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A MOLECULAR PHYLOGENY OF THE HETEROKONT ALGAE BASED ON ANALYSES OF CHLOROPLAST-ENCODED *rbc*L SEQUENCE DATA[1]

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ABSTRACT

*Nearly complete ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) sequences from 27 taxa of heterokont algae were determined and combined with* rbc*L sequences obtained from GenBank for four other heterokont algae and three red algae. The phylogeny of the morphologically diverse heterokont algae was inferred from an unambiguously aligned data matrix using the red algae as the root. Significantly higher levels of mutational saturation in third codon positions were found when plotting the pairwise substitutions with and without corrections for multiple substitutions at the same site for first and second codon positions only and for third positions only. In light of this observation, third codon positions were excluded from phylogenetic analyses. Both weighted-parsimony and maximum-likelihood analyses supported with high bootstrap values the monophyly of the nine currently recognized classes of heterokont algae. The Eustigmatophyceae were the most basal group, and the Dictyochophyceae branched off as the second most basal group. The branching pattern for the other classes was well supported in terms of bootstrap values in the weighted-parsimony analysis but was weakly supported in the maximum-likelihood analysis (<50%). In the parsimony analysis, the diatoms formed a sister group to the branch containing the Chrysophyceae and Synurophyceae. This clade, characterized by siliceous structures (frustules, cysts, scales), was the sister group to the Pelagophyceae/Sarcinochrysidales and Phaeo-/Xantho-/Raphidophyceae clades. In the latter clade, the raphidophytes were sister to the Phaeophyceae and Xanthophyceae. A relative rate test revealed that the* rbc*L gene in the Chrysophyceae and Synurophyceae has experienced a significantly different rate of substitutions compared to other classes of heterokont algae. The branch lengths in the maximum-likelihood reconstruction suggest that these two classes have evolved at an accelerated rate. Six major carotenoids were analyzed cladistically to study the usefulness of carotenoid pigmentation as a class-level character in the heterokont algae. In addition, each carotenoid was mapped onto both the* rbc*L tree and a consensus tree derived from nuclear-encoded small-subunit ribosomal DNA (SSU rDNA) sequences. Carotenoid pigmentation does not provide unambiguous phylogenetic information, whether analyzed cladistically by itself or when mapped onto phylogenetic trees based upon molecular sequence data.*

Key index words: carotenoids; chromophytes; evolution; heterokont algae; maximum-likelihood; parsimony; phylogeny; rbc*L; red algae; RuBisCo; stramenopiles*

The heterokont algae (= chromophytes, autotrophic stramenopiles) consist of nine major taxonomic groups: bacillariophytes, chrysophytes, dictyochophytes, eustigmatophytes, pelagophytes, phaeophytes, raphidophytes, synurophytes, and xanthophytes. The term "Heterokontae" was first applied taxonomically to algae now classified in the Xanthophyceae (= Tribophyceae) and Raphidophyceae (Luther 1899). The term "heterokont" has also been used to define flagellar morphology; cells with two often unequally long flagella that beat differently. More recently, the term "heterokont" has been used to define protists that have tripartite flagellar hairs (e.g. Patterson 1989), and this definition will be used in this paper. The heterokont algae are characterized by chloroplasts with similar ultrastructure and pigmentation, by certain flagellar features, and by an unusual carbohydrate storage product (Van den Hoek et al. 1995). Chloroplasts typically have lamellae comprised of three appressed thylakoids, a girdle lamella, and two additional surrounding membranes (chloroplast endoplasmic reticulum) (Dodge 1973). The chloroplasts have an abundance of carotenoids, providing most cells with a brownish color (Bjørnland and Liaaen-Jensen 1989). The long, immature flagellum has tripartite tubular hairs (= mastigonemes *sensu* Deflandre 1934, Leadbeater 1989), and in many cases, the transitional region of the flagellum bears a helical structure (Hibberd 1979, Preisig 1989). The tubular flagellar hairs constitute a synapomorphic character that unites the heterokont algae with other heterokont protists (Patterson 1989). Finally, the carbohydrate storage product is not starch but a small (degree of polymerization < 40) β-1,3-linked glucan that is stored as a solute in a vacuole (Craigie 1974, Wang and Bartnicki-Garcia 1974).

With few exceptions, the early literature on heterokont algae is almost devoid of phylogenetic discussions (see Blackman 1900, Bourrelly 1957). Except for a few attempts based upon ultrastructural

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and biochemical observations (e.g. Andersen 1991, Williams 1991a, b), most phylogenetic analyses have been based on small-subunit ribosomal DNA (SSU rDNA) gene sequences (e.g. Ariztia et al. 1991, Bhattacharya et al. 1992, Andersen et al. 1993, Leipe et al. 1994, 1996, Bhattacharya and Medlin 1995, Saunders et al. 1995, 1997, Cavalier-Smith et al. 1995, Cavalier-Smith and Chao 1996, Van de Peer et al. 1996). It now appears that the heterokont algae, as a group, are most closely related to certain protozoan groups (e.g. actinomonads, bicosoecids, labyrinthulids, oomycetes, slopalinids, and thraustochytrids; see Patterson 1989, Leipe et al. 1994, 1996). Despite these many studies and the establishment of a natural group of heterokont protists, the deep-branching relationships of the heterokont algae remain unclear. By combining ultrastructural, biochemical, and SSU rDNA sequence data, Saunders et al. (1995) suggested that those heterokont algae with a highly reduced flagellar apparatus formed a monophyletic cluster comprising diatoms, pelagophytes, and dictyochophytes. Using a new algorithm known as the substitution rate calibration (Van de Peer et al. 1993), which takes into account the significant differences in the evolutionary rate among different sites of the SSU rDNA gene, Van de Peer et al. (1996) showed a fairly well-resolved topology for eight heterokont classes. In that analysis, the diatoms branched off as the most basal group, but the monophyly of the reduced flagellar apparatus group was not supported.

As an independent alternative to the heterokont SSU rDNA data sets, we have determined the nucleotide sequence of the large subunit of the chloroplast-encoded gene, ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L), from 27 taxa of heterokont algae. A phylogenetic reconstruction based on a plastid gene not only provides an alternative for evolutionary studies of the organisms, but it also allows comparisons of phylogenies from host cells and their plastids. Furthermore, phylogenetic hypotheses based on chloroplast-encoded genes make it possible to study the evolutionary origin of algal plastids. In photosynthetic organisms, carotenoids are considered important chemosystematic markers, and with one exception (the Raphidophyceae), the distribution of carotenoids in the heterokont algae is typically congruent with the classification of the group at the class level. Although pigments have long been used to classify algae, phylogenetic analysis using carotenoids alone has not been conducted. We performed a cladistic analysis to evaluate the phylogenetic value of carotenoids, that is, does carotenoid composition define monophyletic groups or have carotenoids been gained or lost repeatedly during the evolution of the heterokont algae. Thus, our objectives were (1) to study the evolutionary history of the heterokont algae by establishing a phylogeny based on a chloroplast gene, (2) to compare the chloroplast phylogeny to phylogenies based on other gene sequences from different organelles (nuclear versus chloroplast), specifically the SSU rDNA gene, (3) to examine carotenoid phylogeny by analyzing six commonly occurring carotenoid pigments, and (4) to map carotenoid distribution on phylogenetic trees derived from nuclear and chloroplast gene sequences.

MATERIALS AND METHODS

Cultures. A list of heterokont algae for which the *rbc*L gene was determined is shown in Table 1. Cultures were obtained from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton (CCMP strain numbers), the Culture Collection of Algae at the University of Texas at Austin (UTEX strain numbers), the Scandinavian Culture Centre of Algae and Protozoa (K strain numbers), and the Sammlung von Algenkulturen der Universität Göttingen (SAG strain numbers).

Extraction, amplification, and sequencing of the rbc*L gene.* Approximately 30 mL of nonaxenic clonal cultures were centrifuged (400 g) for 15 min at room temperature. Pellets were resuspended in 500 μL preheated 2 × cetyltrimethylammonium bromide (CTAB) (2% hexadecyltrimethyl-ammonium bromide) isolation buffer and 1% β-mercapto-ethanol for 1–2 h at 60° C (Doyle and Doyle 1987) and transferred to Eppendorf tubes. Genomic DNA was extracted in 500 μL of 24:1 chloroform:isoamyl alcohol, precipitated with the GENECLEAN *II*® Kit (La Jolla, California) (BIO 101) according to the manufacturer's directions, resuspended in 25 μL H_2O and kept at −20° C. Double-stranded DNA was amplified in 100-μL reaction volumes containing 10 × PCR Buffer II (10 mM Tris-HCl, pH 8.3 [at 25° C]; 50 mM KCl), 200 μM dNTP, 0.2 or 1.0 μM of each primer, 2.5 units of Ampli Taq® DNA Polymerase (Roche Molecular Systems, Inc., Branchburg, New Jersey), and 2.5 mM $MgCl_2$. Amplification conditions were one initial cycle of denaturation at 94° C for 3 min, followed by 30 cycles of denaturation at 94° C for 1 min, annealing at 50° C for 1 min, and extension at 72° C for 2 min. The double-stranded PCR products were visualized in a 0.8% agarose gel containing 0.67 μg/mL ethidium bromide in a TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The resulting bands were cut out with razor blades, transferred to Eppendorf tubes, and melted in 700–900 μL NaI. Subsequent precipitations used the GENECLEAN *II*® Kit. Single-stranded DNA was obtained using the AmpliCycle™ sequencing kit (Perkin Elmer, Branchburg, New Jersey) following the recommendations from the manufacturer. Sequencing primers were biotinylated at the 5′ end. The sequence reactions were run in a 6% Long Ranger gel and transferred to an Immobilon-S membrane (Millipore Corporation, Milford, Massachusetts). The band patterns were detected using the NEBlot™ Phototope™ Kit (New England Biolabs, Beverly, Massachusetts) as recommended by the manufacturer and finally visualized by exposure on X-ray film. The nucleotide sequences of the amplification and sequencing primers used are given in Table 2.

Outgroup. Phylogenetic studies of cyanobacteria, proteobacteria, diverse groups of algae, and green plants have revealed that the red algal chloroplasts form the sister group to the heterokont algal chloroplasts (e.g. Morden et al. 1992, Delwiche and Palmer 1996, Daugbjerg and Andersen 1997). To polarize the ingroup taxa, we used the *rbc*L sequences from three red algae (GenBank accession numbers are given in parentheses): *Porphyra purpurea* (Roth) C. Agardh (U38804), *Porphyridium aerugineum* Geitler (X17597), and *Antithamnion* sp., (X54532).

Sequence availability. The *rbc*L sequences determined in this study have been submitted to GenBank and accession numbers are presented in Table 1. Additionally, we included *rbc*L sequences from the following taxa (with GenBank accession numbers given in parentheses): *Cylindrotheca* sp. strain N1 (M59080), *Odontella sinensis* (Grev.) Grunow (Z67753), *Heterosigma akashiwo* (Hada) Hada *ex* Y. Hara et Chihara (not *Olisthodiscus luteus* N. Carter as stated in older literature, X67918), *Pilayella littoralis* (L.) Kjellm. (X55372), *Ectocarpus siliculosus* (Dillwyn) Lyngb. (X53503).

Alignment and phylogenetic analyses of rbc*L.* The *rbc*L nucleotide

TABLE 1. *List of heterokont algae for which the ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) gene was determined. Strain numbers and GenBank accession numbers are also provided. Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), Scandinavian Culture Centre of Algae and Protozoa (K), Culture Collection of Algae at the University of Texas at Austin (UTEX), Sammlung von Algenkulturen der Universität Göttingen (SAG).*

Algal species	Strain number	GenBank accession no.
Bacillariophyceae		
Rhizosolenia setigera Brightwell	CCMP 1330	AF015568
Skeletonema costatum (Greville) Cleve	CCMP 1332	AF015569
Chrysophyceae		
Chrysolepidomonas dendrolepidota Peters et Andersen	CCMP 293	AF015570
Epipyxis pulchra Asmund et Hilliard	CCMP 382	AF015571
Hibberdia magna (Belcher) Andersen	CCMP 453	AF015572
Dictyochophyceae		
Apedinella radians (Lohman) Campbell	K-0077	AF015573
Pseudopedinella elastica Skuja	CCMP 716	U89899
Rhizochromulina sp.	CCMP 237	AF015574
Eustigmatophyceae		
Eustigmatos magna Hibberd	CCMP 387	AF015575
Nannochloropsis salina (Droop) Hibberd	CCMP 369	AF015576
Nannochloropsis sp.	CCMP 531	AF015577
Nannochloropsis sp.	CCMP 533	AF015578
Vischeria helvetica (Vischer et Pascher) Hibberd	UTEX 49	AF015579
Pelagophyceae		
Pelagococcus subviridis Norris	CCMP 1429	AF015580
Pelagomonas calceolata Andersen et Saunders	CCMP 1214	U89898
Raphidophyceae		
Chattonella subsalsa Biecheler	CCMP 217	AF015581
Vacuolaria virescens Cienkowski	SAG 1195	AF015582
Sarcinochrysidales		
Pulvinaria sp.	CCMP 292	AF015583
Sarcinochrysis marina Geitler	CCMP 770	AF015584
Synurophyceae		
Mallomonas asmundae (Wujek et Van der Veer) Nichols	CCMP 1658	AF015585
Synura uvella Ehrenberg em. Korshikov	CCMP 870	AF015586
Xanthophyceae (=Tribophyceae)		
Botrydiopsis intercedens Vischer et Pascher	UTEX 296	AF015587
Bumilleriopsis filiformis Vischer	UTEX 309	U89900
Tribonema intermixtum Pascher	K-0317	AF015588
Vaucheria bursata (Vauchi) de Candolle	CCMP 1084	AF015589
Incertae sedis		
Unidentified coccoid	CCMP 1395	AF015590
Unidentified coccoid	CCMP 1410	AF015591

sequences were aligned unambiguously by eye using the Eyeball Sequence Editor V. 1.09d (Cabot and Beckenbach 1989) and Compare Sequences V. 3.0 (Siegismund, unpubl.). Mutational saturation of third codon positions in the *rbc*L gene was examined by plotting all pairwise substitutions uncorrected for multiple substitutions against those corrected for multiple substitutions ("uncorrected p" and Kimura-2-parameter model, options available in PAUPstar V. 4.0.0d53, Swofford, unpubl.). For all pairwise combinations, values corrected and uncorrected for multiple substitutions were calculated for first/second codon positions only and third codon positions only. Due to the observed mutational saturation of third codon positions (silent sites), these data points were excluded prior to all analyses, reducing the data matrix to 954 base pairs. Phylogenetic analyses used weighted-parsimony and maximum-likelihood (Felsenstein 1981). Parsimony was performed with PAUP V. 3.1.1 (Swofford 1993) using the heuristic search option with random addition of sequences (100 replicates) and a branch-swapping algorithm (tree bisection-reconnection [TBR]). Nucleotide sites were reweighted (rescaled consistency index over an interval of 1–1000) and then were used as input for a bootstrap analysis. To find the optimal tree in maximum-likelihood analyses, searches were repeated by varying the transition to transversion ratio until the best maximum-likelihood score was reached using fastDNAml V. 1.0.8. (Olsen et al. 1994). Bootstrap analyses with 500 and 100 replications in weighted-parsimony and maximum-likelihood, respectively, were conducted in

TABLE 2. *Oligonucleotide primer sequences used to amplify and sequence heterokont algae. Numbers in parentheses refer to the position in the ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) gene of the brown alga* Pilayella littoralis. *Abbreviations (IUPAC code): S (C/G), H (A/T/C), D (A/T/G, W (A/T), Y (C/T).*

Name of forward primers	Primer sequence (5′—3′)	Name of reverse primers	Primer sequence (5′—3′)
DP*rbc*L1 (12–6)	AAGGAGGAADHHATGTCT	DP*rbc*L7 (23–3; *rbc*S)	AAASHDCCTTGTGTWAGTYTC
ND*rbc*L2 (34–53)	AAAAGTGACCGTTATGAATC	ND*rbc*L8 (1232–1212)	CCAATAGTACCACCACCAAAT
ND*rbc*L3 (43–58)	CGTTACGAATCTGGTG	ND*rbc*L9 (1226–1212)	GTACCACCACCAAAT
ND*rbc*L4 (342–356)	AGGTTCACTAGCTAA	ND*rbc*L10 (983–969)	TGGTCAACACCAGCC
ND*rbc*L5 (635–650)	CACAACCATTCATGCG	ND*rbc*L11 (835–820)	CAGTGTAACCAATTAC
ND*rbc*L6 (953–967)	GTAAATGGATGCGTA	ND*rbc*L12 (527–514)	GCACCTAATAGTGG

TABLE 3. *Distribution of six major carotenoids in the heterokont algae. Based on Bjørnland and Liaeen-Jensen (1989). The 0 = absent, 1 = present.*

	Fucoxanthin	Violaxanthin	Diatoxanthin	Diadinoxanthin	Heteroxanthin	Vaucheria-xanthin
Eustigmatophytes	0	1	0	0	0	1
Dictyochophytes	1	0	1	1	0	0
Chrysophytes and Synurophytes	1	1	0	0	0	0
Bacillariophytes	1	0	1	1	0	0
Freshwater Raphidophytes	0	0	0	1	1	1
Marine Raphidophytes	1	1	0	0	0	0
Xanthophytes	0	0	1	1	1	1
Phaeophytes	1	1	0	0	0	0
Pelagophytes and Sarcinochrysidales	1	0	1	1	0	0

order to find the relative support for the branching pattern (Felsenstein 1985).

Cladistic analysis of carotenoids. A data matrix consisting of six major carotenoids was established for the heterokont algae (Table 3). The freshwater and marine raphidophytes have a different distribution of carotenoids; thus, they were separated in this analysis. Because the carotenoids in the Chrysophyceae and Synurophyceae are identical, these two classes were combined. The tree was rooted using the red algae, which do not possess significant amounts of any of the carotenoids included in this analysis. In PAUP V. 3.1.1, all characters were treated as unordered and were analyzed using the branch and bound search option, which ensures finding all most parsimonious solutions (Swofford 1993).

Relative rate test. The computer program PHYLTEST (V. 2.0, Kumar 1995) was applied to test for the presence of a molecular clock (= equal rates of substitutions) in the different monophyletic clusters of heterokont algae and used the red algae as the outgroup lineage. The relative rate test is based on the two-tailed test of Takezaki et al. (1995). Distances were estimated using Kimura-2-parameter model. If the Z-statistic is smaller than 1.96, then rate constancy is not rejected at the 5% level, that is sequences in the predefined monophyletic clusters do not evolve with a significantly different speed.

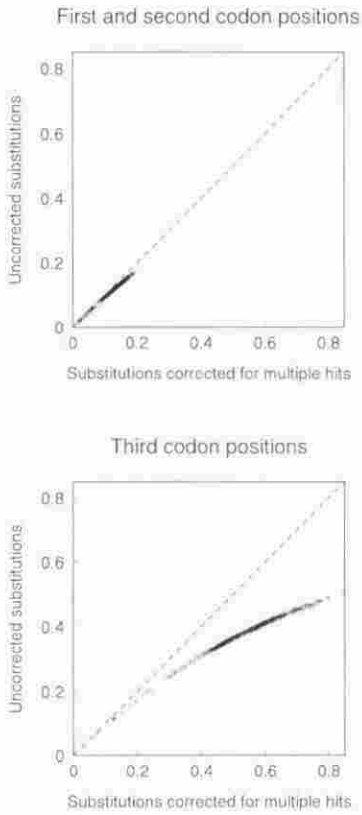

FIG. 1. Plots of pairwise substitutions without corrections for multiple hits against pairwise substitutions corrected for multiple hits for first and second codon positions (A) and third codon positions (B). Corrected distances were estimated using the Kimura-2-parameter model and uncorrected distances were estimated using the "uncorrected p" in PAUPstar V. 4.0.0d53 (Swofford, unpubl.). The almost linear relationship (with a slope of 1) indicates that first and second codon positions are probably not mutationally saturated, whereas the deflection from linearity (curves to the right) of third codon positions strongly implies that multiple substitutions at this site are occurring more rapidly.

RESULTS

Among the 32 taxa of heterokont algae and three species of red algae included in this study, more than half (62%) of the total number of substitutions in the *rbc*L gene were encountered at third codon positions. The frequency of substitutions at first and second codon positions was 26% and 12%, respectively. To test for mutational saturation at third codon positions, nucleotide substitutions uncorrected for multiple substitutions were plotted against the differences with corrections for multiple substitutions (Fig. 1). The almost linear relationship for first and second codon positions suggests that these positions are not mutationally saturated (Fig. 1A). Thus, the observed differences were a good estimate of the actual substitutions occurring. The nonlinear relationship (curves to the right) for third codon positions strongly suggests that multiple substitutions occur at this site. For distantly related species, one observes fewer than half of the substitutions that actually occur (Fig. 1B).

The three centric diatoms *Odontella sinensis, Rhizosolenia setigera,* and *Skeletonema costatum* and the pennate diatom *Cylindrotheca* sp. possess a two-amino-acid insertion at the homologous position, 1321–1326, in the *rbc*L sequence relative to the brown alga *Pilayella littoralis* (Assali et al. 1990). The eustigmatophytes included here are characterized by being one amino acid shorter at the C terminus of the *rbc*L gene when compared to the other heterokont algae. Apart from these apomorphic character states, the *rbc*L sequences determined were unambiguously

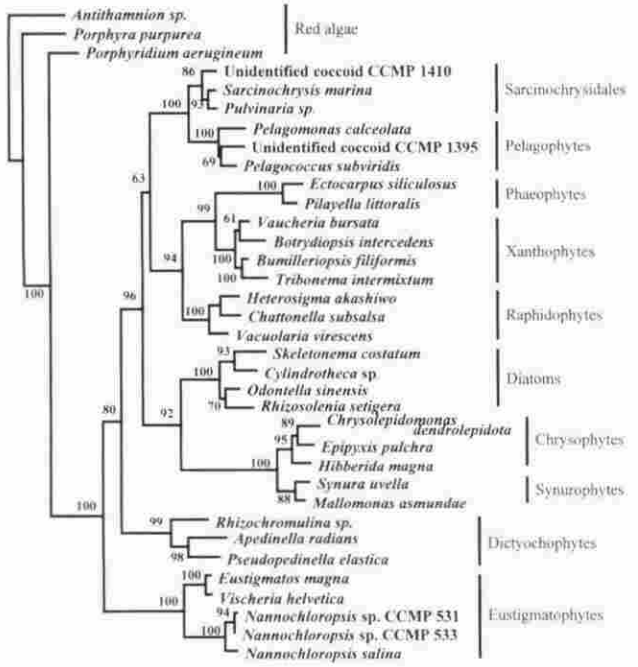

FIG. 2. Phylogeny of 32 taxa of heterokont algae inferred from plastid-encoded ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) nucleotide sequences including first and second codon positions only (954 base pairs). The three red algal taxa were used to root the tree. The reconstruction was based on the weighted-parsimony method (rescaled consistency index over an interval of 1–1000) with the heuristic search option and 100 random additions of taxa in PAUP V. 3.1.1. The single-most parsimonious tree using the rescaled consistency index had a consistency index = 0.73 and a retention index = 0.84. The branch lengths are proportional to the number of character changes. Bootstrap values ≥50% (500 replications) are shown at internodes.

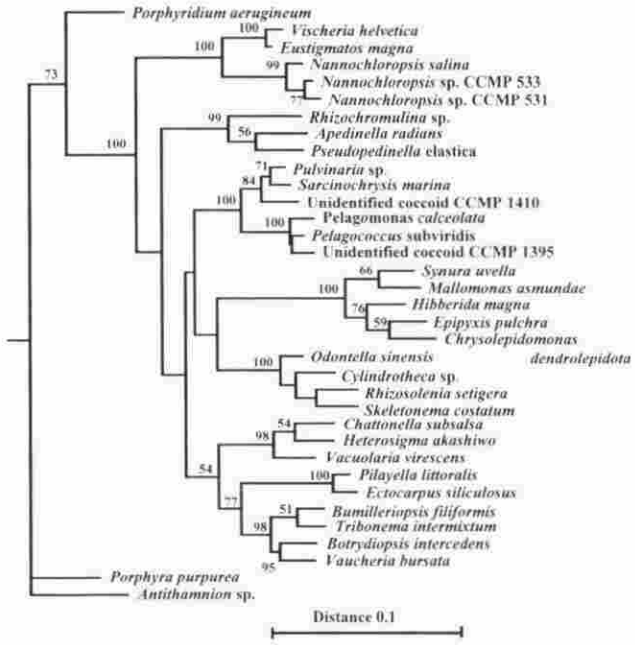

FIG. 3. Phylogeny of 32 taxa of heterokont algae inferred from plastid-encoded ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) nucleotide sequences of 954 unambiguously aligned first and second codon positions. Homologous sequences from three species of red algae were used to root the tree. The reconstruction was based on the maximum-likelihood method (fastDNAml V. 1.0.8) and the best log-likelihood score (−7062.54) was obtained with a transition to transversion ratio of 0.8. Bootstrap values ≥50% (100 replications) are given at internodes. No value is shown if a node was not present in the maximum-likelihood bootstrap consensus tree.

aligned starting with position 43 in the *rbc*L gene in *Pilayella littoralis*.

Sequence divergence. An extremely low sequence-divergence value of 1.6% was found between *Eustigmatos magna* and *Vischeria helvetica*, two genera in the Eustigmatophyceae. The sequence divergence value for the three *Nannochloropsis* spp. ranged from 3.1–7.3%; thus, these values were 1.9–4.6 times as great. Estimates of sequence divergence among the five eustigmatophytes ranged from 14.0–15.6% and are slightly higher than the estimates for the four diatom (9.6–12%) and the four xanthophyte (7.2–10.9%) taxa.

Phylogeny based on rbc*L sequences.* Both the weighted-parsimony and maximum-likelihood phylogenetic reconstructions (Figs. 2 and 3, respectively) support the monophyletic status of all presently recognized classes of heterokont algae. The eustigmatophytes occupy the most basal position among the phototrophic heterokonts, and the Dictyochophyceae (*sensu* Moestrup 1995) branch off as the secondmost basal group. The branching pattern for the remaining classes was resolved well (high bootstrap values) in the weighted-parsimony analysis, but deep branches were not well supported in the maximum-likelihood analysis (compare Figs. 2 and 3).

In both weighted-parsimony and maximum-likelihood reconstructions, the Sarcinochrysidales (*Pulvinaria* sp. and *Sarcinochrysis marina*) are closely related to the Pelagophyceae. One of the two coccoid picoplankton strains (CCMP 1395) is related to the coccoid pelagophyte *Pelagococcus subviridis*, whereas the other (CCMP 1410) is the sister to Sarcinochrysidales. The sister group relationship between the Xanthophyceae (= Tribophyceae) and Phaeophyceae (= Fucophyceae) is well supported in both the weighted-parsimony and maximum-likelihood analyses (bootstrap = 99% and 77%, respectively). The relationship of the Raphidophyceae to the Xanthophyceae/Phaeophyceae clade is well established in the weighted-parsimony analysis (bootstrap = 94%), but the relationship is supported weakly by the maximum-likelihood bootstrap analysis (bootstrap = 54%). Relationships within the Raphidophyceae, especially between the marine (*Heterosigma akashiwo* and *Chattonella subsalsa*) and freshwater (*Vacuolaria virescens*) taxa, are not established with confidence. The centric diatom species do not form a monophyletic group as *Skeletonema* joins with the pennate *Cylindrotheca* in the weighted-parsimony analysis (Fig. 2). The synurophytes and chrysophytes are sister taxa in both analyses, and there is a well-supported relationship between these classes and

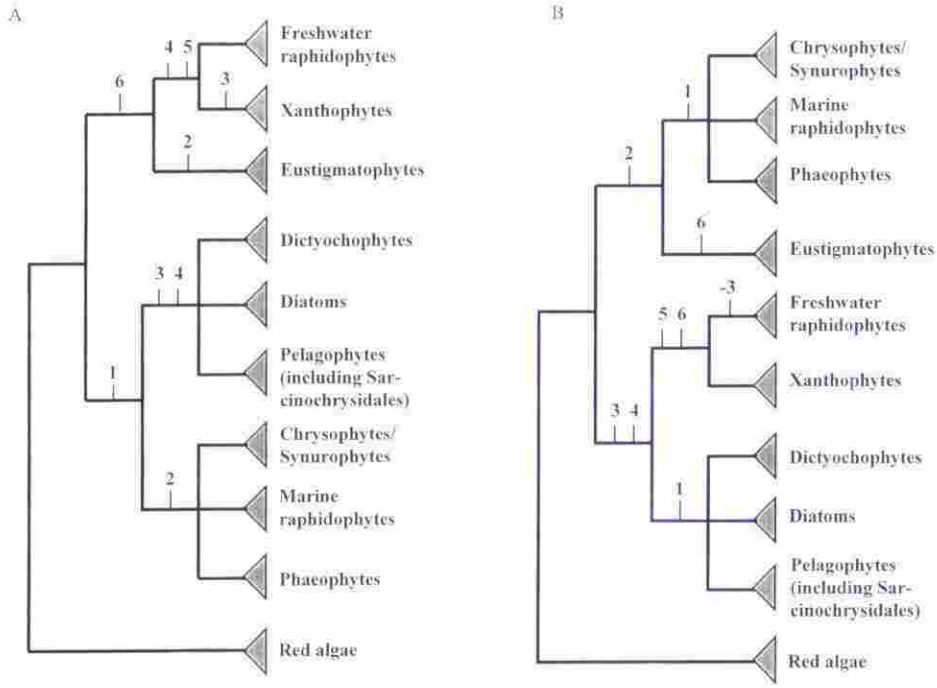

FIG. 4. A cladistic analysis of six major carotenoids in the heterokont algae produced two most parsimonious trees (A and B). Each tree was nine steps long and had a consistency index = 0.67 and retention index = 0.81. The most parsimonious distribution of characters is shown on both cladograms. Explanation of characters 1–6: 1 = fucoxanthin, 2 = violaxanthin, 3 = diatoxanthin, 4 = diadinoxanthin, 5 = heteroxanthin, 6 = vaucheriaxanthin.

the diatoms in the weighted-parsimony analysis (bootstrap = 92%).

Cladistic analysis of carotenoids. The cladistic analysis of six major carotenoids (see Table 3) revealed two most parsimonious trees (9 steps long), each with two polychotomies (Fig. 4A, B). The character state changes are mapped on the trees.

Relative rate test. The results from the relative rate test for first and second codon positions only are shown in Table 4. For the 10 algal groups (see Table 4) 17 of 45 pairwise comparisons showed a heterogeneous rate of substitution. Fourteen (≈82%) of the significant values were observed when comparing either chrysophytes or synurophytes to other groups of autotrophic heterokonts. Only the pairwise comparisons between synurophytes/eustigmatophytes and synurophytes/pelagophytes did not produce significant results. Constraining chrysophytes and synurophytes to one group prior to a comparison to the other groups produced highly significant values in all possible combinations. The results of the relative rate test strongly indicate that the *rbc*L gene in the Chrysophyceae and Synurophyceae has evolved at a different rate relative to the other lineages. The branch lengths from the maximum-likelihood reconstruction (Fig. 3) demonstrate that spe-

TABLE 4. *Results from a relative rate test based on Z-statistics. Three red algae were used as the outgroup in all pairwise comparisons between major groups of heterokont algae.*

	Eustigmatophytes	Dictyochophytes	Synurophytes	Chrysophytes	Synurophytes and Chrysophytes	Diatoms	Raphidophytes	Xanthophytes	Phaeophytes	Pelagophytes	Sarcinochrysidales
Eustigmatophytes	—	1.69	1.87	1.97[a]	2.01[a]	1.13	1.97[a]	2.82[a]	1.78	0.05	1.12
Dictyochophytes		—	3.76[a]	3.70[a]	3.90[a]	0.54	0.17	0.91	0.08	1.80	0.56
Synurophytes			—	0.24	—	3.41[a]	4.12[a]	4.47[a]	3.53[a]	1.91	3.50[a]
Chrysophytes				—	—	3.42[a]	4.10[a]	4.44[a]	3.52[a]	1.99[a]	3.33[a]
Synurophytes and Chrysophytes					—	3.61[a]	4.33[a]	4.66[a]	3.68[a]	2.04[a]	3.52[a]
Diatoms						—	0.81	1.54	0.44	1.26	0.08
Raphidophytes							—	0.90	0.25	1.92	0.81
Xanthophytes								—	1.11	2.60[a]	1.52
Phaeophytes									—	1.43	0.40
Pelagophytes										—	1.94
Sarcinochyrsidales											—

[a] Significant values at the 5% level (= acceptance of an unequal substitution rate in the *rbc*L gene for the two groups compared).

cies belonging to the Chrysophyceae and Synurophyceae have evolved at an accelerated rate relative to other heterokonts.

DISCUSSION

These results make up the first extensive molecular data set that offers an alternative to the SSU rDNA data sets for understanding the phylogeny of the heterokont algae. Our analyses of the *rbc*L nucleotide sequences corroborate certain phylogenetic relationships that were found during earlier molecular studies (based upon SSU rDNA), and they suggest different phylogenetic relationships for other groups. For example, the *rbc*L-based analyses support the close relationship of the phaeophytes and xanthophytes, as first reported by Ariztia et al. (1991) and later corroborated by others (e.g. Bhattacharya et al. 1992, Andersen et al. 1993, Leipe et al. 1994, Saunders et al. 1995, Van de Peer et al. 1996, Potter et al. 1997). Thus, these nuclear- and chloroplast-encoded genes indicate a common origin between the Phaeophyceae and Xanthophyceae. However, this relationship is challenged by differences in the cell wall composition, pyrenoid structure, and distribution of carotenoids (Bold and Wynne 1985).

A recent molecular study by Potter et al. (1997) showed that the nuclear-encoded SSU rDNA genes in marine and freshwater raphidophytes cluster together when compared to homologous sequences from other heterokonts. Both raphidophyte groups have a similar three-dimensional cellular organization (including the configuration of the flagellar apparatus), but the freshwater species contain diadinoxanthin, dinoxanthin, neoxanthin, heteroxanthin, and vaucheriaxanthin, whereas the marine taxa contain fucoxanthin, violaxanthin, and zeaxanthin (Bjørnland and Liaaen-Jensen 1989). The carotenoids of the freshwater raphidophytes are very similar to those of the Xanthophyceae and Eustigmatophyceae, whereas the pigments of marine raphidophytes resemble those of the Phaeophyceae, Chrysophyceae, and Synurophyceae. Thus, the carotenoids found in the Raphidophyceae are very diverse, with pigment profiles resembling other groups of heterokont algae. The hypothesis that the freshwater and marine raphidophytes obtained their chloroplast via two separate endosymbiotic events cannot be rejected by the SSU rDNA phylogeny. The *rbc*L-based phylogenetic tree corroborates the results from the SSU analysis that both the freshwater and marine raphidophytes form a monophyletic group (bootstrap = 100% and 98% in Figs. 2 and 3, respectively) and that they are related to the xanthophytes and phaeophytes. Therefore, we conclude that despite the presence of marked differences in the composition of carotenoids, the plastid gene analysis fails to support a separate endosymbiotic origin for freshwater and marine raphidophyte chloroplasts.

Also, the sarcinochrysidalean algae are sister taxa to the Pelagophyceae in the *rbc*L analysis, corroborating the recent SSU rDNA results of Saunders et al. (1997). The Sarcinochrysidales have zoospores which, in gross morphology, resemble those of the brown algae (Gayral and Billard 1977), leading Billard (1984) and Pedersen (1984) to propose that the Phaeophyceae originated from a sarcinochrysidaleanlike ancestor. This relationship was modified when the Sarcinochrysidales *sensu stricto* were shown to have a flagellar apparatus and other features unlike the phaeophytes (O'Kelly 1989). Conversely, some taxa previously classified in the Sarcinochrysidales *sensu lato* are now placed in a separate order (Chrysomeridales), and their flagellar apparatus resembles that of brown algae (O'Kelly 1989). Therefore, the relationship between the Sarcinochrysidales *sensu stricto* and the Pelagophyceae is now supported by ultrastructure, chloroplast pigments, SSU rDNA, and *rbc*L nucleotide sequences.

Within taxonomic classes, we also found some interesting relationships. The centric diatoms do not form a monophyletic group, that is, *Skeletonema* and *Cylindrotheca* are sister taxa (Fig. 2). Although we have a limited number of taxa, this branching pattern corroborates a more detailed study based on nuclear SSU rDNA that showed the paraphyly of centric diatoms (Medlin et al. 1996). Within the eustigmatophytes, the sequence divergence value of *Vischeria helvetica* and *Eustigmatos magna* indicates that these taxa might be identical and their identity and taxonomy should be reinvestigated. A close relationship between *Vischeria* and *Eustigmatos* also was observed in the nuclear-encoded SSU rDNA sequences where only two base substitutions occur (Andersen, pers. observ.).

Finally, the suggested relationship among the four xanthophytes in the *rbc*L trees agrees with an analysis based on SSU rDNA sequences (Potter et al. 1997). The branching of taxa in both molecular analyses do not support Hibberd's (1990) ordinal classification based upon the dominant life forms (i.e. coccoid, flagellate, filamentous, or siphoneous), an idea originally proposed by Pascher (1914, 1931). Therefore, both the SSU rDNA and the *rbc*L data suggest that the Xanthophyceae are in need of taxonomic revision.

Our analyses also show several substantial differences relative to the SSU rDNA analyses. The most apparent difference is that the Eustigmatophyceae are the most deeply divergent group in the *rbc*L trees, whereas the diatoms frequently are the most deeply divergent group in SSU rDNA trees (e.g. Bhattacharya et al. 1992, Andersen et al. 1993, Leipe et al. 1994, Saunders et al. 1995, Van de Peer et al. 1996). Based upon the unusual photoreceptor–eyespot apparatus found in eustigmatophytes, Hibberd (1979) speculated that they diverged early in the evolutionary lineage of heterokont algae. The photoreceptor apparatus of eustigmatophytes includes a

large, extraplastidal red eyespot that is associated with a T-shaped swelling at the proximal end of the longer, immature flagellum. Furthermore, the eustigmatophytes lack a chloroplast girdle lamella (Hibberd 1979) and also differ from all other heterokont algae by completely lacking chlorophyll *c* (Jeffrey 1989). The photoreceptors in the Chrysophyceae, Phaeophyceae, and Xanthophyceae consist of a swollen region on the shorter, mature flagellum. Also, the flagellar swelling is typically associated with a red eyespot located inside the chloroplast. Although Hibberd's (1979) hypothesis for an early divergence of eustigmatophytes is supported by the *rbc*L data, the evolution of the photoreceptor–eyespot apparatus in other heterokont algae is difficult to explain based upon *rbc*L data or based upon SSU rDNA data. The reconstruction shown in Figures 2 and 3 suggests that if the Chryso-/Phaeo-/Xanthophyceae-type of photoreceptor–eyespot apparatus was derived from the Eustigmatophyceae, there must have been numerous losses of the photoreceptor apparatus during the evolutionary history of the heterokont algae. That is, neither type of photoreceptor is found in the diatoms, dictyochophytes, pelagophytes (including the Sarcinochrysidales), raphidophytes, or synurophytes. Alternatively, the type of photoreceptor–eyespot apparatus found in the chrysophyte and phaeophyte/xanthophyte clades may have evolved independently. However, this also seems improbable given its identical structure in both algal groups and given the complexity of the photoreceptor apparatus itself.

The reduced flagellar apparatus lineage, which was identified when SSU rDNA data and traditional morphological and biochemical data were combined (Saunders et al. 1995, 1997, Potter et al. 1997), was not present in the weighted-parsimony analysis of *rbc*L sequences. Due to the lack of bootstrap support for the deep-branching pattern, no definitive conclusion can be drawn from the maximum-likelihood analysis. Indeed, the reduced flagellar clade is more fragmented in the *rbc*L trees than in the SSU rDNA trees. Organisms with a reduced flagellar apparatus have the following characteristics: no transitional helix above the transitional plate, microtubular flagellar roots are absent (except in Sarcinochrysidales, see Saunders et al. 1997), the photoreceptor–eyespot complex is lacking, and there is only one emergent flagellum (except for *Dictyocha speculum* where a second, stubby flagellum is present in the skeleton stage, although absent in the naked stage, Moestrup and Thomsen 1990). The lack of flagellar roots most likely represents a secondary reduction as the deeply diverging heterokont protists (bicosoecids, labyrinthulids, and oomycetes) possess flagellar roots homologous to those observed in the phototrophic heterokonts (Andersen 1991). Ringlike structures below the transitional plate also characterize organisms with a reduced flagellar apparatus (*Apedinella, Dictyocha, Pelagomonas, Pteridomonas, Rhizochromulina, Sulcochrysis*; Larsen 1985, Moestrup and Thomsen 1990, Andersen et al. 1993, Honda et al. 1995, O'Kelly and Wujek 1995, Saunders et al. 1995). The *rbc*L-based phylogeny suggests that these reduced flagellar apparatus features may have evolved independently three times.

A phylogenetic relationship between the chrysophyte/synurophyte clade and the diatoms has not, to our knowledge, been described previously in molecular analyses. More than 60 years ago, a number of prominent phycologists speculated on the origin of diatoms, and the consensus was that diatoms were closely related to the chrysophytes (e.g. Pascher 1921, Korsikov 1930). Electron microscopic observations also point toward a relationship between the two groups (Takahashi 1964, Round and Crawford 1981, Round 1986, Andersen 1987). Round and Crawford (1981) hypothesized that the prediatom ancestor acquired a coating of siliceous scales and that the valves and girdle bands of the ur-diatom stage were formed by a differentiation of the siliceous body scales. Because numerous chrysophyte and all synurophyte taxa have silica scales, the relationship of the chrysophyte/synurophyte with the diatoms in the *rbc*L analysis gives molecular support to this hypothesis.

Evolution of carotenoids. The heterokont algae are particularly rich in carotenoid diversity, and there appears to be little variability within specific classes. Therefore, carotenoids are useful chemosystematic markers to help define algal classes (Bjørnland and Liaaen-Jensen 1989). The various carotenoids are not always independent of each other, because basic biosynthetic pathways provide an enzymatic means for isomerization and molecular modification (Hager and Stransky 1970, Bjørnland and Liaaen-Jensen 1989). Thus, the stability of carotenoids within classes and the commonality of biosynthetic pathways among classes suggest that carotenoids should be good characters for phylogenetic analyses. A character-limited, cladistic analysis of carotenoids in the heterokont algae gave two equally parsimonious trees (Fig. 4A, B). The most parsimonious distribution of carotenoid characters are mapped on the two trees. The consensus cladogram (not shown) reveals a topology of unresolved relationship for the four clusters comprising the following: 1) eustigmatophytes, 2) dictyochophytes/diatoms/pelagophytes (including the sarcinochrysidalean species), 3) chrysophytes/synurophytes/marine raphidophytes/phaeophytes, and 4) xanthophytes/freshwater raphidophytes. As an attempt to study further the evolution of carotenoids in the autotrophic heterokonts, we mapped each of the six carotenoids on phylogenetic trees constructed from *rbc*L and SSU rDNA nucleotide sequences (Fig. 5A, B, respectively). Mapping of carotenoids on gene trees was performed by assuming that secondary losses were more likely to occur than independent evolutionary

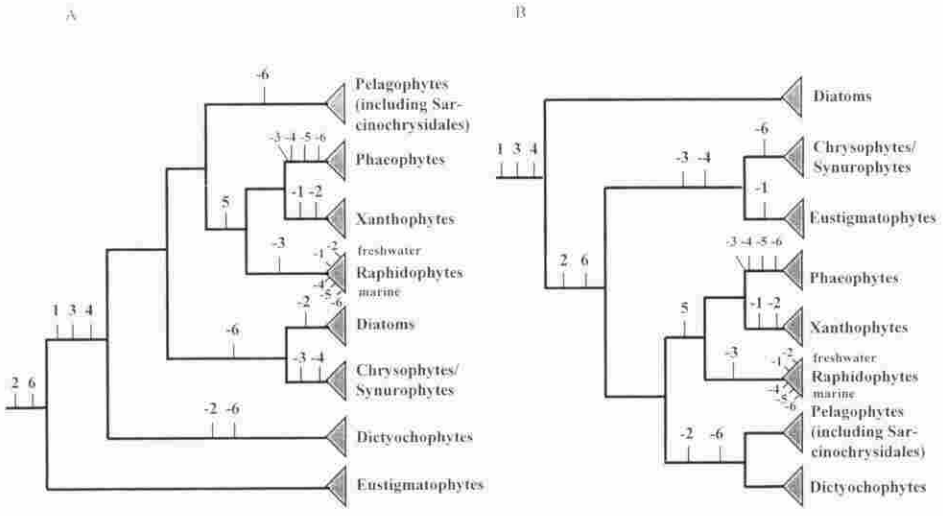

FIG. 5. The evolution of major photosynthetic pigments (carotenoids) as best accounted for by the weighted-parsimony reconstruction based on ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) (A) and nuclear-encoded small-subunit ribosomal DNA (SSU rDNA) (b) nucleotide sequences. The consensus SSU rDNA reconstruction was based on the method of Van de Peer et al. (1996). The hypothesized distribution of the six carotenoids was based on the assumption that character losses are more likely than origin of the same character more than once. Explanation of characters 1–6: 1 = fucoxanthin, 2 = violaxanthin, 3 = diatoxanthin, 4 = diadinoxanthin, 5 = heteroxanthin, 6 = vaucheriaxanthin.

gains. For the six major carotenoids, a total of 19 losses occur in the *rbc*L tree, and 18 losses occur in the SSU rDNA consensus tree (compare Fig. 5A, B). Particularly, the distribution of vaucheriaxanthin (character 6) appears complex because it is lost five times in the *rbc*L tree and four times in the SSU rDNA consensus tree. Alternatively, vaucheriaxanthin may have evolved independently more than once, or both the *rbc*L and SSU rDNA trees are incorrect. The distribution of certain other carotenoids (i.e. diatoxanthin, diadinoxanthin, heteroxanthin) involves only one or two losses on the gene trees. If any of the carotenoid distributions illustrated in Figure 5 represents the true evolution of carotenoids in heterokont algae, it must follow that only one can be correct. Thus, the evolution of carotenoids appears complex, and the use of carotenoids for phylogenetic inference must be done with caution. A better understanding of carotenoid biosynthetic pathways may shed new light on this problem.

CONCLUSION

The *rbc*L data presented here strengthen the proposed phylogenetic relationships for some heterokont algae, but the incongruencies between *rbc*L and SSU rDNA data indicate that a consensus of relationships among heterokont algal groups is not yet at hand. In particular, the earliest diverging branch of heterokont algae is unclear. If this earliest branch can be established, then polarization of characters may provide a more sound means for hypothesizing subsequent evolutionary changes. The congruence of some branches on both nuclear-encoded and plastid-encoded gene trees makes it tempting to suggest that plastids arose once in the heterokont algal lineage, and in turn, that phylogenies based upon plastids are similar to those based upon host cells. Data from other plastid and nuclear genes should confirm or reject this hypothesis. Substantial diversity has been discovered within the heterokont algal lineage during the past few decades, and a continued effort to discover any hidden diversity may also help to resolve the evolutionary history of this group.

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