG/A CA/T A/C T	variable	variable
1023	A	T (rarely C)	variable	variable
1283	G	T	T/G/A (rarely C)	variable
1354	A	C or G	C (rarely T)	variable
1749	A	G	G	G (T only in *Trichomonas*)
1773	A	G	G	usually G
2322	T	G	G (except) *Chilomonas*)	G (rarely A/C/T)
2649	G	T	T	variable
2911	A	G	usually G	usually G
3383	A	T or G	T or A	A/T/G (rarely C)
3439	T	G, A (rarely C)	A (rarely C)	usually A
3829	C	T	T	T (rarely G, very rarely A or C)
3972	G	A	A	usually A
2903	A	C or T (rarely G)	T (except G in *Emiliania*)	C/T (G in *Entamoeba*, A in *Tritrichomonas*)
Oomycetes				
750	G	T	T or A	usually T or A (rarely C or G)
804	T	G	G	G (T only in *Entamoeba*)
1781[a]	T	A	usually A	variable
2106	A	G	A/C/G	G (rarely A or C)
2114	C	T	C or T	variable
2141–2142	CG	TT	TT, TC, CC (rarely CA)	TG, TT, CG
2151	T	C	C/T/G	C (rarely T or G)
2154	G	A	A	usually A
2157	T	A (rarely T or G)	T or A	A/T/G (rarely C)
2160	G	T	T	A or T (rarely G)
2903	T	A, C (rarely G)	T (rarely G)	T or C (rarely G)
2909	G	C	C	C or G (rarely A or T)
3439	A	G, T (rarely C)	A (rarely C)	A or G (rarely C or T)
3832	C	T	T	T/C/G (rarely A)
3969	G	A	A	A/G/C (rarely T)

Table 2. Continued

alignment position	sequences in			
	the taxon shown below	other heterokonts	other chromists	other metakaryotes
Ochrista				
2560	A	G	G	G (rarely A)
Diatomea				
1897	G	A or T	A or G (rarely T)	G (rarely A or T)
3766	A	G	G (except A in *Chlorarachnion*)	A/G/T (rarely C)
3768	C	T	T (rarely G)	T or C (rarely A or G)
Pennatea				
934	G	A or T (rarely C)	T or —	variable
2313	A	T	T	T (rarely C, very rarely A)
2472	T	A (rarely G)	A	usually A or G
2503	C	—	—	usually — (rarely T or C)
3357	C	T	T (rarely G)	variable
3386	G	T	T or C	variable
3437	A	G (rarely T or A)	T or C	variable
Centricea				
72	G	C	C	C or G (rarely A)
Chrysophycea				
131	—	T	T	T (rarely —)
3753	T	C	C	C (rarely T or A)
3772	G	A	G or A	G (rarely A)
Synuridae				
2218	T	G	G (C only in *Goniomonas*)	usually G

[a] Our alignment differs from Neefs *et al.* in this region; the numbering is that for *Skeletonema costatum*, the only heterokont in their table.

evidence that the ancestral heterokont was non-photosynthetic as some authors assume (Patterson & Sogin 1992). First, there are several major groups of photosynthetic heterokonts for which no 18s rRNA sequences are known (e.g. Raphidomonadea, Silicoflagellatea, Flavoretea, Sarcinochrysidales, Chrysomeridales; for a good recent treatment of the ultrastructural diversity of chromobiote algae see Andersen (1991)). Conceivably one or more of these might branch lower down than thraustochytrids. Second, even if none of them prove to, one should bear in mind that the sister group of the Heterokonta on the present trees is either the photosynthetic Chlorarachniophyta or the Alveolata (which include the dinoflagellates, which when photosynthetic have chlorophyll c, like heterokont algae) while on other recent trees that exclude thraustochytrids (Cavalier-Smith 1994; Cavalier-Smith *et al.* 1994) the sister group is either the Haptophyta, which also have chloroplasts with chlorophyll c, or the Alveolata (Bhattacharya *et al.* 1992). Because of the inconsistency between the trees they do not clearly establish which one of these three major taxa is the sister group of the Heterokonta. Since however there are three synapomorphies uniquely shared between Heterokonta and Haptophyta (fluorescent carotenoid in one of the two cilia; fucoxanthin in their chloroplasts; absence of nucleomorphs in the periplastid space – since such loss must require the positive transfer of genes for chloroplast proteins into the nucleus it is not a purely negative character), they are probably sister groups (Cavalier-Smith 1986, 1989, 1994) and are classified together in the infrakingdom Chromobiota (Cavalier-Smith 1986, 1989, 1993*a*). Therefore it is highly probable that the ancestral chromobiote and heterokont were both photophagotrophs possessing fucoxanthin-containing chloroplasts located within a smooth periplastic membrane in the lumen of the perinuclear cisterna. If so, then all plastidless heterokonts must have lost their chloroplasts. The topology of most of our trees makes it probable that such loss occurred independently in the ancestors of labyrinthists and pseudofungi. If, however, the topology of the three chromobiote trees that grouped thraustochytrids and oomycetes together were really the correct one, then only a single loss of chloroplasts would have occurred in the common ancestor of the two groups. Possibly sequences from related groups, notably hyphochytrids and labyrinthulids, would help to establish more firmly the correct branching order in this part of the heterokont tree. Chloroplasts must also have been lost independently in some pedinellids and probably also in bicoecids (neither yet on published rRNA trees). Thus chloroplasts have probably been lost at least four times within the Heterokonta.

Total loss of plastids (as opposed to the conversion of chloroplasts to non-photosynthetic leucoplasts as in non-photosynthetic chrysophytes) is only possible if the host has not yet become dependent on them for some function other than photosynthesis. Thus they are never lost from green plants (Cavalier-Smith 1993*b*) because they are the only sites for synthesis of fatty acids, starch and certain amino acids. Therefore one should expect that examples of the total loss of chloroplasts from heterokont algae will be concentrated in the earlier part of the heterokont tree, before the host became dependent on them for non-photosynthetic functions. This consideration makes it highly improbable that the purely phagotrophic bicoecids were derived from chrysophytes (as their common classification in Chrysophyceae (e.g. Starnach 1985) tends to imply), since all phagotrophic non-photosynthetic chrysophytes have leucoplasts, unlike bicoecids; this was a major reason for placing bicoecids, like labyrinthists and pseudofungi, in a separate heterokont subphylum (Cavalier-Smith 1989).

Our molecular phylogenetic analysis has now shown that at least three of the four heterokont subphyla form a single clade, and thus gives independent support to the use of rigid tubular hairs (retronemes) on the anterior cilium only as the synapomorphy defining the Heterokonta. Our present trees are also consistent with the monophyly of the kingdom Chromista (phyla Heterokonta, Chlorarachniophyta, Haptophyta and Cryptista) (Cavalier-Smith *et al.* 1994). Although the distance trees, unlike the parsimony and maximum likelihood trees, do not group all the four chromist phyla together they do not group them with any other taxa with strong bootstrap support.

There is never any tendency for thraustochytrids (or any Heterokonta) to group with the Fungi. Fungi consistently group with choanoflagellates and animals as a predicted clade called Opisthokonta (Cavalier-Smith 1987), as demonstrated by Wainright *et al.* (1993). Therefore it is highly undesirable to use such names as Labyrinthulomycota or Heterokontimycotina, which have distinct fungal connotations, for the major taxa that include thraustochytrids.

Our results also show that Labyrinthista are definitely not Mycetozoa, as was once thought for labyrinthulids (Zopf 1892). There is equally no justification for treating *Labyrinthula* as a protozoan phylum (Levine *et al.* 1980; Lee *et al.* 1985): such taxonomic inflation is quite unnecessary when their ultrastructure shows that they can be properly grouped with the thraustochytrids within a single subphylum (Labyrinthista) of the phylum Heterokonta. Clearly the usually non-phagotrophic labyrinthists (a recent observation of bacterivory (Raghukumar 1982), however, suggests that phagotrophy may be more widespread than previously thought) are neither protozoa nor fungi, but are heterotrophic heterokont chromists. They are abundant on or in seaweeds: more common than fungi, but have been very little studied. Perhaps now that their correct taxonomic position is clear they will be less neglected by marine ecologists.

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Note added in proof (24 October 1994): Leipe *et al.* (1994) have now sequenced the 18s rRNA of a third thraustochytrid genus, *Labyrinthuloides*; their conclusions as to the affinities of thraustochytrids fully agree with ours. Their findings that the bicoecean *Cafeteria* also diverges near the base of the heterodont tree supports our argument that Biocoecea must be excluded from Ochrista.

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