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L.K. Medlin · A. Cooper · C. Hill
S. Wrieden · U. Wellbrock

Phylogenetic position of the Chromista plastids based on small subunit rRNA coding regions

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Abstract The kingdom Chromista contains eukaryotic organisms with tubular mastigonemes on the leading flagellum of their bi-flagellated stages, and plastids within a chloroplast endoplasmic reticulum (CER). The complex series of events leading to the formation of the CER is hypothesized to have occurred only once. Thus, all organisms with plastid-CER connections are believed to be monophyletic and derived from a single secondary endosymbiotic event. Analyses of sequence data from the 16*s* rRNA gene from three of the four Chromista pigmented classes indicate that these algae are not monophyletic. The validity of the kingdom Chromista and the number of secondary plastid endosymbioses are questioned.

Key words Plastid evolution · *Emiliania* · *Skeletonema* · Chromista

Introduction

The kingdom Chromista as originally defined by Cavalier-Smith (Cavalier-Smith 1981, 1986) contains eukaryotic organisms (Cryptophyta, Heterokonta and Haptophyta) whose bi-flagellated stages have a leading flagellum with tubular tripartite hairs, termed mastigonemes, capable of conferring reverse thrust movement to the cell. Its photosynthetic members have plastids contained within the chloroplast endoplasmic reticulum [CER, (Gibbs 1993)] and possess chlorophyll-*a* + *c* (Heterokonta and Haptophyta) or chlorophyll-*a* + *c* + phycobilins (Cryptophyta). Cavalier-Smith (1993) has recently re-defined the Chromista to include the Chlorarachniophyta. Of its present members, the Haptophyta do not possess mastigonemes on either flagellum and the Chlorarachniophyta have chlorophyll-*a* + *b*; all members possess a CER with four membranes surrounding the plastids.

The CER results from the fusion of the outer nuclear membrane of the host cell with the fourth membrane surrounding the plastid. The presence of four membranes surrounding the plastids of all pigmented Chromista is widely accepted to provide evidence for the phagocytosis of a photosynthetic eukaryotic alga, i.e., a secondary endosymbiotic event (see review in Whatley 1993 a,b). The first two membranes are presumed to be those of the original cyanobacterium. The third membrane is interpreted to be the former plasmalemma of the engulfed eukaryote, and the fourth the phagosomal membrane of the host cell. Algae with only two membranes surrounding their plastid are presumed to be descendants of the primary endosymbiotic event.

Critical to the circumscription of the Chromista is the hypothesis that the secondary endosymbiotic event, which is manifested by the CER, occurred only once (Cavalier-Smith 1982) and, as such, all host cells and their plastids resulting from this event would share a monophyletic origin. Sequence data from nuclear-encoded 18*s* rRNA suggest that the host cells of the Chromista (as originally defined and subsequently including the Chlorarachniophyta) are not a natural group (Bhattacharya et al. 1992; Cavalier-Smith et al. 1994; Bhattacharya and Medlin 1995; Saunders et al. 1995). We present a phylogenetic analysis of sequence data from the plastid-encoded 16*s* rRNA of pigmented representatives of the Chromista to address the question of the monophyly of the Chromista plastids.

L.K. Medlin (✉) · S. Wrieden · U. Wellbrock
Alfred-Wegener Institute, Am Handelshafen 12, D-27570 Bremerhaven, Germany
A. Cooper · C. Hill
School of Biological Sciences, University of Bristol, Bristol BS8 1UG, UK

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Materials and methods

Cultures and DNA methods. The diatom *Skeletonema costatum* (Grev.) Cleve (UBC clone 18/C) and *S. pseudocostatum* Medl. (CSIRO clone CS-76) and the haptophyte *Emiliania huxleyi* (Lohm.) Hay et Mohl. (A and B morphotypes, PML clones "L" and 92D) were grown as described in Medlin et al. (1986) or Barker et al. (1994), respectively. Nucleic acids were extracted following Medlin et al. (1988) or with a 3% CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle and Doyle 1990). The 16*s* ssu rDNA gene from *S. pseudocostatum* and from two different morphotypes of *E. huxleyi* were amplified (PCR, Saiki et al. 1988) with biotin-labelled primers, either in the forward or the reverse orientation, to produce single-stranded templates (Dynabeads M-280 Streptavidin, DYNAL A.S. Oslo, Norway) for sequencing. Both coding and non-coding strands were completely sequenced (Sanger et al. 1977) with a T7 sequencing kit (Pharmacia) using primers complementary to internal conserved regions of the 16*s* rRNA (Huss personal communication). For biotin-labelled single-stranded sequencing, six PCR reactions were pooled for each orientation. That of *S. costatum* was sequenced from a 9-kb *Hin*dIII fragment cloned into pUC12.

Sequence analysis. The 16*s* rRNA gene sequences from four algal plastids were manually aligned with previously published plastid, cyanobacterial and eubacterial sequences (Neefs et al. 1991; Larsen et al. 1993) using maximum primary- and secondary-structural similarity and deposited in GenBank (X82154, X82155, X82156). Secondary structures were constructed for each isolate modified from Gutell (1993) to aid sequence alignment. Positional homology was assumed for 1504 positions, of which 528 were informative. Twenty-two taxa were selected for the final data set. Maximum-parsimony analyses were implemented with the PAUP program (Swofford 1993). Introduced gaps were treated as missing data, and informative characters as multistate unordered. Unweighted maximum-parsimony trees were obtained using the tree-bisection-reconnection (TBR)-branch swapping option and the heuristic search. The most-parsimonious tree (MPT) and the data matrix were entered into MacClade (Maddison and Maddison 1992) to produce a weighted data set in which the frequency of nucleotide substitutions at each position was inversely related to the weight of that position (scale 1–100). The type of substitution (i, j) at each position was also weighted (K_{ij}) as the cost of going from one state to another: $[K_{ij} = -\ln(X_{ij}/X_i)]$ (where X_{ij} is the number of $i \rightarrow j$ changes, X_i is the number of changes from i to any state, and X is the number of changes on the tree (scale 1–100). These weightings greatly enhance the ability of the maximum-parsimony analyses to recover the correct tree when multiple substitutions have occurred (Hillis et al. 1994). These constraints were used to generate weighted maximum-parsimony trees. The stability of the branching order was estimated using a 50% majority rule-bootstrap analysis for 100 replicates (Felsenstein 1985). Distance analysis was performed using PHYLIP (Felsenstein 1993). Dissimilarity values (Fitch and Margoliash 1967), based on pairwise comparisons of sequences, were transformed into distances using the Kimura two-parameter model (Kimura 1980). Distance matrices were converted into trees using the neighbor-joining option in PHYLIP. Branching-order stability was estimated by bootstrap analysis (100 replicates, Felsenstein 1985). Maximum-likelihood (ML) analyses were performed using the fast DNAml program v. 1.0 (Larsen et al. 1993) with the global search option. LogDet transformations were performed to correct for base compositional bias known for plastid data (Lockhart et al. 1994). Phylogenies inferred with this method are more likely to reflect the evolutionary history of the group rather than the nucleotide composition of the sequences.

Results and discussion

Secondary structures of the 16*s* rRNA molecule of *S. costatum* and *E. huxleyi* are presented in Figs. 1 and 2. The two morphotypes of *E. huxleyi* are identical, whereas the two closely related species of *Skeletonema* differ only by two positions. One of these involves a compensatory base change.

A phylogeny inferred from maximum-likelihood analysis of 16*s* rRNA coding regions is presented in Fig. 3. As in other 16*s* rRNA analyses (Giovanonni et al. 1988, 1993; Douglas and Turner 1991; Martin et al. 1992; Morden et al. 1992) the plastids are shown to be rooted in the cyanobacteria. From this analysis, two lineages emerge. These two lineages are also recovered in the neighbour-joining and weighted-parsimony analyses, with high bootstrap support. The first plastid lineage comprises the green algae/land plants, whose plastids were derived from the primary endosymbiotic event in which a cyanobacterial-like ancestor was engulfed by a eukaryote (Gibbs 1993). Polyphyletic origins of the primary endosymbiotic event have been proposed but most recent evaluations of this theory have been rejected (see review in Bhattacharya and Medlin 1995). The second plastid lineage is defined by the Rhodophyta and the plastids derived from secondary endosymbioses in which a eukaryote was engulfed (Gibbs 1981; Cavalier-Smith 1982). The Chromista belong to this latter group. A third separate lineage, represented by the cyanelles (Helmchen et al. 1995), is not included in these analyses. The euglenophytes are also not included because their position within the lineage containing the Rhodophyta and other plastids derived from the secondary endosymbioses in the rRNA trees (Douglas et al. 1991; Markowicz and Loiseaux de Goër 1991; Giovanonni et al. 1993), is influenced by base composition (Lockhart et al. 1994). A detailed analysis of their position using the LogDet-transformation analysis is presented elsewhere (Bhattacharya and Medlin 1995).

Three of the main groups of algae belonging to the kingdom Chromista (the Cryptophyta, Heterokonta, and the Haptophyta) are included in our analyses. Sequence data from the Chlorarachniophyta are not available. Each of the groups of the Chromista included in our analysis is a monophyletic group with high bootstrap support. The Cryptophyta are an early divergence within the second lineage. Analysis of the nuclear-encoded ssu rRNAs indicates that the nucleomorph (the vestigial nucleus contained within the periplastid space of the cryptophytes) is a early divergence in the lineage leading to the Rhodophyta (Douglas et al. 1991; Ragan et al. 1994; Bhattacharya and Medlin 1995), and their early divergence in the plastid lineage probably reflects the primitive nature of the red-algal precursor, which has been shown to be the eukaryotic endosymbiont in cryptophytes (Douglas et al. 1991). The Haptophyta form a monophyletic group sister group to the lineage containing the Heterokonta and the two unusual unicellular Bangiophycean algae (primitive red algae) in the maximum-likelihood and weighted maximum-parsimony analyses. In the distance analysis, the Haptophyta and Cryptophyta are

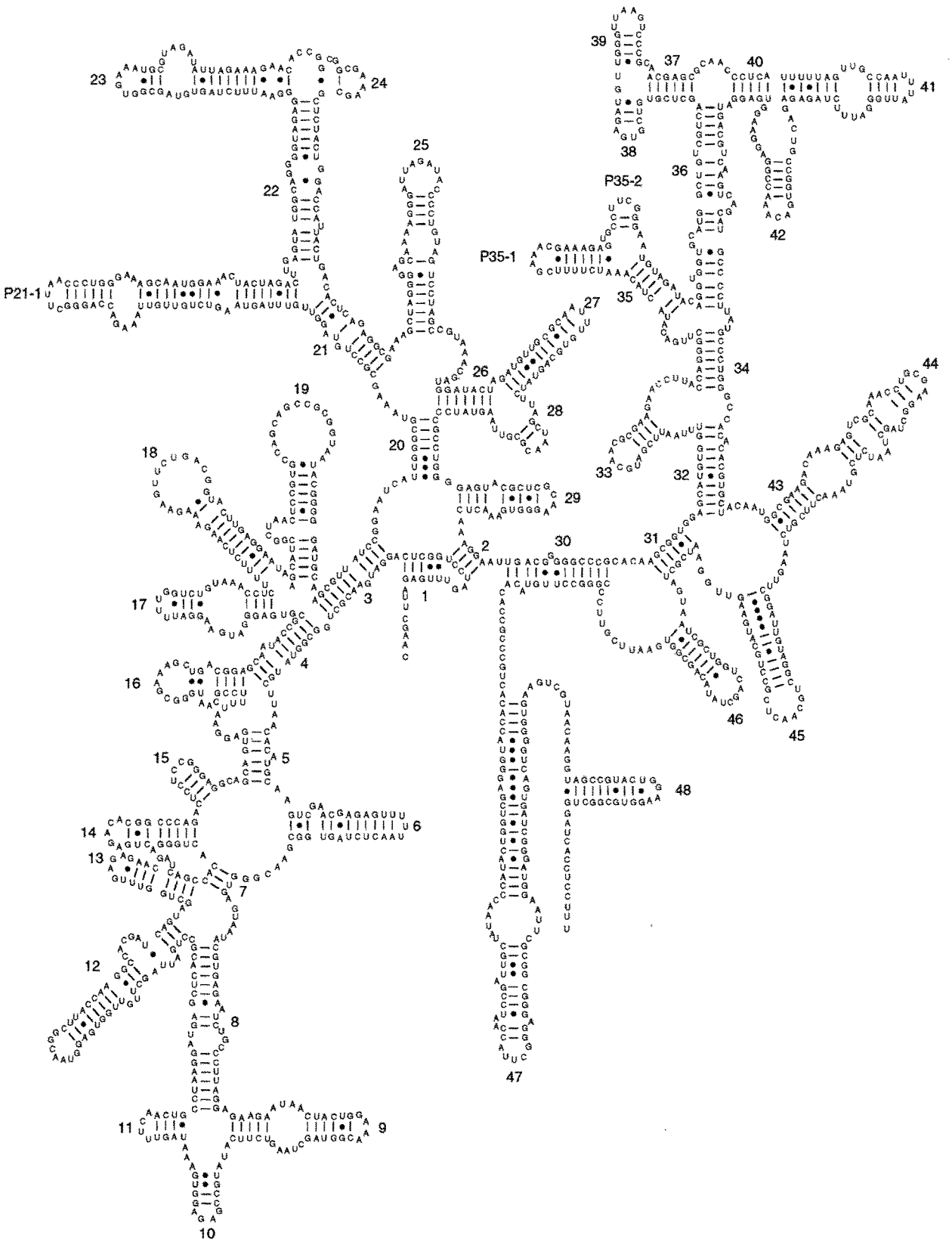

Fig. 1 Secondary structure model of the plastid small subunit rRNA of *E. huxleyi*

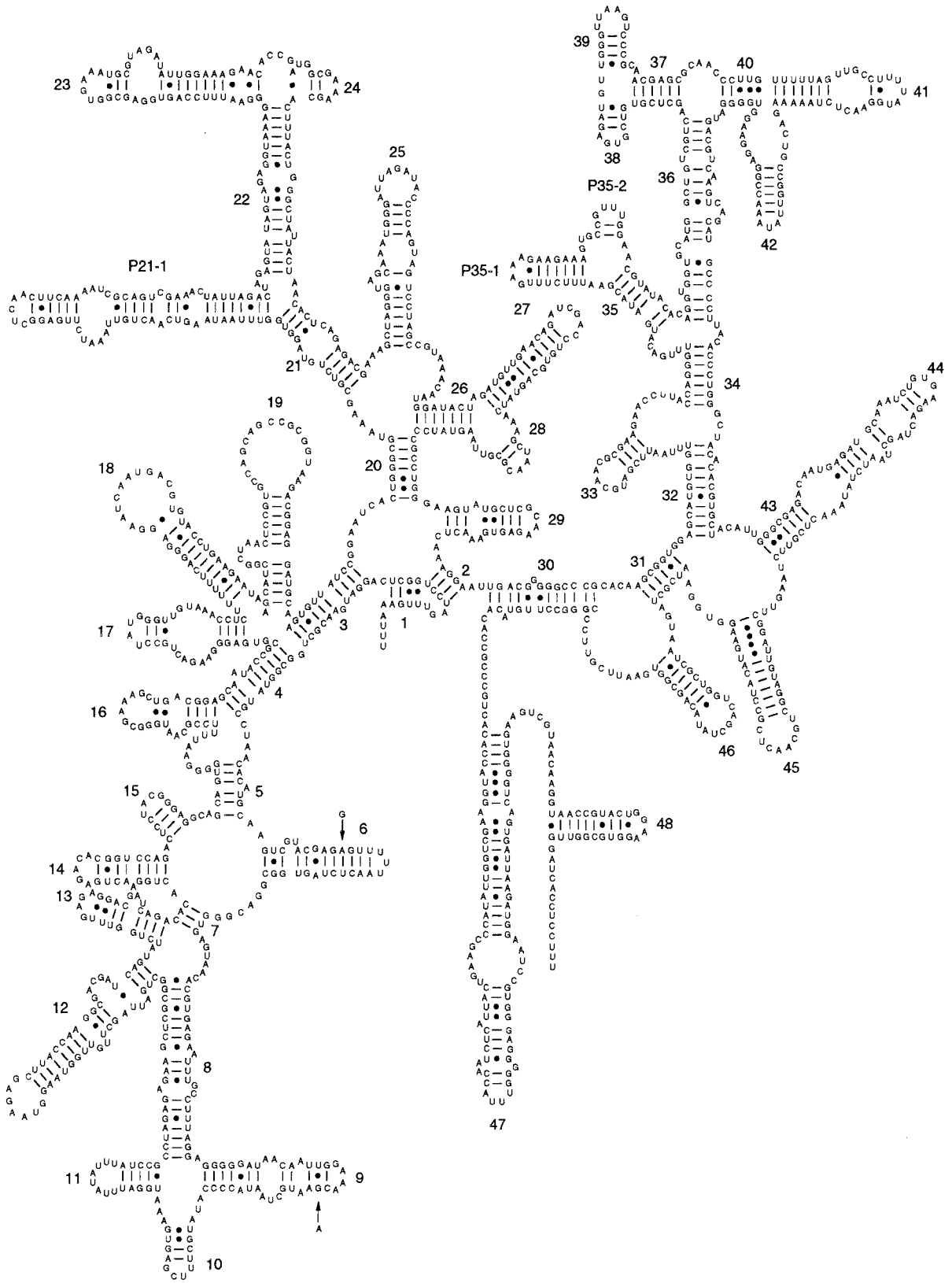

Fig. 2 Secondary structure model of the plastid small subunit rRNA of *S. costatum* and *S. pseudocostatum* (*arrows*)

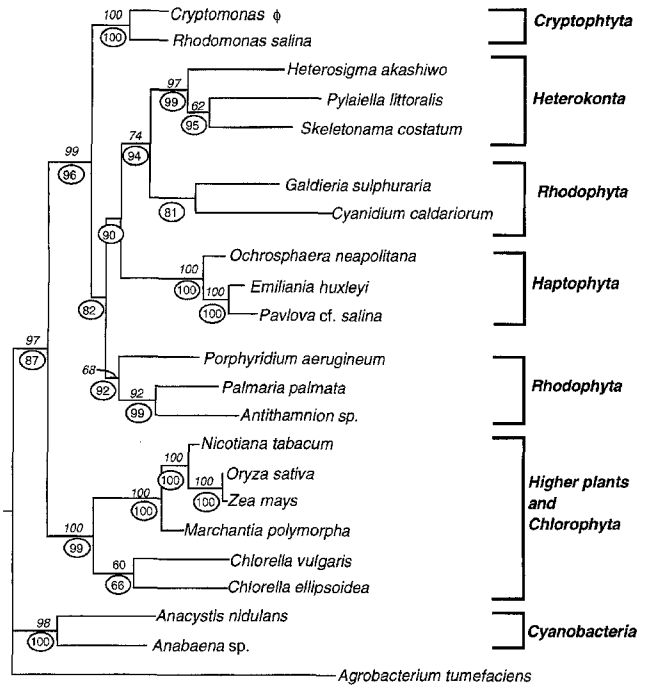

Fig. 3 Phylogeny of 16*s* rRNA sequences based on a comparison of 1504 unambiguously aligned positions inferred with the maximum-likelihood method. Bootstrap values [100 replications, obtained with the neighbor-joining method using the Kimura model (Kimura 1980)] appear above the internal nodes (*italics*), whereas those inferred from a weighted parsimony analysis are shown *encircled* below the internal nodes. Nodes with missing bootstrap values were not recovered in the distance analysis. A single most-parsimonious tree was recovered in both the unweighted and weighted analysis. Both the frequency that a site changed and the state change at that site were weighted over the entire data set [MacClade, 3.0 (Maddison and Maddison 1992)]. All trees were rooted on the branch length leading to the *A. tumefaciens* 16*s* rRNA sequence

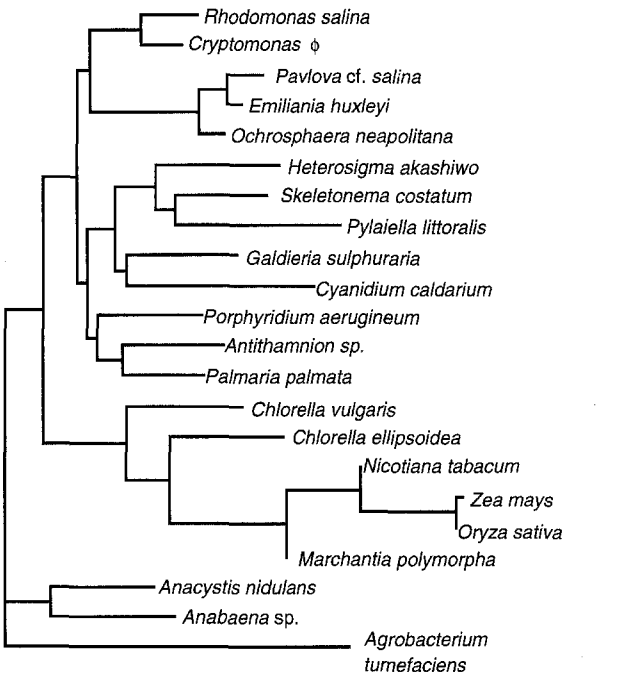

Fig. 4 Phylogeny of 16*s* rRNA sequences inferred with the neighbor-joining method (Saitou and Nei 1987) using a LogDet matrix (Lockhart et al. 1994) as input. Only informative sites were used in the LogDet analysis and gaps were excluded. Bootstrap analyses are not available with this data transformation

grouped together. The different plastid ultrastructure (Gibbs 1962) and the unusually large plastid genome size (Engel et al. 1993) of the haptophyte algae are supported by their monophyletic position in the rRNA tree.

Re-analysis of these data with the LogDet-transformation method, which corrects for biased base composition, positions the Haptophyta as a sister group to the Cryptophyta (Fig. 4) as in the *rbc*L phylogeny (Fugiwara et al. 1994). These two algal classes are sister groups to the Rhodophyta, the Heterokonta, and the unusual small Bangiophyceae lineage.

These data clearly indicate that the plastids of the algae currently assigned to the kingdom Chromista do not share a monophyletic origin. This implies that the number and timing of the secondary endosymbiosis are not limited to a single event and that the series of events converting an engulfed cell into an integral part of the host cell may not be as difficult as originally hypothesized. That secondary endosymbioses can be recurrent events is supported by the myriad of algal symbionts constituting the plastids of the dinoflagellates (Schnepf 1993) and the regular renewal of algal plastids within vacuoles of certain protozoa (Laval-Peuto 1992). Thus, the Kingdom Chromista appears not to be a natural group and this taxon should be abandoned.

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Note added in proof: The Gen Bank entry of *Pavlova* of *Solina* has been corrected to *Isochrysis*