Chlorophyll c–Containing Plastid Relationships Based on Analyses of a Multigene Data Set with All Four Chromalveolate Lineages

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The chlorophyll c–containing algae comprise four major lineages: dinoflagellates, haptophytes, heterokonts, and cryptophytes. These four lineages have sometimes been grouped together based on their pigmentation, but cytological and rRNA data had suggested that they were not a monophyletic lineage. Some molecular data support monophyly of the plastids, while other plastid and host data suggest different relationships. It is uncontroversial that these groups have all acquired plastids from another eukaryote, probably from the red algal lineage, in a secondary endosymbiotic event, but the number and sequence of such event(s) remain controversial. Understanding chlorophyll c–containing plastid relationships is a first step towards determining the number of endosymbiotic events within the chromalveolates. We report here phylogenetic analyses using 10 plastid genes with representatives of all four chromalveolate lineages. This is the first organellar genome-scale analysis to include both haptophytes and dinoflagellates. Concatenated analyses support the monophyly of the chlorophyll c–containing plastids and suggest that cryptophyte plastids are the basal member of the chlorophyll c–containing plastid lineage. The gene *psbA*, which has at times been used for phylogenetic purposes, was found to differ from the other genes in its placement of the dinoflagellates and the haptophytes, and in its lack of support for monophyly of the green and red plastid lineages. Overall, the concatenated data are consistent with a single origin of chlorophyll c–containing plastids from red algae. However, these data cannot test several key hypothesis concerning chromalveolate host monophyly, and do not preclude the possibility of serial transfer of chlorophyll c–containing plastids among distantly related hosts.

Introduction

Four groups of photosynthetic eukaryotes, namely, dinoflagellates, heterokonts, haptophytes, and cryptophytes, contain chlorophyll c as a major photosynthetic pigment. In recent years three of these, heterokonts, haptophytes, and cryptophytes, have been grouped together based on their common pigmentation in a phylum called Chromophyta *sensu* Cavalier-Smith (1986), although Christensen's original concept of a chromophyte clade also included dinoflagellates (Christensen 1962, 1989). Classification of algae primarily on the basis of plastid pigmentation fell into disfavor when it was understood that these organelles were endosymbiotic and potentially subject to transfer. Based on plastid membrane topology and molecular phylogenies, it has become clear that the chlorophyll c–containing plastids were acquired from another eukaryote in a secondary endosymbiotic event. Moreover, morphological studies and phylogenetic analyses based on nuclear genes showed that nonphotosynthetic organisms were closely related to some of these groups (Van de Peer and De Watcher 1997; Marin, Klinberg, and Melkonian 1998; Gunderson, Goss, and Coats 1999). To reconcile these observations, it has been proposed that the four lineages including the nonphotosynthetic relatives are included in a monophyletic clade called the chromalveolates (Cavalier-Smith 1999, 2002). This hypothesis suggests that a single endosymbiotic event gave rise to the plastids of chromalveolates, after which the four distinct host lineages diverged, and the nonphotosynthetic lineages in the chromalveolates have lost photosynthesis (and in most cases, plastids). Another hypothesis would postulate four separate plastid acquisitions in the four host lineages (Cavalier-Smith, Allsopp, and Chao 1994; Delwiche 1999). Here, we refer to "chromophyte plastids" as heterokont, haptophyte, and cryptophyte plastids and "chromalveolate plastids" or "chlorophyll c–containing plastids" as chromophyte plus dinoflagellate plastids.

Even though there is good evidence that all four groups have plastids ultimately derived from the red algal plastid lineage (Delwiche and Palmer 1997; Durnford et al. 1999; Yoon et al. 2002), plastid relationships within the chromalveolates are still uncertain. Conflicting evidence has been reported concerning the monophyly of chlorophyll c–containing plastids (Daugbjerg and Andersen 1997; Fast et al. 2001; Ishida and Green 2002; Yoon, Hackett, and Bhattacharya 2002; Yoon et al. 2002). Unfortunately, most of previous studies have included only a subset of those four lineages and were based on analyses of one or only a few genes. Peridinin-containing dinoflagellates have been problematic because of their highly modified plastid, which, among other peculiarities, apparently only contains a few plastid genes (Barbrook and Howe 2000; Hiller 2001) with a very high rate of sequence evolution (Zhang, Green, and Cavalier-Smith 1999, 2000; Takishita et al. 2003). The haptophytes have not been thoroughly studied in this regard, and only a few genes from their plastid genome have been available for analysis. Recent evidence suggests a relationship between haptophyte and peridinin-containing dinoflagellate plastids (Yoon, Hackett, and Bhattacharya 2002). However, one of the genes used for this analysis may be biased (Inagaki et al. 2004).

To address the questions of chlorophyll c–containing plastid monophyly and a specific relationship between haptophyte and dinoflagellate plastids, we undertook phylogenetic analyses designed to include all four chlorophyll c–containing plastid lineages and to make use of a number of concatenated plastid genes. We obtained chloroplast genome data from the haptophytes, a key lineage of chromalveolates, missing from most previous studies (Sanchez-Puerta, Bachvaroff, and Delwiche 2005). Ten plastid genes from representatives of each of the four

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groups were analyzed in the context of other plastids, and tests were performed to evaluate support for the monophyly of the chlorophyll c–containing plastids and to understand the phylogenetic relationships among them. These 10 genes represent almost all the protein-coding genes known from peridinin-containing dinoflagellate plastids (Takishita et al. 2003; but see Laatsch et al. 2004); thus this study makes use of almost all of the presumably plastid-encoded data share by these four lineages. To avoid potential problems with individual gene bias, analysis were performed with each gene individually as well as in concatenation.

Materials and Methods

Sequence Acquisition

The complete plastid genome sequence of *Emiliania huxleyi* has been published elsewhere (Sanchez-Puerta, Bachvaroff, and Delwiche 2005).

Alignments

Nucleotide alignments for the 10 genes (*atpA, atpB, petB, petD, psaA, psbA, psbB, psbC, psbD,* and *psbE*) were made by downloading the appropriate sequences from GenBank. GenBank numbers are as follows: *Arabidopsis thaliana* (NC_000932), the peridinin-containing dinoflagellate *Amphidinium operculatum* (AJ311628–AJ311632; AJ250262–AJ250266), *Chaetosphaeridium globosum* (NC_004115), *Cyanidium caldarium* (NC_001840), *Cyanidioschyzon merolae* (NC_004799), *Cyanophora paradoxa* (NC_001675), *E. huxleyi* (AY675516–AY675525), *Guillardia theta* (NC_000926), *Mesostigma viride* (NC_002186), *Nephroselmis olivacea* (NC_000927), *Nostoc* sp. PCC7120 (NC_003272), *Odontella sinensis* (NC_001713), *Porphyra purpurea* (NC_000925), and *Synechocystis* sp. PCC6803 (NC_000911). The sequences were imported into MacClade 4.0 (W. Maddison and P. Maddison 2000) and in most cases were easily aligned by eye. However, genes were also translated, and inferred amino acid sequences were aligned using ClustalW as a guide to the nucleotide alignment.

Analyses

For all single and multigene phylogenetic analyses based on nucleotides, PAUP*4b10 (Swofford et al. 2002) was used, and the third codon position was excluded from all analyses. First a Fitch-Margoliash tree was constructed using LogDet distances, and then maximum likelihood (ML) parameters for the general time-reversible model of evolution with invariant sites and gamma correction (four categories) were estimated from this tree. These parameter estimates were used in the ML heuristic search repeated three times with different random addition order. For bootstrapping, a single heuristic search with full branch swapping (TBR) was used. In addition, maximum parsimony (MP) nucleotide analyses were performed using PAUP*.

The CONSEL package was used to calculate the approximately unbiased (AU) *P* values for seven different trees using individual genes and concatenated data sets (Schimodaira 2000; Schimodaira and Hasegawa 2001). Six constraint trees were compared to the most likely unconstrained tree. The dinoflagellate plastid was constrained with a monophyletic red algal plastid lineage, a monophyletic green plastid lineage, or with a monophyletic cyanobacterial lineage. Three additional hypotheses were tested: dinoflagellate and haptophyte plastid monophyly, dinoflagellate and heterokont plastid monophyly, and chromalveolate plastid monophyly. The most likely tree corresponding to each constraint was determined by searching for the best tree compatible with that constraint using PAUP* with ML parameters found as described above. Site likelihoods for these trees as well as the most likely tree in unconstrained analyses were exported from PAUP* and the AU *P* values were calculated from these data.

ML phylogenetic analyses based on amino acids data were performed using ProML (PHYLIP 3.63) and Tree-Puzzle 5.2 (Schmidt et al. 2002). Tree-Puzzle 5.2 implements a fast tree combination algorithm, quartet puzzling (Strimmer and von Haeseler 1996) to reconstruct the topology, and automatically assigns estimations of support to each internal branch. The model of substitution used for Tree-Puzzle was the WAG model (Whelan and Goldman 2001), and the rate heterogeneity included invariant sites and gamma-distributed rates in eight categories. All parameters were estimated from the data set by ML approaches. Branches showing quartet puzzling support values that range from 90% to 100% can be considered very strongly supported. ProML uses a true heuristic optimization algorithm. For the ProML analyses of concatenated data sets, eight rate categories were used with the parameters estimated by Tree-Puzzle, under the Jones, Taylor, and Thornton (1992) (JTT) model of amino acid substitution. In addition, MP analyses based on amino acid data were performed using PAUP*.

The codeml program in the PAML3.14 package (Yang 1997) was used to estimate site likelihoods for amino acid–based AU tests. For these analyses the most likely tree from Tree-Puzzle was used, and unresolved nodes were manually swapped to find an optimal tree. The same trial and error process was used to find an optimal tree compatible with the six constraint hypotheses. These site likelihoods were then used for the AU test.

Results

Individual Gene Analyses and AU Tests

Individual gene nucleotide phylogenetic analyses are shown in figures 1–3 of the Supplementary Material online, and bootstrap and quartet puzzling support values for key nodes are shown in table 1, where analyses were performed without the dinoflagellate, and in table 2 with the dinoflagellate. All 10 individual trees show extreme branch length leading to the dinoflagellate. While individual genes support cyanobacterial monophyly, and to a lesser extent green plastid lineage monophyly, the monophyly of the red plastid lineage, here defined as red algal plastids and secondary plastids derived from red algae, is sensitive to the addition of the dinoflagellate. When the dinoflagellate is included, bootstrap support for a monophyletic red lineage declines, in some cases substantially (*petD* 85% vs. 78%, *psaA* 85% vs. 54%, *psbB* 100% vs. 69%, and *psbC* 100% vs. 65%).

Table 1
Support Values for Monophyly of Selected Clades with Individual Plastid Genes and Concatenated Data Sets When Dinoflagellates Are Excluded

Gene Name	Type of Analysis	Cyanobacteria	Green Algae	Red Lineage[a]	Chl c Plastids	Haptophyte + Heterokont	Alignment Size
10 genes	PAUP-ML	100	100	100	74	99	7,566 nt
10 genes	PAUP-MP-nt	100	100	100	–[b]	81	7,566 nt
10 genes	Tree-Puzzle	91	100	100	99	100	3,783 aac
10 genes	PAUP-MP-prot	100	100	100	70	98	3,783 aac
10 genes	ProML	100	100	100	99	100	3,783 aac
9 genes[c]	PAUP-ML	100	100	100	88	89	6,904 nt
9 genes[c]	PAUP-MP-nt	100	100	100	53	60	6,904 nt
9 genes[c]	Tree-Puzzle	79	100	99	99	100	3,452 aac
9 genes[c]	PAUP-MP-prot	100	100	100	80	94	3,452 aac
9 genes[c]	ProML	100	100	100	100	100	3,452 aac
8 genesA[d]	PAUP-ML	100	100	100	80	83	6,466 nt
8 genesA[d]	Tree-Puzzle	78	100	100	99	100	3,233 aac
8 genesA[d]	PAUP-MP-prot	100	100	100	67	95	3,233 aac
8 genesB[e]	PAUP-ML	100	100	100	87	95	5,964 nt
8 genesB[e]	Tree-Puzzle	80	100	99	97	99	2,982 aac
8 genesB[e]	PAUP-MP-prot	100	100	100	86	97	2,982 aac
7 genes[f]	PAUP-ML	100	100	100	71	92	5,522 nt
7 genes[f]	PAUP-MP-nt	100	100	100	–[b]	71	5,522 nt
7 genes[f]	Tree-Puzzle	80	100	99	91	98	2,761 aac
7 genes[f]	PAUP-MP-prot	100	100	100	71	96	2,761 aac
atpA	PAUP-ML	75	96	51	–[b]	–[b]	1,010 nt
atpA	Tree-Puzzle	97	93	57	–[b]	–[b]	505 aac
atpB	PAUP-ML	100	76	–[b]	–[b]	–[b]	942 nt
atpB	Tree-Puzzle	96	91	–[b]	–[b]	–[b]	471 aac
petB	PAUP-ML	99	–[b]	–[b]	70	–[b]	440 nt
petB	Tree-Puzzle	99	–[b]	–[b]	89	–[b]	220 aac
petD	PAUP-ML	56	–[b]	85	–[b]	–[b]	308 nt
petD	Tree-Puzzle	79	88	99	–[b]	73	154 aac
psaA	PAUP-ML	100	97	85	75	66	1,464 nt
psaA	Tree-Puzzle	77	100	87	99	99	732 aac
psbA	PAUP-ML	100	–[b]	–[b]	–[b]	–[b]	662 nt
psbA	PAUP-MP-nt	70	–[b]	–[b]	–[b]	–[b]	662 nt
psbA	Tree-Puzzle	92	58	–[b]	–[b]	90	331 aac
psbA	PAUP-MP-prot	85	67	–[b]	–[b]	73	331 aac
psbA	ProML	84	63	–[b]	–[b]	79	331 aac
psbB	PAUP-ML	100	100	100	–[b]	80	1,020 nt
psbB	Tree-Puzzle	81	100	100	72	69	510 aac
psbC	PAUP-ML	100	100	100	+[g]	97	908 nt
psbC	Tree-Puzzle	78	99	100	–[b]	62	454 aac
psbD	PAUP-ML	100	100	100	52	99	678 nt
psbD	Tree-Puzzle	100	100	83	–[b]	–[b]	339 aac
psbE	PAUP-ML	85	50	–[b]	+[g]	–[b]	136 nt
psbE	Tree-Puzzle	98	56	–[b]	–[b]	58	68 aac

NOTE.—Analyses using PAUP-ML are nucleotide (nt) based, without the third codon position. Analyses with Tree-Puzzle and ProML are amino acid (aac) based.

[a] Defined as red algal plastids and secondary plastids derived from them.

[b] Indicates that the feature is absent from the best tree.

[c] Analyses based on nine genes, excluding *psbA*.

[d] Analyses based on eight genes, excluding *psbA* and *petB*.

[e] Analyses based on eight genes, excluding *psbA* and *atpB*.

[f] Analyses based on seven genes, excluding *psbA*, *atpB*, and *petB*.

[g] Indicates that the feature is present on the best tree but the support value is $< 50\%$.

A similar pattern is observed regarding chlorophyll c–containing plastid monophyly; individual genes provide poor bootstrap support for chlorophyll c–containing plastid monophyly, and this support declines when dinoflagellates are included (tables 1 and 2; figs. 1–3 of the Supplementary Material online).

The *psbA* tree is incongruent with the other single-gene trees not only because the branching pattern is unusual but also because there is high bootstrap support for these unusual branches in nucleotide analyses. In *psbA* analyses the red algal plastid lineage is not monophyletic, and the cryptophyte *Guillardia* and the red alga *Porphyra* are placed within the green algal plastid lineage with 81% bootstrap support in the nucleotide analyses including (fig. 2) or excluding the dinoflagellate (fig. 1). The *psbA* tree also shows a strong support for a haptophyte + dinoflagellate relationship (fig. 2).

Individual gene analyses using Tree-Puzzle with amino acids are similar to the nucleotide-based analyses; in most cases the trees are less well resolved (data not shown). Notably *petB* (89%), *psaA* (99%), and *psbB* (72%) support chlorophyll c–containing plastid monophyly when

Table 2
Support Values for Monophyly of Selected Clades with Individual Plastid Genes and Concatenated Data Sets When Dinoflagellates Are Included

Gene Name	Type of Analysis	Cyanobacteria	Green Algae	Red Lineage[a]	Chl c Plastids	Dinoflagellate + Haptophyte	Dinoflagellate + Heterokont
10 genes	PAUP-ML	100	100	100	73	73	–[b]
10 genes	PAUP-MP-nt	99	98	93	–[b]	93	–[b]
10 genes	Tree-Puzzle	82	100	100	61	84	–[b]
10 genes	PAUP-MP-prot	100	100	79	69	+[c]	–[b]
10 genes	ProML	100	100	100	99	83	–[b]
9 genes[d]	PAUP-ML	100	100	98	89	58	–[b]
9 genes[d]	PAUP-MP-nt	98	87	75	+[c]	71	–[b]
9 genes[d]	Tree-Puzzle	77	100	100	62	61	–[b]
9 genes[d]	PAUP-MP-prot	100	100	79	59	+[c]	–[b]
9 genes[d]	ProML	100	100	100	100	70	–[b]
8 genesA[e]	PAUP-ML	100	100	100	78	72	–[b]
8 genesA[e]	Tree-Puzzle	76	100	100	–[b]	60	–[b]
8 genesA[e]	PAUP-MP-prot	100	100	74	+[c]	+[c]	–[b]
8 genesB[f]	PAUP-ML	100	100	100	85	52	–[b]
8 genesB[f]	Tree-Puzzle	78	99	100	53	–[b]	–[b]
8 genesB[f]	PAUP-MP-prot	100	100	88	76	–[b]	+[c]
7 genes[g]	PAUP-ML	100	100	100	69	59	–[b]
7 genes[g]	PAUP-MP-nt	97	88	78	–[b]	75	–[b]
7 genes[g]	Tree-Puzzle	76	100	99	–[b]	–[b]	–[b]
7 genes[g]	PAUP-MP-prot	100	100	81	57	–[b]	+[c]
atpA	PAUP-ML	81	79	+[c]	+[c]	+[c]	–[b]
atpA	Tree-Puzzle	97	73	60	–[b]	–[b]	75
atpB	PAUP-ML	100	+[c]	+[c]	–[b]	–[b]	–[b]
atpB	Tree-Puzzle	97	78	–[b]	–[b]	92	–[b]
petB	PAUP-ML	90	–[b]	–[b]	+[c]	–[b]	+[c]
petB	Tree-Puzzle	96	–[b]	–[b]	83	–[b]	92
petD	PAUP-ML	+[c]	–[b]	78	–[b]	+[c]	–[b]
petD	Tree-Puzzle	75	82	99	–[b]	–[b]	–[b]
psaA	PAUP-ML	96	75	54	+[c]	–[b]	–[b]
psaA	Tree-Puzzle	73	75	–[b]	–[b]	–[b]	–[b]
psbA	PAUP-ML	92	–[b]	–[b]	–[b]	95	–[b]
psbA	PAUP-MP-nt	55	–[b]	–[b]	–[b]	95	–[b]
psbA	Tree-Puzzle	85	62	–[b]	–[b]	72	–[b]
psbA	PAUP-MP-prot	71	61	–[b]	–[b]	64	–[b]
psbA	ProML	82	69	–[b]	–[b]	78	–[b]
psbB	PAUP-ML	88	99	69	–[b]	+[c]	–[b]
psbB	Tree-Puzzle	77	100	95	–[b]	–[b]	–[b]
psbC	PAUP-ML	96	91	65	–[b]	–[b]	–[b]
psbC	Tree-Puzzle	65	99	69	–[b]	–[b]	–[b]
psbD	PAUP-ML	98	100	90	–[b]	–[b]	–[b]
psbD	Tree-Puzzle	99	100	72	–[b]	–[b]	–[b]
psbE	PAUP-ML	95	+[c]	–[b]	–[b]	–[b]	–[b]
psbE	Tree-Puzzle	93	67	–[b]	–[b]	–[b]	–[b]

NOTE.—Analyses using PAUP-ML are nucleotide based, without the third codon position. Analyses with Tree-Puzzle and ProML are amino acid based.

[a] Defined as red algal plastids and secondary plastids derived from them.

[b] Indicates that the feature is absent from the best tree.

[c] Indicates that the feature is present on the best tree but the support value is < 50%.

[d] Analyses based on nine genes, excluding *psbA*.

[e] Analyses based on eight genes, excluding *psbA* and *petB*.

[f] Analyses based on eight genes, excluding *psbA* and *atpB*.

[g] Analyses based on seven genes, excluding *psbA*, *atpB*, and *petB*.

the dinoflagellate is excluded, and *petB* (83%) supports monophyly with the dinoflagellate included. In the ProML analysis of *psbA*, high support values are still found for a *Guillardia* + *Porphyra* relationship, but the support for a haptophyte + dinoflagellate relationship declines when amino acids are used (95% for nucleotides vs. 78% with amino acids, fig. 2*C* and *F*).

The AU test was used on the nucleotide alignments to assess the signal in the individual genes by comparing the most likely tree with trees generated from six specific hypotheses (enumerated in Materials and Methods) and the results are shown in table 3. Although several individual genes reject alternate placements of the dinoflagellate plastid with the green algae or cyanobacteria, only *psbA* rejects a placement of the dinoflagellate in the red plastid lineage using nucleotides (*P* value 0.031), but not using amino acids (*P* value 0.191). The AU test was also used on the *psbA* without the dinoflagellate where the haptophyte was constrained to either the cyanobacterial (*P* value 0.936) or red plastid lineage (*P* value 0.064). The *psbA* tree is the only gene that rejects chlorophyll c–containing plastid monophyly (table 3). For these reasons *psbA* was removed

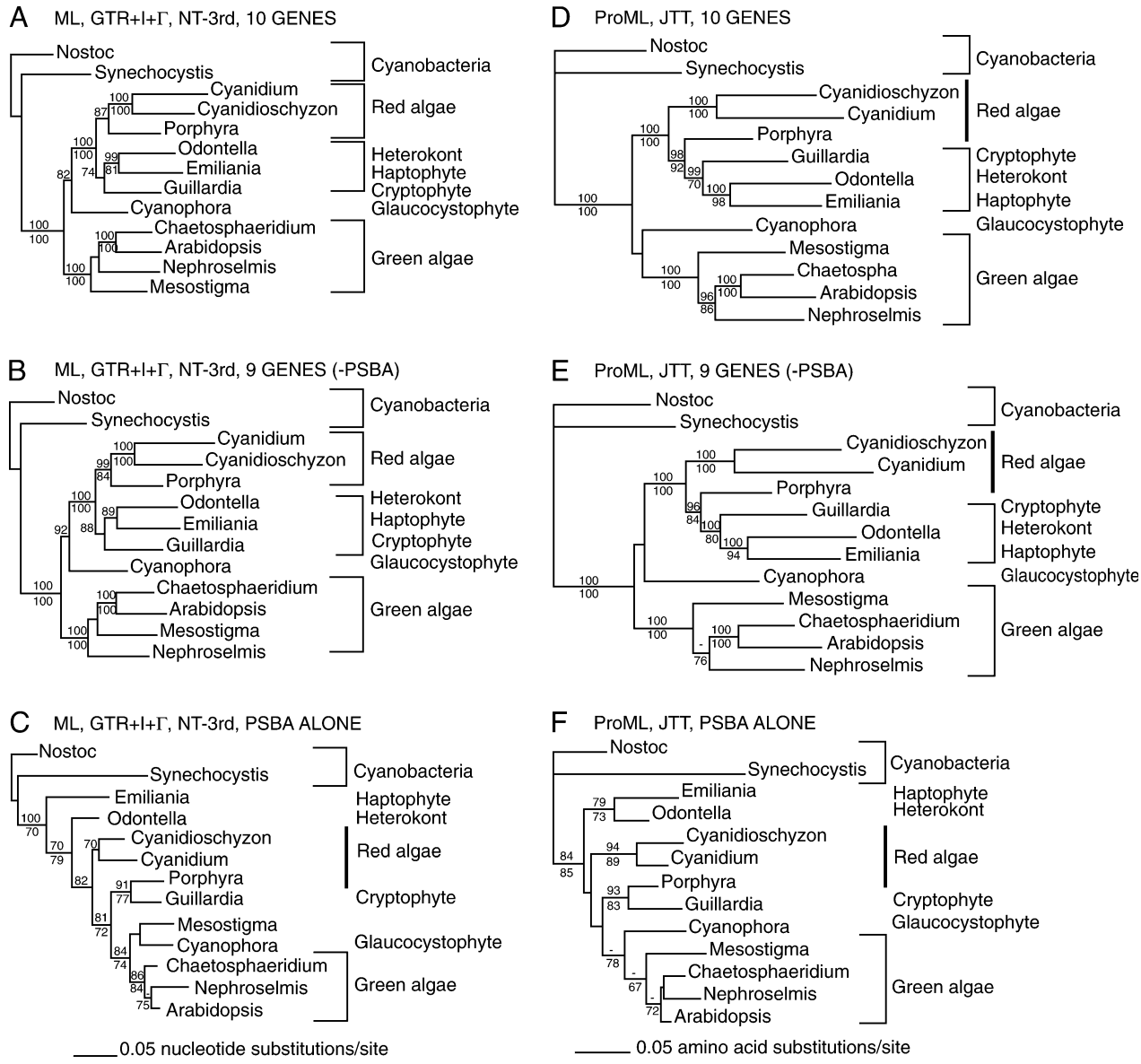

FIG. 1.—Phylogenetic analyses–based plastid genes without the dinoflagellate. Square brackets indicate monophyletic lineages, and bold lines indicate paraphyletic lineages. (*A–C*) Maximum likelihood trees based on nucleotides using PAUP* and excluding the third codon position. ML bootstrap proportions are shown when over 65% and are indicated above the branches, and MP bootstrap values are below the branches. (*D–F*) Likelihood trees based on amino acid data using ProML under the JTT model of amino acid substitution with eight rate categories. ProML bootstrap support values are shown above the branches, and MP bootstrap values are below the branches. (*A*, *D*) Phylogenetic trees based on 10 concatenated genes. (*B*, *E*) Phylogenetic trees based on nine concatenated genes without *psbA*. (*C*, *F*) Single-gene phylogenetic analyses of *psbA* alone. Other single-gene analyses are included in the Supplementary Material online.

from some concatenated analyses creating a nine-gene data set.

Concatenated Gene Analyses

The concatenated 10-gene data set supports a monophyletic chlorophyll c–containing plastid lineage (tables 1 and 2). Removing the *psbA* gene increases support for monophyletic chlorophyll c–containing plastids in nucleotide analysis (74% vs. 88% without dinoflagellate, and 73% vs. 89% with dinoflagellate), as well as in amino acid analyses (99% vs. 100% with dinoflagellate; tables 1 and 2). After careful review of the individual gene trees, concatenated data sets were constructed where *petB* and *atpB* were also excluded, as well as *psbA*, producing two eight-gene data sets: 8 genesA (no *psbA* and *petB*) and 8 genesB (no *psbA* and *atpB*) as well as a seven-gene data set (no *psbA*, *petB*, and *atpB*). The results for these data sets are similar to previous analyses in that inclusion of the dinoflagellate decreases support for chlorophyll c–containing plastid monophyly (figs. 4 and 5 of the Supplementary Material online). Monophyly of the cryptophyte, heterokont, and haptophyte

plastids is highly supported in all concatenated analyses, and chlorophyll c–containing plastid monophyly is only moderately supported in nucleotide-based analyses (tables 1 and 2; figs. 4 and 5 of the Supplementary Material online). Also, the seven-gene data set places the dinoflagellate next to the *Cyanidium* + *Cyanidioschyzon* group when Tree-Puzzle is used (figs. 4 and 5 of the Supplementary Material online). The AU test results for the concatenated data sets also show a decrease in the *P* value for chlorophyll c–containing plastid monophyly as the amount of data is reduced (table 3). Maximum parsimony analyses show generally lower support values, especially when nucleotides are used.

Notably, nucleotide analyses of the concatenated data sets find a single red algal plastid clade, with the chlorophyll c–containing plastids sister to this clade (figs. 1, 2*A–C*; figs. 4 and 5 of the Supplementary Material online). Amino acid analyses find a tree where the chlorophyll c–containing plastids are embedded within the red algae, sister to *Porphyra*, with *Cyanidium* + *Cyanidioschyzon* forming another clade (figs. 1, 2*D–F*; figs. 4 and 5 of the Supplementary Material online).

Relationships Within the Chlorophyll c–Containing Plastids

In concatenated analyses the cryptophyte *Guillardia* is the deepest branching chlorophyll c–containing plastid. This branching pattern is strongly supported in all concatenated nucleotide and amino acid trees without dinoflagellates, but when dinoflagellates are included support declines. The haptophyte plastid is sister taxon to the dinoflagellate plastid when using most concatenated data sets, but this feature is only supported when *psbA* is included (table 2). A haptophyte + dinoflagellate plastid relationship is not seen in five single-gene analyses, while four genes show this relationship with low bootstrap support (table 2). A heterokont + dinoflagellate plastid relationship is found in two individual gene tree analyses and two concatenated gene analyses (table 2).

Discussion

A good phylogeny of chlorophyll c–containing plastids is an important first step in evaluating the number of endosymbiotic events within the chromalveolates, as well as in inferring the evolution of chloroplast characters like pigmentation and membrane topology. Chlorophyll c–containing plastid monophyly is a prerequisite for chromalveolate host monophyly. The analyses presented here support chlorophyll c–containing plastid monophyly and place the cryptophytes as the basal chlorophyll c–containing plastid, but the relationships among the other chlorophyll c–containing plastids are not well resolved. Because chlorophyll c–containing plastids were acquired via secondary symbiosis, monophyly of these organelles would be consistent with a single endosymbiotic event, but alternative hypotheses might explain the data as well or better. For example, incongruities between host and plastid phylogenies could be explained by independent endosymbiotic events in unrelated hosts. Distinguishing among such hypotheses requires careful analyses of sometimes contradictory data.

The Plastid Gene *psbA* Undergoes Aberrant Evolution

Independent analyses of single genes and comparison to the concatenated analysis suggested a possible incongruence of *psbA* with the other data. Analyzed independently, *psbA* provided strong support for an unusual branching pattern not seen with the other data. To better understand the signal in this gene, the AU test was used to evaluate alternative tree topologies. The AU test complements standard phylogenetic analysis and bootstrapping by providing an AU measure of the probability that a tree with a given test topology would be observed given the data and phylogenetic method (Schimodaira 2000; Schimodaira and Hasegawa 2001). In both nucleotide and amino acid analyses this individual gene favored a tree where *Odontella*, *Emiliania*, and *Amphidinium* were sister to the cyanobacteria (table 3, fig. 2*C* and *F*). Such a phylogeny is inconsistent with the other data presented here and with a variety of structural and molecular data that indicate that these plastids are secondarily derived from those of red algae. The AU test results using nucleotides for this single gene rejected a relationship of the dinoflagellate with the red lineage and revealed that the *psbA* gene is incongruent with chlorophyll c–containing plastid monophyly, both with and without dinoflagellates (table 3).

In addition, it was recently shown that phylogenetic analyses based on the gene *psbA* are influenced by codon usage heterogeneity of the amino acids, leucine, serine, and arginine that may produce phylogenetic artifacts (Inagaki et al. 2004). We confirmed these observations for the gene *psbA*, although our data set does not include fucoxanthin-containing dinoflagellates. Codon usage heterogeneity of these amino acids was not identified in the other nine genes considered in this study. The apparent aberrant signal from the gene *psbA* was not completely ameliorated by using amino acid analysis, with both methods favoring a cyanobacterial relationship with the dinoflagellate, haptophyte, and heterokont group. Although the signal from this gene clearly unites the dinoflagellate and haptophyte, it is difficult to assess whether this is due to genuine shared evolutionary history; the unusual characteristics of this gene makes this observation suspect and difficult to evaluate.

Assessing Monophyly of Chlorophyll c–Containing Plastids

Chlorophyll c–containing plastid phylogeny remains controversial largely because of conflict between different data sets and methods of analysis. For example, analysis using the complete plastid genomes of the heterokont *Odontella* and the cryptophyte *Guillardia* did not recover a monophyletic chromophyte plastid lineage, although only two chromophyte lineages were sampled (Martin et al. 2002). Chlorophyll c–containing plastids were not monophyletic with better taxon sampling using the gene *rbcL* (Daugbjerg and Andersen 1997) or in concatenated *psaA* and *psbA* analysis (Yoon, Hackett, and Bhattacharya 2002). However, two analyses based on five genes that excluded dinoflagellates showed monophyletic chromophyte plastids embedded within the red algae (Yoon et al. 2002, 2004). The nuclear-encoded plastid gene *psbO* found

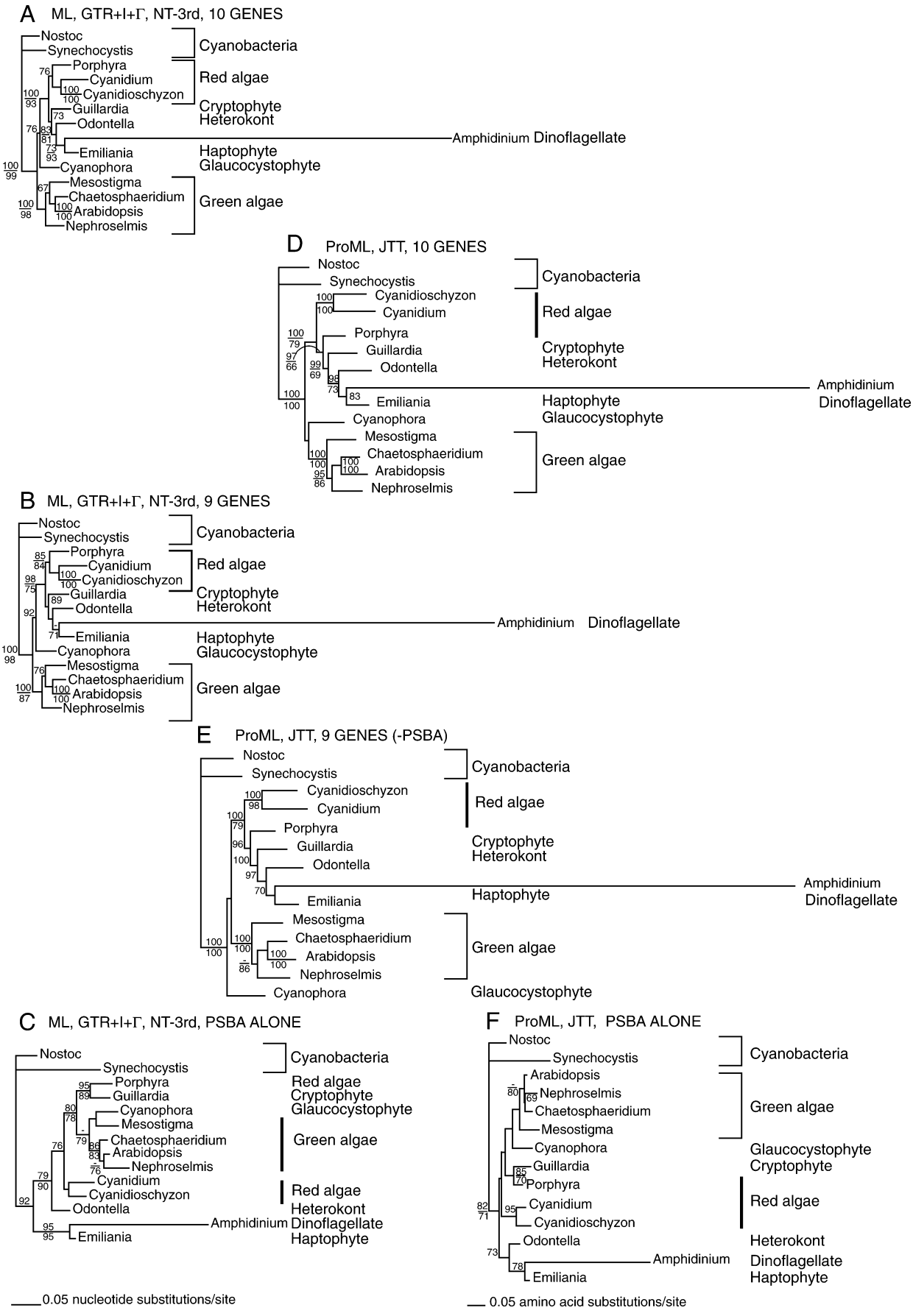

Table 3
Approximately Unbiased Test *P* Values Using ML Site Likelihoods Generated by PAUP* or PAML for Several Alternate Placements of the Dinoflagellate

Gene Name	Best Tree[a]	*Amphidinium* + Red Lineage	*Amphidinium* + Cyanobacteria	*Amphidinium* + Green	Chl c Plastids + Monophyly	*Amphidinium* + *Emiliania*	*Amphidinium* + *Odontella*
atpA	*0.951*	*0.951*	0.085	0.106	*0.951*	*0.951*	**0.045**
atpB	0.597	0.509	**0.034**	0.576	0.210	0.509	0.188
petB	*0.605*	0.402	0.425	0.120	*0.605*	0.592	*0.605*
petD	*0.700*	*0.700*	**0.006**	0.180	0.608	0.686	0.201
psaA	*0.752*	*0.752*	0.377	0.165	*0.752*	0.380	0.377
psbA	*0.977*	**0.031**	0.078	**0.002**	**0.001**	*0.977*	**0.004**
psbB	*0.589*	*0.589*	0.512	**0.035**	0.459	*0.589*	0.302
psbC	*0.800*	*0.800*	**0.043**	0.437	0.451	0.178	0.273
psbD	0.628	*0.704*	0.228	0.081	0.609	**0.009**	0.265
psbE	0.687	0.450	0.073	0.222	0.378	0.378	0.146
10 genes	*0.962*	*0.962*	**0.007**	**0.007**	*0.962*	*0.962*	0.063
9 genes	0.674	0.470	0.076	0.187	0.395	0.395	0.118
8 genesB[b]	0.870	*0.870*	0.135	0.136	*0.870*	*0.870*	0.249
8 genesA[c]	0.892	*0.892*	**0.012**	**0.012**	*0.892*	*0.892*	0.158
7 genes	*0.542*	*0.651*	**0.016**	**0.016**	*0.651*	*0.542*	0.110
10 genes[d]	0.570	*0.570*	**0.012**	**0.005**	*0.570*	0.497	*0.570*
psbA[d]	0.926	0.191	*0.926*	0.091	**0.008**	*0.926*	0.269

[a] *P* value of the best tree produced by PAUP; in cases where the best tree is compatible with a constraint the values will be equal. Boldface type indicates $P < 0.05$.
[b] Test based on eight concatenated genes, excluding *psbA* and *petB*.
[c] Test based on eight concatenated genes, excluding *psbA* and *atpB*.
[d] Test using PAML site likelihoods with amino acids.

a monophyletic chlorophyll c–containing lineage without conspicuously long branches leading to the dinoflagellates, although cryptophytes were not present in this study (Ishida and Green 2002). Analyses based on the nuclear-encoded, plastid-targeted gene GAPDH and fructose 1,6 bisphosphate aldolase (FBA) including all four chromalveolate plastid lineages supported chlorophyll c–containing plastid monophyly (Fast et al. 2001; Harper and Keeling 2003; Patron, Rogers, and Keeling 2004).

Here, available data from peridinin-containing dinoflagellate minicircle genes and new data from a haptophyte plastid were used to construct phylogenies with all four chlorophyll c–containing plastid lineages. Chlorophyll c–containing plastid monophyly is strongly supported with ML nucleotide analyses using nine concatenated genes (*psbA* excluded) and moderately supported when the anomalous *psbA* gene is included. Moreover this result is also strongly supported when the dinoflagellate is excluded. This observation was consistent in both nucleotide and amino acid analyses. Some individual gene analyses (for example *atpB*, *psbB*, and *petD*) find weakly supported trees without chlorophyll c–containing plastid monophyly, but only *psbA* strongly refutes it (table 3). In ProML analyses, chlorophyll c–containing plastid monophyly is strongly supported whether or not *psbA* is included (tables 1 and 2). A notable difference between amino acid- and nucleotide-based concatenated analyses is in the monophyly of the red algae in nucleotide analyses, whereas in amino acid analyses *Porphyra* is placed at the base of the chlorophyll c–containing plastid clade (figs. 1 and 2; *A*,*B* vs. *D*,*E*). Given that chlorophyll c–containing plastids are thought to be derived from those of red algae, monophyly of reds is not necessarily expected in this context.

Chlorophyll c–Containing Plastid Relationships

The relationships among chlorophyll c–containing plastids are hard to resolve. In most concatenated analyses, the plastid of the cryptophyte *Guillardia* is found sister to the other chlorophyll c–containing plastids, and this has good support. This contrasts sharply with the placement of the haptophyte plastid at the root of the chlorophyll c–containing plastid clade found using the LHC, GAPDH, FBA, and *rbcL* genes (Daugbjerg and Andersen 1997; Dunford et al. 1999; Harper and Keeling 2003; Patron, Rogers, and Keeling 2004).

In previous studies, dinoflagellate plastids were grouped with heterokont plastids in analyses of nuclear-encoded, plastid-associated GAPDH and LHC gene sequences; in these analyses the branches leading to dinoflagellates are less extreme (Dunford et al. 1999; Fast et al. 2001). However, the LHC gene trees are now known to

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FIG. 2.—Phylogenetic analyses based–plastid genes with all four chlorophyll c–containing plastid lineages. Square brackets indicate monophyletic lineages, and bold lines indicate paraphyletic lineages. (*A–C*) Maximum likelihood trees based on nucleotides using PAUP* and excluding the third codon position. ML bootstrap proportions are shown when over 65% and are indicated above the branches, and MP bootstrap values are below the branches. (*D–F*) Likelihood trees based on amino acid data using ProML under the JTT model of amino acid substitution with eight rate categories. ProML bootstrap support values are shown above the branches, and MP bootstrap values are below the branches. (*A*, *D*) Phylogenetic trees based on 10 concatenated genes. (*B*, *E*) Phylogenetic trees based on nine concatenated genes without *psbA*. (*C*, *F*) Single-gene phylogenetic analyses of *psbA* alone. Other single-gene analyses are included in the Supplementary Material online.

have sampled only a single gene from a complex gene family, and further work is needed to assess the paralogy of these genes (Bachvaroff et al. 2004). The branching order within chromalveolate plastids based on GAPDH analyses is incongruent with the present analyses. In GAPDH trees, haptophyte, and apicomplexans form a clade that is sister to all other chromalveolate plastids (Harper and Keeling 2003). Although GAPDH was originally a cytosolic gene, it can now be seen as a marker for plastid evolution like other nuclear-encoded plastid-targeted genes (see below). In this study, in analyses based on concatenated nucleotide data, the haptophyte and dinoflagellate plastids are sister taxa, although moderate bootstrap support is present only when *psbA* is included. The presence of a single dinoflagellate and single haptophyte in these analyses means that we are unable to test the hypothesis that peridinin-containing dinoflagellate plastids are derived from those of haptophytes. While our data do not clearly resolve the branching order of haptophytes, heterokonts, and dinoflagellates, the present study does not strongly support a placement of the haptophyte plastid as sister to the other chlorophyll c–containing plastids.

Implications and Models of Chloroplast Evolution

Conflicting hypotheses on the number of plastid acquisitions within the chromalveolate algae are supported by different interpretations of the same data. A single origin of the chlorophyll c–containing plastid from red algae (Yoon et al. 2002; Harper and Keeling 2003) has been assumed to correspond to host phylogeny (Baldauf 2003; Palmer 2003) and has been interpreted as support for the chromalveolate hypothesis (Cavalier-Smith 1986, 1989, 1999; Fast et al. 2001; Delwiche 2004). This hypothesis is appealing because secondary endosymbiosis is thought to require extensive gene transfer from one eukaryote to another (Bachvaroff et al. 2004) along with subsequent retargeting of these genes to the new chloroplast and consequently has been presumed to be rare (Cavalier-Smith 1999). Another potentially complex process is the removal, adaptation, or fusion of the food vacuole of the host and the outer membranes of the endosymbiont into stable membranes that can efficiently import and export metabolites and gene products. For these reasons, explanations that minimize the number of endosymbiotic events have been favored. If the chromalveolate hypothesis is correct, however, multiple independent losses of photosynthesis (and plastids) in the chromalveolates are required to explain the distribution of photosynthesis in these groups. A prediction of this hypothesis would be that there are some relict plastid-associated genes in these nonphotosynthetic chromalveolates (Andersson and Roger 2002), and the complete genomes of the ciliate *Tetrahymena* as well as the heterokont *Phytophthora* may show this.

There is no doubt that secondary endosymbiosis involves a complicated series of events. However, it is not clear how parsimony can be applied in this case—either the number of endosymbiotic events can be minimized or plastid loss and conflict between host and plastid trees can be minimized by invoking independent plastid acquisitions. Only when the conflict between chloroplast and host phylogeny is well established, as in the case of *Euglena,* for example (Delwiche 1999), can independent endosymbiotic events be invoked with certainty.

There are at least two alternative hypotheses that postulate multiple independent endosymbiotic events within the chromalveolates. The parallel model of plastid evolution invokes multiple plastid acquisitions within the chromalveolates from closely related red algae, with convergent development of similar pigmentation, given that chlorophyll c is not present in known red algae (Delwiche 1999). However, the GAPDH data argue against the parallel acquisition of plastids by multiple chromist lineages from red algae. The cyanobacterial GAPDH plastid gene that red algae use has been replaced by a cytosolic version that has been targeted to the plastid in all chromalveolate lineages (Fagan, Hastings, and Morse 1998; Fast et al. 2001; Harper and Keeling 2003). Therefore, the cytosolic GAPDH gene would have to have been independently acquired and targeted to the plastid in each of the four chromalveolate lineages. The type of FBA gene present only in chlorophyll c–containing algae (Patron, Rogers, and Keeling 2004) also argues against this hypothesis, although the evolutionary history of this gene is not completely understood.

An alternative, serial model of chlorophyll c–containing plastid evolution would postulate that the chlorophyll c–containing plastid originated once from red algae and was then passed among cryptophytes, heterokonts, haptophytes, and dinoflagellates. Under this hypothesis some of the chlorophyll c–containing plastids would be tertiary (or even quaternary) in origin. This is incompatible with naive parsimony but seems more likely given the tertiary association of diatoms and cryptomonads with dinoflagellates (Chesnick et al. 1997; Schnepf and Elbrächter 1999). There are several minor variants of this hypothesis. For example, after cryptophytes acquired their plastid from a red alga, cryptophytes themselves could have been engulfed by a heterokont, haptophyte, and/or dinoflagellate (Cavalier-Smith, Allsopp, and Chao 1994). Cryptophyte plastids with the reduced red algal nucleus and phycobiliproteins are more similar to those of red algae than any of other chlorophyll c–containing plastids and would make a biologically plausible out-group to the other chromalveolate plastids. This model of plastid transfer within the chromalveolates is compatible with plastid trees showing chlorophyll c–containing plastid monophyly and with the GAPDH data. If the substitution of the GAPDH gene by a cytosolic version had already occurred in the cryptophyte before it was engulfed by another chromalveolate host, then this gene would be passed on to the next host just like all of the other nuclear-encoded plastid-targeted genes. In addition, the serial transfer of plastids within chromalveolates could explain why basal clades of cryptophytes (Marin, Klinberg, and Melkonian 1998), heterokonts (Van de Peer and De Watcher 1997), and dinoflagellates (Saldarriaga et al. 2003) are apparently aplastidic because the plastids could have been acquired after the host lineages diverged. A prediction of this model is that membrane fusion or loss must have occurred during the transfer of plastids within chromalveolates because the haptophytes, heterokonts, and cryptophytes share a similar four-membrane topology while dinoflagellates have only three.

Multiple plastid acquisitions can also explain the apparent incongruence between host and plastid phylogeny. While plastid gene trees show monophyly of cryptophytes, haptophytes, heterokonts, and dinoflagellates (figs. 1 and 2), host gene trees based on rRNA and mitochondrial genes do not support chromalveolate host monophyly (Van de Peer and De Watcher 1997; Sanchez-Puerta, Bachvaroff, and Delwiche 2004).

The data presented here support a monophyletic chlorophyll c–containing plastid clade, while phylogenetic relationships within the chlorophyll c–containing plastids remain unclear. This study calls into question the support for a sibling-taxon relationship between haptophyte and dinoflagellate plastids. Some constructive statements can be made about the evolution of chlorophyll c–containing plastids. A serial model of plastid evolution has some advantages over monophyletic and parallel hypothesis. The boundaries of the problem are defined by a single origin from red algae, with cryptophytes as the most likely starting point. From there, at the very least, the modern dinoflagellate peridinin-type plastid is unlikely to have given rise either to that of haptophytes or heterokonts because of their anomalous rubisco (Morse et al. 1995), massive plastid to nucleus gene transfer (Bachvaroff et al. 2004; Hackett et al. 2004), and unique carotenoid, peridinin. What remains unknown or uncertain at present is the order and number of times that plastids were acquired and transferred within the haptophytes, heterokonts, and cryptophytes as well as the genuine proximal source of the dinoflagellate plastid.

Supplementary Material

Supplementary figures 1–5 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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