J. Phycol. **37,** 1110–1120 (2001)

PIGMENT COMPOSITION AND *rbc*L SEQUENCE DATA FROM THE SILICOFLAGELLATE *DICTYOCHA SPECULUM*: A HETEROKONT ALGA WITH PIGMENTS SIMILAR TO SOME HAPTOPHYTES[1]

Niels Daugbjerg[2]

Department of Phycology, Botanical Institute, University of Copenhagen, Øster Farimagsgade 2D, DK-1353 Copenhagen K, Denmark

and

Peter Henriksen

Department of Marine Ecology, National Environmental Research Institute, Frederiksborgvej 399, DK-4000 Roskilde, Denmark

Monophyly of plastids in the morphologically diverse heterokont algae has rarely been questioned. However, HPLC analysis revealed that the pigment composition of the silicoflagellate *Dictyocha speculum* Ehrenberg is similar to that observed in a group of haptophytes ("type 4" *sensu* Jeffrey and Wright 1994). *Dictyocha speculum* and type 4 haptophytes possess acylfucoxanthins (19′-butanoyloxy- and 19′-hexanoyloxyfucoxanthin) in addition to fuco-, diadino-, and diatoxanthin and chl *a*, *c*, and c_3. The pigment composition of two pedinellids (*Apedinella radians* [Lohmann] Campbell and *Mesopedinella arctica* Daugbjerg), a sister group to *D. speculum*, deviates from *D. speculum* by lack of chl c_3 and acylfucoxanthins. The distinct pigment composition suggested that plastid evolution in *D. speculum* differs from that of other heterokont algae. This prompted determination of the plastid-encoded *rbc*L gene from *D. speculum* to gain further insight into the evolutionary history of plastids in heterokont algae and haptophytes. A phylogenetic inference based on parsimony, maximum likelihood, and LogDet transformation methods included 35 heterokonts, 19 haptophytes, 8 red algae, and 1 cryptomonad. Three proteobacteria possessing type I RUBISCO were used to root the tree. In phylogenetic analyses, *D. speculum* was closely related to *Rhizochromulina* sp. and pedinellids, despite the latter possessing a different pigment composition. Surprisingly, the Dictyochophyceae clustered outside the lineage of heterokont algae but not within the haptophytes. Hence, analyses deduced from *rbc*L sequences indicated that the plastids in heterokont algae might have a more complex evolutionary history and that the shared pigment composition in *D. speculum* and type 4 haptophytes could be explained by convergent evolution or gene transfer. The pigment composition in *D. speculum* may have implications for pigment-based characterization of phytoplankton community structure in natural samples.

Key index words: **acylfucoxanthins; *Apedinella radians*; *Dictyocha speculum*; haptophytes heterokont algae; *Mesopedinella arctica*; pigment composition; plastid evolution; *rbc*L**

Siliceous skeletons from silicoflagellates are common in fossil deposits dating back to the middle Cretaceous (circa 120 million years ago). The skeletal remains in fossil material also indicate that species diversity reached a peak in the Miocene (circa 23–25 million years ago), with more than 100 described species (Tappan 1980). Diversity of silicoflagellates has decreased thereafter, leaving behind only a single genus *Dictyocha* with approximately five recognized species (Throndsen 1993). One of these, *D. speculum*, possesses six slender spines attached to the outer of two different sized hexagonal rings interconnected by bars and is a well-recognized component of the marine phytoplankton. The life history of *D. speculum* is complex and not yet fully understood. In addition to the skeleton-bearing stage, naked cells (without skeleton), large spherical cells, and multinucleate cells have been described (Moestrup and Thomsen 1990, Henriksen et al. 1993). *Dictyocha speculum* has a worldwide distribution, but the skeleton-bearing stage seems to occur more frequently in polar and temperate waters, often with relatively low abundances. Since 1983, high cell numbers of the naked stage have recurred as spring blooms in the western Baltic, thus prompting studies of its ultrastructure, autecology, and toxicology (Jochem and Babenerd 1989, Moestrup and Thomsen 1990, Henriksen et al. 1993). The fine structural studies showed that both stages possess tripartite tubular flagellar hairs (= mastigonemes), a synapomorphic character that unites the heterokont protists (= chromophytes, stramenopiles). Moestrup and Thomsen (1990) also identified some unusual features relating to the flagellar apparatus, the plastids and the cisternae of the Golgi apparatus. These characters might imply a somewhat isolated position of *D. speculum* within the heterokont algae. Nevertheless, phylogenetic studies based on nuclear-encoded small subunit (SSU) rDNA revealed that *D. speculum* forms a sister taxon to pedinellids and *Rhizochromulina* (e.g. Andersen et al. 1993, Cavalier-Smith et al. 1995, Guillou et al. 1999). This relationship is consistent with a systematic revision of the Dictyochophyceae (Moestrup 1995)

[1]Received 27 March 2001. Accepted 19 July 2001.

[2]Author for correspondence: e-mail nielsd@bot.ku.dk.

TABLE 1. List of species, with GenBank accession numbers, included in the phylogenetic analyses.

Taxon	GenBank accession no.
Proteobacteria (with type I RUBISCO)	
Ralstonia eutropha (Davis) Yabuuchi, Kosako, Yano, Hotta *et* Nishiuchi (β)	M17744
Rhodobacter sphaeroides (van Niel) Imhoff, Truper *et* Pfenning (α)	M64624
Xanthobacter flavus Malik and Claus (α)	X17252
Rhodophyta	
Antithamnion sp.	X54532
Cyanidium caldarium Geitler (strain RK-1)	Z21723
Cyanidium caldarium Geitler	X55524
Galdieria partite Tokara	AB018008
Mastocarpus papillatus (C. Agardh) Kützing	U04028
Palmaria palmata (L.) Kuntze	U28421
Porphyra purpurea (Roth) C. Agardh	U38804
Porphyridium aerugineum Geitler	X17597
Heterokontophyta	
Apedinella radians (Lohman) Campbell	AF015573
Aureococcus anophagefferens Hargraves *et* Sieburth	AF117906
Aureoumbra lagunensis Stockwell, De-Yoe, Hargraves *et* Johnson	AF117786
Bolidomonas mediterranea Guillou *et* Chrétiennot-Dinet	AF333977
Bolidomonas pacifica var. *eleuthera* Guillou *et* Chrétiennot-Dinet	AF333978
Botrydiopsis intercedens Vischer *et* Pascher	AF015587
Botrydium stoloniferum Mitra	AF064743
Bumilleriopsis filiformis Vischer	U89900
Chattonella subsalsa Biecheler	AF015581
Chromulina nebulosa Cienkowski	AF155876
Cylindrotheca sp.	M599080.1
Detonula confervacea (Cleve) Gran	AB018006
Dictyocha speculum Ehrenberg	AY043280
Elachista fucicola (Velley) Areschoug	AF055398
Eustigmatos magna Hibberd	AF015575
Heterosigma akashiwo (Hada) Hada ex Sournia	X61918
Hibberdia magna (Belcher) Andersen	AF015572
Mallomonas asmundae (Wujek *et* van der Veer) Nichols	AF015585
Nannochloropsis salina (Droop) Hibberd	AF015576
Odontella sinensis (Greville) Grunow	Z67753
Pelagococcus subviridis Norris	AF015580
Pelagomonas calceolata Andersen *et* Saunders	U89898
Petalonia fascia (O.F. Müller) Kuntze	AB022243
Phaeoschizochlamys mucosa Lemmerman	AF064747
Phaeothamnion confervicola Lagerheim	AF064746
Pseudopedinella elastica Skuja	U89899
Punctaria plantaginea (Roth) Greville	AF055410
Rhizochromulina sp.	AF015574
Sarcinochrysis marina Geitler	AF015585
Sphaerotrichia divaricata	AF055412
Stichogloea globosa Starmach	AF155584
Synura uvella Ehrenberg em. Koshikov	AF015586
Thalassiosira nordenskioeldii Cleve	AB018007
Vacuolaria virescens Cienkowski	AF015582
Vischeria helvetica (Vischer *et* Pascher) Hibberd	AF015579
Haptophyta	
Calcidiscus leptoporus (Murray *et* Blackman) Loeblich Jr *et* Tappan	AB043690
Calyptrosphaera sphaeroidea Schiller	AB043628
Chrysochromulina alifera Parke *et* Manton	AB043695
Chrysochromulina hirta Manton	AB043632
Chrysochromulina parva Lackey	AB043694
Chrysochromulina spinifera (Fournier) Pienaar *et* Norris	AB043700
Chrysochromulina sp. (strain TKB8936)	BAB20787
Cruciplacolithus neohelis (McIntyre *et* Bé) Reinhardt	AB043689
Emiliania huxleyi (Lohmann) Hay *et* Mohler	AB043631
Exanthemachrysis gayraliae Lepailleur	AB043701
Helicosphaera carteri (Wallich) Kamptner	AB043692
Imantonia rotunda Reynolds emend. Green *et* Pienaar	AB043696
Isochrysis galbana Parke emend. Green *et* Pienaar	AB043693
Rebecca salina (Carter) Green	AB043633
Platychrysis sp.	AB043699
Pleurochrysis carterae (Braarud *et* Fagerland) Christensen	D11140
Pleurochrysis haptonemofera (Inouye *et* Chihara) Gayral *et* Fresnel	AB043688
Prymnesium parvum N. Carter emend. Green, Hibberd *et* Pienaar	AB043698
Umbilicosphaera sibogae var. *foliosa* (Kamptner) Okada *et* McIntyre	D45843
Cryptophyta	
Guillardia theta Hill *et* Wetherbee	AF041468
Insertae sedis	
Unidentified endosymbiont of *Peridinium foliaceum*	U31876

and a cladistic analysis using 33 morphological characters (Daugbjerg 1996). The current classification of the Dictyochophyceae includes Dictyochales, Pedinellales, and Rhizochromulinales.

With the development of analytical methods using HPLC, pigment analyses have been increasingly used in the description of phytoplankton community structure. For silicoflagellates, only preliminary results on pigment composition of *Dictyocha fibula* have been published (van Valkenburg 1980) and at a time when HPLC techniques were not developed to allow pigment separation and identification to the extent possible today. The objective of this work was to report the pigment composition, using HPLC, of selected members of the Dictyochophyceae (i.e. *Dictyocha speculum*, *Apedinella radians*, and *Mesopedinella arctica*) and to determine the plastid-encoded *rbc*L gene from *D. speculum* to gain further insight into the evolutionary history of plastids and accessory pigments in heterokont algae and haptophytes.

MATERIALS AND METHODS

Cultures. The naked stages of *D. speculum* (strain K-0036), *M. arctica* (strain K-0508; unfortunately the culture has since died), and *A. radians* (strain K-0077) were obtained from the Scandinavian Culture Collection of Algae and Protozoa, University of Copenhagen. The cultures were grown at 4° C under ≈50 μmol photons·m^{-2}·s^{-1} irradiance and a 16:8-h light:dark cycle.

HPLC pigment analyses. Dense cultures were filtered onto 25-mm Advantec GF 75 glass fiber filters (Toyo Roshi Kaisha, Japan) that were stored in liquid nitrogen. Pigments were extracted by sonication on ice (15 min) of filters in 3 mL acetone, followed by extraction for 24 h at 4° C. The extracts were subsequently filtered (0.2 μm) into HPLC vials containing 1 mL water and were analyzed on a Shimazu LC 10A system (Holm and Halmby, Denmark) with a Supercosil C18 column (250 × 4.6 mm, 5 μm) using the method of Wright et al. (1991), modified according to Schlüter and Havskum (1997). Pigments were identified by retention times and absorption spectra (recorded by photodiode array detection, 400–800 nm) identical to those of authentic standards, which were purchased from the International Agency for ^{14}C Determination (Hørsholm, Denmark). The same standards were used as external standards for the quantification of pigments. Chl c_3 and diatoxanthin were identified by comparison of absorption spectra with those of reference cultures (*Chrysochromulina polylepis* Manton *et* Parke and *Ditylum brightwellii* [West] Grunow, respectively), and diatoxanthin was quantified using the HPLC response factor for diadinoxanthin. Chl c_1 and c_2 were not separated by the HPLC method used. Thus, in the following, chl *c* refers to chl c_1 and/or c_2.

DNA extraction and determination of rbc*L.* An exponentially growing nonaxenic culture (*ca.* 30 mL) was harvested by centrifugation (1500 rpm) for 10 min at 4° C. Most of the supernatant was discarded, and the pellet was resuspended and transferred to a 1.5-mL Eppendorf tube. After 2 days at −20° C, total DNA was extracted as outlined in Daugbjerg et al. (1994). Total genomic

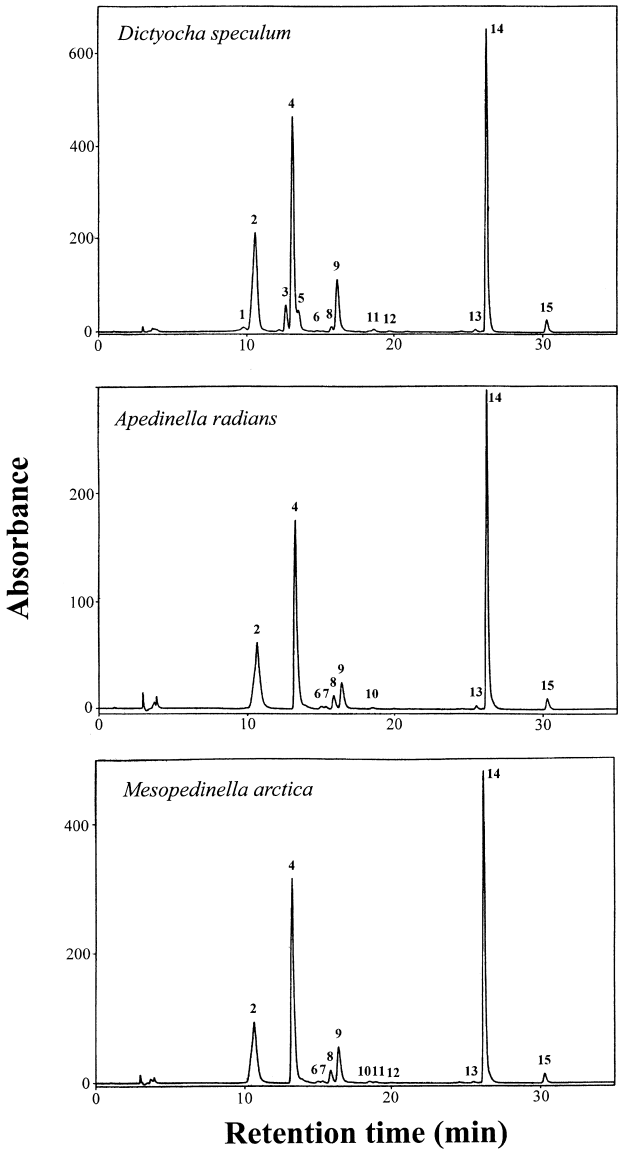


FIG. 1. HPLC absorbance chromatograms (436 nm) of *Dictyocha speculum* (top), *Apedinella radians* (middle), and *Mesopedinella arctica* (bottom). 1, chl c_3; 2, chl *c*; 3, 19′-butanoyloxyfucoxanthin; 4, fucoxanthin; 5, 19′-hexanoyloxyfucoxanthin; 6, violaxanthin; 7, unidentified carotenoid; 8, unidentified carotenoid; 9, diadinoxanthin; 10, antheraxanthin; 11, diatoxanthin; 12, zeaxanthin; 13, chl *a* allomer; 14, chl *a*; 15, β,β-carotene. Antheraxanthin identified from comparison of carotenoid elution order and absorption spectrum by Jeffrey et al. (1997).

TABLE 2. Carotenoids of *Dictyocha speculum*, *Apedinella radians*, and *Mesopedinella arctica* given as percentage of total identified and quantified carotenoids.

	D. speculum	*A. radians*[a]	*M. arctica*[a]
Fucoxanthin	66.9	71.1	80.0
19′-Butanoyloxyfucoxanthin	9.8	nd	nd
19′-Hexanoyloxyfucoxanthin	1.8	nd	nd
Violaxanthin	0.3	0.9	0.7
Diadinoxanthin	16.2	23.8	14.9
Diatoxanthin[b]	0.9	nd	0.5
Zeaxanthin	0.3	0.1	0.2
β,β-Carotene	3.8	4.1	3.7

[a] Trace amounts of antheraxanthin identified from carotenoid retention order and absorption spectrum given in Jeffrey et al. (1997).

[b] Diatoxanthin quantified using the HPLC response factor of diadinoxanthin.

nd, not detected.

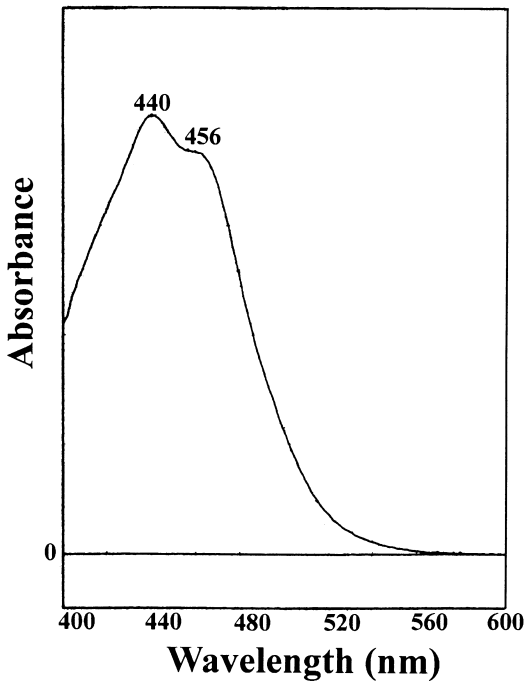


FIG. 2. Absorption spectrum (400–600 nm) of unidentified carotenoid eluting before diadinoxanthin (peak 8 in Fig. 1).

DNA was used as a template to amplify ≈1400 base pairs of the *rbc*L gene using terminal primers PrL1 (Fujiwara et al. 1994, 2001) and ND*rbc*S (5′-TCAAATAATGGWARACCC-3′, note two degenerate sites). The PCR amplification volume was 50 μL (67 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 200 μM dNTP, 0.5 μM of each primer, and 0.2 units of Taq DNA polymerase; Amersham Pharmacia Biotech, Buckinghamshire, UK). The amplification temperature profile was one initial cycle of denaturation at 94° C for 3 min, followed by 32 cycles of denaturation at 94° C for 1 min, annealing at 50° C for 1 min, and extension at 72° C for 3 min. The amplification profile was completed by a final extension at 72° C for 6 min. After a check in a 2% agarose gel, PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Nucleotide sequences were determined according to manufacturer's recommendations for the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA). Cycle-sequence reactions were run on an ABI PRISM 377 DNA Sequencer (Perkin Elmer). Terminal and internal primers (Daugbjerg and Andersen 1997a) were used to determine more than 97% of the *rbc*L gene in both directions. We used BioEdit version 5.0.2 (Hall 1999) for the unambiguous alignment.

Phylogenetic analyses. In phylogenetic analyses of coding DNA sequences, third positions are often down-weighted or omitted from the data matrix. The rationale is that third positions evolve at a higher rate than first and second positions and may therefore over time become saturated (a balance between forward and backward substitutions is reached). Recent studies have addressed the assumption that third positions (= saturated sites) are noisy characters comprising a larger amount of misleading information and therefore are likely to distort the phylogenetic signal (e.g. Björklund 1999, Källersjö et al. 1997, 1999, Wenzel and Siddall 1999). Contrary to what is often presumed by molecular systematists, these studies conclude that there is no justification for down-weighting or excluding third positions. Källersjö et al. (1999) even observed that homoplasy increased the phylogenetic structure in a data matrix comprising 2538 *rbc*L sequences from green algae and higher plants. Hence, all codon positions were weighted equally. The computer program PAUP* version 4.0b6 (Swofford 1998) was implemented for parsimony, maximum likelihood (ML), and LogDet transformation. All analyses included positions 100–1473 relative to the complete *rbc*L sequence of the diatom *Odontella sinensis* and the data matrix comprised a diverse assemblage of chl *a*+*c*-containing algae (Table 1). Alignment gaps were treated as missing data.

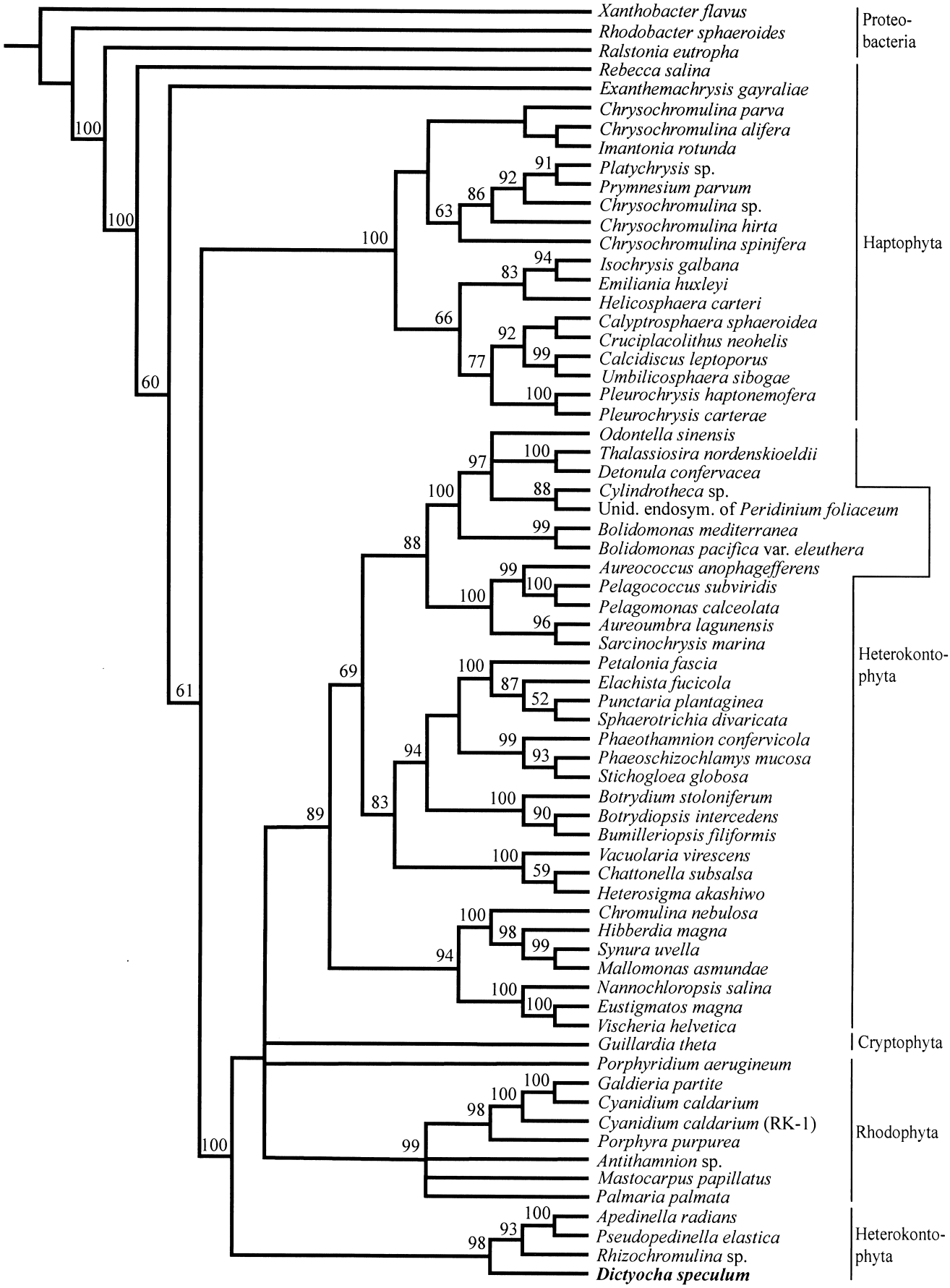
Parsimony analyses used the heuristic search option with branch swapping (tree bisection reconnection) and random addition of sequences (1000 replications). Bootstrap analysis with 1000 replications was applied to assess the robustness of clades (Felsenstein 1985), and the pseudo-samples were weighted according to the rescaled consistency index (over an interval of 1–1000, Bhattacharya 1996). Only bootstrap values supporting a particular node in at least 50% of the pseudo-samples are shown. In ML analysis the F84 model was used. The transition to transversion ratio was set to 0.7 (based on an empirical estimation) and equal divergence rates over all sites. Sequences were added randomly, and five replications were conducted. Bootstrap analysis was not performed in ML analysis due to the large number of taxa included. LogDet transformation (Lockhart et al. 1994) was applied to avoid artificial clustering due to divergence rate differences and a biased nucleotide content of the *rbc*L sequences included (see Daugbjerg and Andersen 1997a).

Outgroup. The RUBISCO operon has undergone a complex evolutionary history involving most likely a horizontal gene transfer of proteobacterial RUBISCO to the ancestral red alga and numerous horizontal transfers in the proteobacteria (for details, see Delwiche and Palmer 1996, 1997). Hence, we used *rbc*L sequences from three proteobacteria with type I RUBISCO as the outgroup.

RESULTS

HPLC analyses revealed that all three species examined contained chl *a* and *c* and fucoxanthin as the main carotenoid (Fig. 1). Additionally, diadinoxanthin, an unidentified carotenoid eluting before diadinoxanthin (Fig. 2), violaxanthin, zeaxanthin, and β,β-carotene were detected in all species (Table 2). Diatoxanthin was only observed in *D. speculum* and *M. arctica*, whereas *A. radians* and *M. arctica* contained small amounts of antheraxanthin, as identified from the carotenoid retention order and absorption spectrum given in Jeffrey et al. (1997). *Dictyocha speculum* differed from the two pedinellids by the additional possession of chl c_3 and the fucoxanthin derivatives 19′-butanoyloxy-fucoxanthin (19′-but) and 19′-hexanoyloxy-fucoxanthin (19′-hex) (Fig. 1, Table 2). In *D. speculum* 19′-but constituted almost 10% of the total amount of identified carotenoids and dominated over 19′-hex by a factor of 5.4 (Table 2).

The *rbc*L sequence of *D. speculum* was PCR amplified using a forward primer designed for haptophytes and a reverse primer designed for heterokonts and unambiguously aligned with 66 other *rbc*L sequences available in GenBank (Table 1). The data matrix was compiled to include a diverse assemblage of chl *a*+*c*-containing algae. In parsimony, ML, and LogDet transformation analyses, *D. speculum* formed a sister taxon to the clade containing *Rhizochromulina* sp., *Apedinella radians*, and *Pseudopedinella elastica* (Figs. 3–5). The monophyly of Dictyochophyceae was well supported in terms of bootstrap values in parsimony


Xanthobacter flavus
Rhodobacter sphaeroides
Ralstonia eutropha
Rebecca salina
Exanthemachrysis gayraliae
Chrysochromulina parva
Chrysochromulina alifera
Imantonia rotunda
Platychrysis sp.
Prymnesium parvum
Chrysochromulina sp.
Chrysochromulina hirta
Chrysochromulina spinifera
Isochrysis galbana
Emiliania huxleyi
Helicosphaera carteri
Calyptrosphaera sphaeroidea
Cruciplacolithus neohelis
Calcidiscus leptoporus
Umbilicosphaera sibogae
Pleurochrysis haptonemofera
Pleurochrysis carterae
Odontella sinensis
Thalassiosira nordenskioeldii
Detonula confervacea
Cylindrotheca sp.
Unid. endosym. of Peridinium foliaceum
Bolidomonas mediterranea
Bolidomonas pacifica var. eleuthera
Aureococcus anophagefferens
Pelagococcus subviridis
Pelagomonas calceolata
Aureoumbra lagunensis
Sarcinochrysis marina
Petalonia fascia
Elachista fucicola
Punctaria plantaginea
Sphaerotrichia divaricata
Phaeothamnion confervicola
Phaeoschizochlamys mucosa
Stichogloea globosa
Botrydium stoloniferum
Botrydiopsis intercedens
Bumilleriopsis filiformis
Vacuolaria virescens
Chattonella subsalsa
Heterosigma akashiwo
Chromulina nebulosa
Hibberdia magna
Synura uvella
Mallomonas asmundae
Nannochloropsis salina
Eustigmatos magna
Vischeria helvetica
Guillardia theta
Porphyridium aerugineum
Galdieria partite
Cyanidium caldarium
Cyanidium caldarium (RK-1)
Porphyra purpurea
Antithamnion sp.
Mastocarpus papillatus
Palmaria palmata
Apedinella radians
Pseudopedinella elastica
Rhizochromulina sp.
Dictyocha speculum
Proteo-bacteria
Haptophyta
Heterokonto-phyta
Cryptophyta
Rhodophyta
Heterokonto-phyta

analyses (98%) but received only moderate support in LogDet transformation (64%). Surprisingly however, it clustered outside the lineage comprising the other heterokont algae (i.e. diatoms/bolido-/pelago-/phaeo-/phaeothamnio-xantho-/raphido-/chryso-/synuro-/eustigmatophytes). Instead, the Dictyochophyceae formed a sister group to the "other" heterokont algae and the red algae (Figs. 3–5). The Haptophyta formed the earliest diverging branch in these analyses, with *Rebecca* and *Exanthemachrysis* forming a sister group to the remaining haptophytes. The Haptophyte taxa assigned to clade B and C *sensu* Edvardsen et al. (2000) formed monophyletic clusters. In terms of bootstrap values, the branching order was generally not well resolved for the deepest nodes (low bootstrap values in parsimony and LogDet transformation analyses, Figs. 3 and 5, respectively), but importantly the heterokont algae (excluding Dictyochophyceae) received relatively high bootstrap support in parsimony analyses (89%) and moderate support in LogDet transformation (68%). In the heterokont lineage (excluding the Dictyochophyceae), the Eustigmatophyceae/Chrysophyceae (including *Synura* and *Mallomonas*) formed one clade (94% in parsimony and 79% in LogDet), the pelagophytes/diatoms/bolidophytes formed a clade (bootstrap $\geq$ 88%), and the raphido-/xantho-phaeo-/phaeothamniophytes also formed a clade (83% bootstrap support in parsimony, no bootstrap support for this in LogDet transformation).

The unexpected position of the dictyochophyte clade outside the heterokont lineage was studied further by performing a parsimony analysis that excluded third positions despite having no justification for this *a priori* weighting scheme (see comments on the use of third codon positions in Materials and Methods). Parsimony analyses produced 195 equally parsimonious trees, each 2194 steps (consitency index [CI] = 0.334 and retention index [RI] = 0.667). This analysis revealed that the dictyochophyte clade still clustered outside the main heterokont lineage but now as the sister group to the haptophytes. In general, the tree topology was less resolved compared with the parsimony analysis, including all positions, but the major branching pattern for the remaining heterokont taxa was almost the same. The topology for the haptophytes was not well resolved (data not shown). Hence, the position of the dictyochophytes outside the main heterokont lineage cannot be explained due to the inclusion of third positions.

DISCUSSION

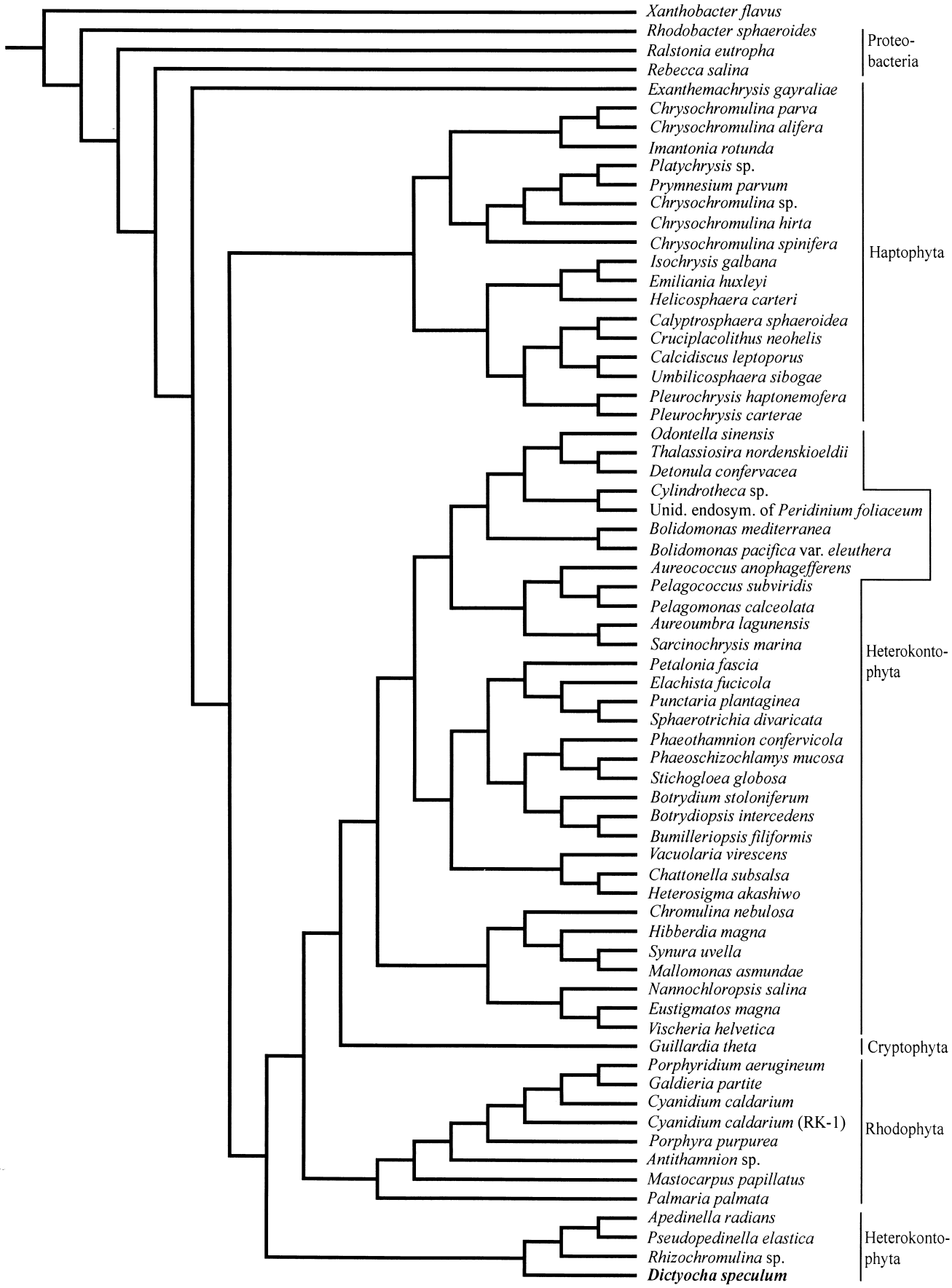
The carotenoid composition of *D. speculum*, *A. radians*, and *M. arctica*, characterized by the predominance of fucoxanthin and diadinoxanthin, and with trace amounts of diatoxanthin detected in *D. speculum* and *M. arctica*, corresponds to that described for *Rhizochromulina marina* and *Sarcinochrysis marina* (Bjørnland and Liaaen-Jensen 1989). Unlike what was found for *S. marina* (Withers et al. 1981), the dictyochophytes examined here contained minor amounts of violaxanthin, zeaxanthin, and, presumably, antheraxanthin, a carotenoid combination characteristic of the chrysophytes and synurophytes (Withers et al. 1981, Bjørnland and Liaaen-Jensen 1989).

The pigment composition of *D. speculum* is particularly interesting, because it also comprises chl c_3 and the acylfucoxanthins 19′-but and 19′-hex. Thus, the pigmentation of this species shows similarities to the pelagophytes *Pelagomonas calceolata* (Andersen et al. 1993, Bidigare 1989), *Pelagococcus subviridis* (Vesk and Jeffrey 1987), and *Aureococcus anophagefferens* (Bidigare 1989). However, the pelagophytes do not possess 19′-hex. Based on pigment composition of 29 species, Jeffrey and Wright (1994) described four types of haptophytes, of which "type 4" was characterized by the presence of chl c_3, 19′-but, 19′-hex, and fucoxanthin, similarly to that found in *D. speculum*. In a preliminary pigment analysis of another silicoflagellate, *Dictyocha fibula*, van Valkenburg (1980) found chl *a* and *c*, fucoxanthin, diadinoxanthin, diatoxanthin, lutein, and carotenes. This study was undertaken before the characterization of 19′-but and 19′-hex and the identified fucoxanthin therefore possibly could have contained minor amounts of the acylfucoxanthins. The pigment composition of *D. fibula* should be reexamined using modern techniques.

Deducing phytoplankton community structure from pigment signatures of natural samples is less time consuming than traditional examinations using the microscope (Schlüter and Havskum 1997). However, the pigment composition of *D. speculum* may have implications for qualitative and quantitative studies of phytoplankton communities based solely on HPLC analyses. The presence of 19′-but in *D. speculum* (up to 10% of the total amount of carotenoids) has undoubtedly resulted in overestimation of the biomass of other 19′-but possessing phytoplankton groups such as haptophytes or pelagophytes in cases where the silicoflagellate was present. In addition to silicoflagellates, several dinoflagellates contain 19′-but and 19′-hex (e.g. Hansen et al. 2000). Future studies using pigment signatures to account for the biomass of phytoplankton groups need to consider the diverse distribution of the acylfucoxanthins for proper estimations of group-specific contributions to chl *a*.

The phylogenetic analyses consistently grouped *D. speculum* with the other dictyochophytes but outside the lineage comprising the other classes of heterokont

FIG. 3. Parsimony analysis (heuristic search option and 1000 random additions of sequences) of a 1389-base pair fragment of the plastid-encoded *rbc*L gene produced five most parsimonious trees (tree length, 7569 steps; consistency index = 0.237 and retention index = 0.561). Bootstrap values (≥50%) from 1000 replications were inferred from maximum parsimony analysis using a weighted rescaled consistency index over an interval of 1–1000. The proteobacteria were used to root the tree.


Xanthobacter flavus
Rhodobacter sphaeroides
Ralstonia eutropha
Rebecca salina
Proteo-bacteria
Exanthemachrysis gayraliae
Chrysochromulina parva
Chrysochromulina alifera
Imantonia rotunda
Platychrysis sp.
Prymnesium parvum
Chrysochromulina sp.
Chrysochromulina hirta
Chrysochromulina spinifera
Isochrysis galbana
Emiliania huxleyi
Helicosphaera carteri
Calyptrosphaera sphaeroidea
Cruciplacolithus neohelis
Calcidiscus leptoporus
Umbilicosphaera sibogae
Pleurochrysis haptonemofera
Pleurochrysis carterae
Haptophyta
Odontella sinensis
Thalassiosira nordenskioeldii
Detonula confervacea
Cylindrotheca sp.
Unid. endosym. of Peridinium foliaceum
Bolidomonas mediterranea
Bolidomonas pacifica var. eleuthera
Aureococcus anophagefferens
Pelagococcus subviridis
Pelagomonas calceolata
Aureoumbra lagunensis
Sarcinochrysis marina
Petalonia fascia
Elachista fucicola
Punctaria plantaginea
Sphaerotrichia divaricata
Phaeothamnion confervicola
Phaeoschizochlamys mucosa
Stichogloea globosa
Botrydium stoloniferum
Botrydiopsis intercedens
Bumilleriopsis filiformis
Vacuolaria virescens
Chattonella subsalsa
Heterosigma akashiwo
Chromulina nebulosa
Hibberdia magna
Synura uvella
Mallomonas asmundae
Nannochloropsis salina
Eustigmatos magna
Vischeria helvetica
Heterokonto-phyta
Guillardia theta
Cryptophyta
Porphyridium aerugineum
Galdieria partite
Cyanidium caldarium
Cyanidium caldarium (RK-1)
Porphyra purpurea
Antithamnion sp.
Mastocarpus papillatus
Palmaria palmata
Rhodophyta
Apedinella radians
Pseudopedinella elastica
Rhizochromulina sp.
Dictyocha speculum
Heterokonto-phyta

algae. Although the branching pattern for some of the deepest nodes was unresolved in terms of bootstrap values, major clades such as haptophytes, red algae, Dictyochophyceae, and the heterokont algae (excluding the dictyochophytes) were observed.

Because red algae possess neither 19′-but nor 19′-hex and *D. speculum* and type 4 haptophytes (e.g. *Imantonia rotunda*) are not closely related, it can be speculated that the possession of acylfucoxanthins and chl c_3 evolved separately after the uptake of red algal endosymbionts. Alternatively, genetic transfer via a vector could also explain the identical pigment profile in *D. speculum* and type 4 haptophytes. Despite a different pigment composition in *D. speculum* and the two pedinellids (Fig. 1), they form sister groups (Figs. 3–5). Hence, the phylogenetic framework presented here indicates that a different pigment profile may occur even among closely related taxa. The marine and freshwater raphidophytes constitute another example (e.g. Bjørnland and Liaaen-Jensen 1989, Daugbjerg and Andersen 1997b). The evolutionary history of carotenoid biosynthetic pathways is complex with multiple origins and secondary losses (see also Daugbjerg and Andersen 1997b), and the use of carotenoids as reliable phylogenetic markers is dubious.

If the *rbc*L-based tree mirrors plastid evolution as it occurred in the evolutionary history of the heterokonts, it offers an admittedly complex explanation. An ancestral heterotrophic heterokont engulfed and retained a red alga (i.e. monophyletic origin of plastids in heterokonts) and later diverged into the lineages present today. One of these, the dictyochophyte lineage, secondarily lost its plastids but later captured another red alga. This scenario requires two secondary endosymbiotic events (plastid gains) and a single loss of plastids. Nuclear-encoded SSU rDNA analyses have revealed that the Dictyochophyceae diverged relatively late in the evolution of the heterokont algae (e.g. Cavalier-Smith and Chao 1996, van de Peer et al. 1996). Analyses of SSU rDNA have also convincingly demonstrated that the dictyochophytes *Ciliophrys infusionum* Cienkowski and *Pteridomonas danica* Patterson *et* Fenchel lost their plastids independently (Cavalier-Smith et al. 1995, Cavalier-Smith and Chao 1996). This illustrates that secondary loss of plastids in the heterokont lineage has occurred (probably also in the chrysophytes and diatoms). The nuclear-encoded SSU rDNA data do not reject the rather complex scenario for the evolution of plastids outlined above. Establishing new symbiont relationships might involve fewer genes being transferred from the plastid of the "recently" engulfed symbiont to the host nucleus if it is related to that lost secondarily. The genes controlling plastid division and function may already have been incorporated in the host nucleus. For a symbiont to become part of the host organism, numerous genetic rearrangements are required. Despite such a complex series of events, secondary endosymbioses have occurred multiple times in the dinoflagellates alone.

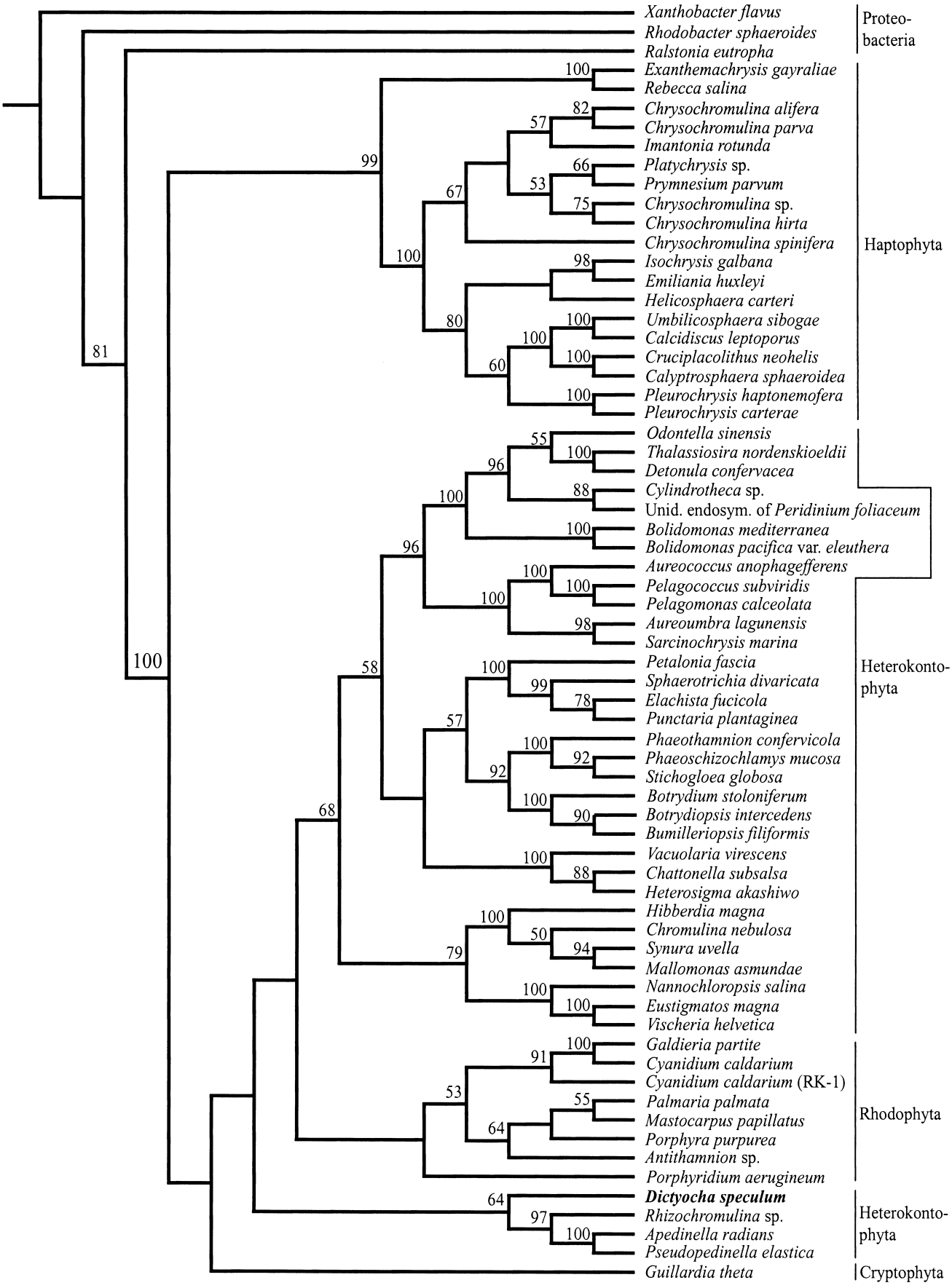
Based on a comparison of the gene order in the plastid genome of the brown alga *Dictyota dichotoma* and in the centric diatom *Odontella sinensis*, Kowallik (1997) suggested an independent origin of the plastids from different red algal symbionts. Phylogenetic inferences using plastid-encoded genes (*rbc*L, *tuf*A, and SSU rDNA) do not suggest that diatoms and brown algae have acquired their plastids separately (e.g. Daugbjerg and Andersen, 1997b, Medlin et al. 1997). Instead, the plastid genome in these heterokont algae has probably undergone a higher rate of rearrangements compared with red algae.

Because of the minute size of phagotrophic heterokonts, it is reasonable to speculate that the red alga engulfed by the ancestor to the heterokont algae was single-celled rather than multicellular. Only few single-celled red algae are known. Three genera were included in the *rbc*L-based phylogenetic inference to examine if one showed a relationship to the plastids in the heterokont algae. The single-celled species *Cyanidium caldarium* and *Galdieria partite* formed a sister group relationship within the multicellular red algae, suggesting that they are secondarily reduced. *Porphyridium aerugineum*, the third single-celled species included, was the most divergent red alga, but it did not cluster with dictyochophytes or the other heterokont algae. The phylogenetic trees of the unicellular red algae obtained by us are incongruent with a recent analysis based on plastid-encoded SSU rDNA by Oliveira and Bhattacharya (2000). Their analyses suggested that *C. caldarium* (strain RK-1) is related to the direct ancestor of plastids in heterokonts. To further elucidate the evolutionary history of the plastid in heterokont algae, future molecular studies should include a higher number of single-celled red algae and additional genetic markers.

CONCLUSIONS

In this study we show that the silicoflagellate *D. speculum* possesses a pigment composition that differs from the closely related pedinellids and *Rhizochromulina* and instead resembles type 4 haptophytes. Qualitative studies of phytoplankton composition in natural samples using HPLC may therefore have masked the occurrence of *D. speculum* and overestimated the biomass of type 4 haptophytes or pelagophytes. If the clustering of the Dictyochophyceae outside the other heterokont algae in *rbc*L trees is correct, it implies that the evolutionary history of plastids in these organisms is more complex than previously believed. Determina-

FIG. 4. Maximum likelihood analysis of a 1389-base pair fragment of the plastid-encoded *rbc*L gene. A transition-to-transversion ratio of 0.7 was used to estimate the ln likelihood score (−38246.916). The proteobacteria were used to root the tree. Because of the large number of taxa, bootstrap analysis was not performed.


Xanthobacter flavus
Rhodobacter sphaeroides
Ralstonia eutropha
Proteo-bacteria
Exanthemachrysis gayraliae
Rebecca salina
Chrysochromulina alifera
Chrysochromulina parva
Imantonia rotunda
Platychrysis sp.
Prymnesium parvum
Chrysochromulina sp.
Chrysochromulina hirta
Chrysochromulina spinifera
Isochrysis galbana
Emiliania huxleyi
Helicosphaera carteri
Umbilicosphaera sibogae
Calcidiscus leptoporus
Cruciplacolithus neohelis
Calyptrosphaera sphaeroidea
Pleurochrysis haptonemofera
Pleurochrysis carterae
Haptophyta
Odontella sinensis
Thalassiosira nordenskioeldii
Detonula confervacea
Cylindrotheca sp.
Unid. endosym. of Peridinium foliaceum
Bolidomonas mediterranea
Bolidomonas pacifica var. eleuthera
Aureococcus anophagefferens
Pelagococcus subviridis
Pelagomonas calceolata
Aureoumbra lagunensis
Sarcinochrysis marina
Petalonia fascia
Sphaerotrichia divaricata
Elachista fucicola
Punctaria plantaginea
Phaeothamnion confervicola
Phaeoschizochlamys mucosa
Stichogloea globosa
Botrydium stoloniferum
Botrydiopsis intercedens
Bumilleriopsis filiformis
Vacuolaria virescens
Chattonella subsalsa
Heterosigma akashiwo
Hibberdia magna
Chromulina nebulosa
Synura uvella
Mallomonas asmundae
Nannochloropsis salina
Eustigmatos magna
Vischeria helvetica
Heterokonto-phyta
Galdieria partite
Cyanidium caldarium
Cyanidium caldarium (RK-1)
Palmaria palmata
Mastocarpus papillatus
Porphyra purpurea
Antithamnion sp.
Porphyridium aerugineum
Rhodophyta
Dictyocha speculum
Rhizochromulina sp.
Apedinella radians
Pseudopedinella elastica
Heterokonto-phyta
Guillardia theta
Cryptophyta

tion of more plastid-encoded genes is pivotal to further elucidate the origin of plastids in heterokont algae. Also, the arrangement of genes in the plastid genome of dictyochophytes would set the stage for an improved understanding of their plastid evolution.

We thank Winnie Martinsen and Susanne Hemmingsen from the National Environmental Research Institute and Charlotte Hansen from the Botanical Institute for technical assistance at various stages of this work. Prof. Øjvind Moestrup kindly commented on an earlier version of the manuscript. Supported by the EU MAST III project MIDAS (contract no. MAS3-CT97-0154) and the Danish Science Foundation (grant no. 96701499 to ND).

Andersen, R. A., Saunders, G. W., Paskind, M. P. & Sexton, J. P. 1993. Ultrastructure and 18S rRNA gene sequence for *Pelagomonas calceolata* gen. et sp. nov. and the description of a new algal class. *J. Phycol.* 29:701–15.

Bhattacharya, D. 1996. Analysis of the distribution of bootstrap tree lengths using the maximum parsimony method. *Mol. Phyl. Evol.* 6:339–50.

Bidigare, R. R. 1989. Photosynthetic pigment composition of the brown tide alga: unique chlorophyll and carotenoid derivatives. *In* Cosper, E. M., Bricelj, V. M. & Carpenter E. J. [Eds.] *Novel Phytoplankton Blooms: Causes and Impacts of Recurrent Brown Tides and Other Unusual Blooms.* Springer Verlag, New York, pp. 57–75.

Björklund, M. 1999. Are third positions really that bad? A test using vertebrate cytochrome b. *Cladistics* 15:191–7.

Bjørnland, T. & Liaaen-Jensen, S. 1989. Distribution patterns of carotenoids in relation to chromophyte phylogeny and systematics. *In* Green, J. C., Leadbeater, B. S. C. & Diver, W. L. [Eds.] *The Chromophyte Algae: Problems and Perspectives.*. Clarendon Press, Oxford, pp. 37–60.

Cavalier-Smith, T., Chao, E. E. & Allsopp, T. E. P. 1995. Ribosomal RNA evidence for chloroplast loss within Heterokonta: pedinellid relationships and a revised classification of Ochristan algae. *Arch. Protisenkd.* 145:209–20.

Cavalier-Smith, T. & Chao, E. E. 1996. 18S rRNA sequence of *Heterosigma carterae* (Raphidophyceae) and the phylogeny of heterokont algae (Ochrophyta). *Phycologia* 35:500–10.

Daugbjerg, N., Moestrup, Ø. & Arctander, P. 1994. Phylogeny of the genus *Pyramimonas* (Prasinophyceae, Chlorophyta) inferred from the *rbc*L gene. *J. Phycol.* 30:991–9.

Daugbjerg, N. 1996. *Mesopedinella arctica* (Pedinellales) II. Phylogeny of *Mesopedinella*, including a cladistic analysis of Dictyochophyceae. *Phycologia* 35:563–8.

Daugbjerg, N. & Andersen, R. A. 1997a. Phylogenetic analyses of the *rbc*L sequences from haptophytes and heterokont algae suggest their chloroplasts are unrelated. *Mol. Biol. Evol.* 14: 1242–51.

Daugbjerg, N. & Andersen, R. A. 1997b. A molecular phylogeny of the heterokont algae based on analyses of chloroplast-encoded *rbc*L sequence data. *J. Phycol.* 33:1030–41.

Delwiche, C. F. & Palmer, J. D. 1996. Rampant horizontal transfer and duplication of Rubisco genes in eubacteria and plastids. *Mol. Biol. Evol.* 13:873–82.

Delwiche, C. F. & Palmer, J. D. 1997. The origin of plastids and their spread via secondary symbiosis. *Plant Syst. Evol.* 11 (Suppl.):53–86.

Edvardsen, B., Eikrem, W., Green, J. C., Moon-van der Staay, S. Y., Andersen, R. A. & Medlin, L. K. 2000. Phylogenetic reconstructions of the Haptophyta inferred from 18S ribosomal DNA sequences and available morphological data. *Phycologia* 39:19–35.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–91.

Fujiwara, S., Sawada, M., Someya, J., Minaka, N., Kawachi, M. & Inouye, I. 1994. Molecular phylogenetic analysis of *rbc*L in the Prymnesiophyta. *J. Phycol.* 30:863–71.

Fujiwara, S., Tsuzuki, M., Kawachi, M., Minaka, N. & Inouye, I. 2001. Molecular phylogeny of the Haptophyta based on the *rbc*L gene and sequence variation in the spacer region of the RUBISCO operon. *J. Phycol.* 37:121–9.

Guillou, L., Chrétiennot-Dinet, M.-J., Medlin, L. K. Claustre, H., Loiseaux-de Goër, S. & Vaulot, D. 1999. *Bolidomonas*: a new genus with two species belonging to a new algal class, the Bolidophyceae (Heterokonta). *J. Phycol.* 35:368–81.

Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41:95–8.

Hansen, G., Daugbjerg, N. & Henriksen, P. 2000. Comparative study of *Gymnodinium mikomotoi* and *Gymnodinium aureolum*, comb. nov. (=*Gyrodinium aureolum*) based on morphology, pigment composition, and molecular data. *J. Phycol.* 36:394–410.

Henriksen, P., Knipschildt, F., Moestrup, Ø. & Thomsen, H. A. 1993. Autecology, life history and toxicology of the silicoflagellate *Dictyocha speculum* (Silicoflagellata, Dictyochophyceae). *Phycologia* 32:29–39.

Jeffrey, S. W. & Wright, S. W. 1994. Photosynthetic pigments in the Haptophyta. *In* Green, J. C. & Leadbeater, B. S. C. [Eds.] *The Haptophyte Algae.* Clarendon Press, Oxford, pp. 111–32.

Jeffrey, S. W., Mantoura, R. F. C. & Bjørnland, T. 1997. Data for the identification of 47 key phytoplankton pigments. *In* Jeffrey, S. W., Mantoura, R. F. C. & Wright, S. W. [Eds.] *Phytoplankton Pigments in Oceanography.* UNESCO Publishing, Paris, pp. 447–559.

Jochem, F. & Babenerd, B. 1989. Naked *Dictyocha speculum*—a new type of phytoplankton bloom in the Western Baltic. *Mar. Biol.* 103:373–9.

Källersjö, M., Farris, J. S., Chase, M. W., Bremer, B., Fay, M. F., Humphries, C. J., Petersen, G., Seberg, O. & Bremer, K. 1997. Simultaneous parsimony jacknife analysis of 2538 *rbc*L DNA sequences reveals support for major clades of green plants, land plants, seed plants and flowering plants. *Plant Syst. Evol.* 213: 259–87.

Källersjö, M., Albert, V. A. & Farris, J. S. 1999. Homoplasy increases phylogenetic structure. *Cladistics* 15:91–3.

Kowallik, K. V. 1997. Origin and evolution of chloroplasts: current status and future perspectives. *In* Schenk, H. E. A., Hermann, R. G., Jeon, K. W., Müller, N. E. & Schwemmler, W. [Eds.] *Eukaryotism and Symbiosis.* Springer-Verlag, Berlin, pp. 3–23.

Lockhart, P. J., Steel, M. A. & Penny, D. 1994. Recovering the correct tree under a more realistic model of evolution. *Mol. Biol. Evol.* 11:605–12.

Medlin, L. K., Kooistra, W. H. C. F., Potter, D., Saunders, G. W. & Andersen, R. A. 1997. Phylogenetic relationships of the "golden algae" (haptophytes, heterokont chromophytes) and their plastids. *Plant Syst. Evol.* 11(Suppl.):187–219.

Moestrup, Ø. 1995. Current status of chrysophyte splinter groups: synurophytes, pedinellids, silicoflagellates. *In* Sandgren, C. D., Smol, J. P. & Kristiansen, J. [Eds.] *Chrysophyte Algae.* Cambridge University Press, Cambridge, pp. 75–91.

Moestrup, Ø. & Thomsen, H. A. 1990. *Dictyocha speculum* (Silicoflagellata, Dictyochophyceae), studies an armoured and unarmoured stages. *Biol. Skrift.* 37:1–57.

Oliveira, M. C. & Bhattacharya, D. 2000. Phylogeny of the Bangiophycidae (Rhodophyta) and the secondary endosymbiotic origin of algal plastids. *Am. J. Bot.* 87:482–92.

Schlüter, L. & Havskum, H. 1997. Phytoplankton pigments in relation to carbon content in phytoplankton communities. *Mar. Ecol. Prog. Ser.* 155:55–65.

Swofford, D. L. 1998. *PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods).* Version 4. Sinauer Associates, Sunderland, Massachusetts.

FIG. 5. LogDet transformation of a 1389-base pair fragment of the plastid-encoded *rbc*L gene. Bootstrap values (≥50%) from 1000 replications are shown above nodes. The three proteobacteria were used to root the tree.

Tappan, H. 1980. *The Paleobiology of Plant Protists.* Freeman, San Francisco, 1029 pp.

Throndsen, J. 1993. The planktonic marine flagellates. *In* Tomas, C. R. [Ed.] *Identifying Marine Phytoplankton.* Academic Press, San Diego, pp. 591–729.

van de Peer, Y., van der Auwera, G. & de Wachter, R. 1996. The evolution of Straminopiles and alveolates as derived by "substitution rate calibration" of small ribosomal subunit RNA. *J. Mol. Evol.* 42:201–10.

van Valkenburg, S. D. 1980. Silicoflagellates. *In* Cox, E. R. [Ed.] *Phytoflagellates.* Elsevier, North Holland, pp. 335–50.

Vesk, M. & Jeffrey, S. W. 1987. Ultrastructure and pigments of two strains of the picoplanktonic alga *Pelagococcus subviridis* (Chrysophyceae). *J. Phycol.* 23:322–36.

Wenzel, J. W. & Siddall, M. E. 1999. Noise. *Cladistics* 15:51–64.

Withers, N. W., Fiksdahl, A., Tuttle, R. C. & Liaaen-Jensen S. 1981. Carotenoids of the chrysophyceae. *Comp. Biochem. Physiol.* 68B: 345–9.

Wright, S. W., Jeffrey, S. W., Mantoura, R. F. C., Llewellyn, C. A., Bjørnland, T., Repeta, D. & Welschmeyer, N. 1991. Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Mar. Ecol. Prog. Ser.* 77:183–96.