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# Phylogenetic analyses indicate that the 19'Hexanoyloxy-fucoxanthin-containing dinoflagellates have tertiary plastids of haptophyte origin

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# Phylogenetic Analyses Indicate that the 19'-Hexanoyloxy-fucoxanthin-Containing Dinoflagellates Have Tertiary Plastids of Haptophyte Origin

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The three anomalously pigmented dinoflagellates *Gymnodinium galatheanum*, *Gyrodinium aureolum*, and *Gymnodinium breve* have plastids possessing 19'-hexanoyloxy-fucoxanthin as the major carotenoid rather than peridinin, which is characteristic of the majority of the dinoflagellates. Analyses of SSU rDNA from the plastid and the nuclear genome of these dinoflagellate species indicate that they have acquired their plastids via endosymbiosis of a haptophyte. The dinoflagellate plastid sequences appear to have undergone rapid sequence evolution, and there is considerable divergence between the three species. However, distance, parsimony, and maximum-likelihood phylogenetic analyses of plastid SSU rRNA gene sequences place the three species within the haptophyte clade. *Pavlova gyrans* is the most basal branching haptophyte and is the outgroup to a clade comprising the dinoflagellate sequences and those of other haptophytes. The haptophytes themselves are thought to have plastids of a secondary origin; hence, these dinoflagellates appear to have tertiary plastids. Both molecular and morphological data divide the plastids into two groups, where *G. aureolum* and *G. breve* have similar plastid morphology and *G. galatheanum* has plastids with distinctive features.

## Introduction

Chloroplasts (more generally called plastids) are derived from previously free living prokaryotes through endosymbiosis between a cyanobacterium and a eukaryote (for insights and reviews, see Merezkhovskiy 1905; Douglas 1994; Van De Peer 1996; Martin et al. 1998; Palmer and Delwiche 1998; Delwiche 1999). The plastids of rhodophytes, chlorophytes, and glaucophytes are surrounded by two membranes and probably directly derived from a cyanobacterial ancestor and are thus called primary plastids. Whether or not these three plastid lineages themselves are the result of one single endosymbiosis event is not known, and there are contradictory data concerning the number of primary endosymbiosis events (Gibbs 1981; Lockhart et al. 1992; Palmer 1993; Palmer and Delwiche 1998). A number of other protists acquired their plastids via secondary endosymbiosis. Secondary endosymbiosis is a phenomenon whereby one eukaryote engulfs another eukaryote and permanently retains part of its prey as a degenerate endosymbiont (for discussion, see the articles cited above and McFadden et al. 1994). Most plastids surrounded by more than two membranes are thought to be the result of secondary endosymbiosis events (Gibbs 1981).

Within the dinoflagellates, there are several different plastid types, and many endosymbiotic events have

apparently taken place. The plastids have been acquired from various pigmentation groups (Watanabe et al. 1987; Farmer and Roberts 1990; Elbrächter and Schnepf 1996; Chesnick et al. 1997). Some species have plastids that are contained within an otherwise independent endosymbiont, and some of these may represent transitional states in which the endosymbiont is consistently present but has apparently not been integrated as a stable organelle. All of these have a secondary nucleus associated with "their" plastids (Dodge 1971; Tomas and Cox 1973).

The dinoflagellates with true plastids can roughly be divided into three groups: The most widespread and typical pigmentation pattern is chlorophyll *a* + *c* and peridinin. These plastids are typically bound by three membranes (Dodge 1975) and appear to have a unique organization of the plastid genome ("minicircles"; Zhang, Green, and Cavalier-Smith 1999). Phylogenetic analyses suggest that they are related to red algal plastids, and they are likely to be of secondary origin (Zhang, Green, and Cavalier-Smith 1999). A second group, the *Dinophysis* species, have plastids containing phycobilins and chlorophyll *a* + *c*. These plastids resemble those of cryptophytes and are bound by two membranes only (Schnepf and Elbrächter 1988). Species containing chlorophyll *a* + *c* and fucoxanthin derivatives can be said to constitute a third group (Van den Hoek, Mann, and Jahns 1995). Based on pigmentation data, there appear to be several "subgroups" of the fucoxanthin-containing species (Bjørnland 1990; Whatley 1993), and in this work we focused on a group of dinoflagellates that have 19'-hexanoyloxy-fucoxanthin as their main carotenoid (for simplicity, they will be referred to herein as the fucoxanthin-containing dinoflagellates/plastids). These organisms have no girdle lamella in their plastids (Steidinger, Truby, and Dawes 1978; Kite and Dodge 1985, 1988), and no extra nuclei have been identified (Kite and Dodge 1988; Bjørnland

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**Table 1**  
**Primers Used in the Amplification of Plastid SSU rDNA (16S) Sequences**

Name <sup>a</sup>	Primer Sequence (5'–3')	Specificity	Melting Temperature <sup>b</sup>
1GenF	CCTGGCTCAGGATGAACGCT	General 16S	61.3°C
557GenF	GTGCCAGCAGCCCGGTAA	General 16S	66.3°C
958GenF	AAACTCAAAGGAATTGACGGG	General 16S	56.8°C
394PlaF	GCAGTGAGGAATTTTCCG	Plastid 16S	52.7°C
411PlaF	GCAATGGCGAAAGCCTGACGG	Plastid 16S	65.5°C
804PlaF	CTCAGAGACGAAAGCTA	Plastid 16S	41.9°C
836PlaF	TAGATACCCCTGTAGTCCTA	Plastid 16S	45.2°C
193G_aurF	AACTCTATATGCTAGTGGTC	<i>Gyrodinium aureolum</i>	42.1°C
444G_aur/breveF	TGTGGAGGATGAAGGATCATAAA	<i>Gymnodinium breve</i> / <i>G. aureolum</i>	57.2°C
958GenR	CCCGTCAATTCCTTTGAGTTT	General 16S	56.8°C
1554 GenR	TGACGGGCGGTGTACAAAG	General 16S	61.5°C
542PlaR	CTGGCACGGAGTTAGCCGAT	Plastid 16S	61.1°C
817PlaR	TCTAATCCCATTTGCTCCCTA	Plastid 16S	53.8°C
855PlaR	AGTATCCATCGTTTACGGCTA	Plastid 16S	53.1°C
1209G_aurR	GTCGAGAGACTCTGTTTAC	<i>G. aureolum</i>	44.9°C

<sup>a</sup> The numbers refer to positions in the primer design alignment (available on request).

<sup>b</sup> As measured by Primer Express software (Perkin Elmer, demo version).

1990). The three species investigated were *Gymnodinium galatheanum* Braarud, *Gyrodinium aureolum* Hulburt, and *Gymnodinium breve* Davis. They all have pigment composition (Bjørnland 1990) and plastid ultrastructure (Steidinger, Truby, and Dawes 1978; Kite and Dodge 1985, 1988) resembling that of haptophytes.

By using capillary techniques to isolate algae for single-cell PCR and testing a large number of different primers, full-length plastid SSU rDNA (16S) gene sequences were determined from the three species. Nuclear SSU rDNA (18S) gene sequences were also determined, and various phylogenetic analyses were performed to study the origin and evolution of the fucoxanthin-containing plastids in the dinoflagellates.

## Materials and Methods

### Algal Strains, Culture Conditions, and Microscopy

Cell morphology was studied by standard light microscopy techniques. Autofluorescence of the plastids was generated by blue-light excitation and recorded by photography or video image analyses.

*Gymnodinium galatheanum* and *G. aureolum* were isolated from the Oslofjord by Karl Tangen and obtained from the division of Marine Botany at Department of Biology, University of Oslo, as was a strain of *Pavlova gyrans* Butcher. *Gymnodinium breve* was obtained from CCMP, West Boothbay Harbor, Maine. *Chrysochromulina polylepis* Manton et Parke was a gift from Bente Edvardsen at the division of Marine Botany (University of Oslo). All dinoflagellate species were cultured in Erd-Schreiber natural seawater medium (Føyn 1934, modified) under fluorescent illumination (14/10 h L/D cycle).

### Cloning and Sequencing

To isolate DNA for our nuclear SSU rDNA (18S) sequences, up to  $2 \times 10^6$  exponentially growing cells were collected from the cultures by centrifugation ( $8,000 \times g$  for 5 min). DNA isolation was performed using DNA-binding magnetic beads and ethanol precipitation/washing (Dynabeads DNA DIRECT, Dynal) as

described by Rudi et al. (1997). Nuclear SSU rDNA was PCR-amplified with primers A and B designed for amplification of haptophytes (Medlin et al. 1988).

To avoid amplification of bacterial SSU rDNA (16S) for our plastid 16S sequences, single-cell isolations were performed. Cells were capillary isolated from nonaxenic cultures, washed in sterile medium, and then transferred to 200- $\mu$ l Perkin-Elmer tubes containing sterile distilled water, in which amplification was performed directly.

Primers used to amplify SSU rDNA from the fucoxanthin-containing dinoflagellate plastids were designed with reference to a 16S SSU rDNA alignment including a wide range of both bacterial and plastid sequences (available on request). Both general SSU rDNA and plastid-specific primers were designed (table 1) and used in various combinations to amplify overlapping fragments. In addition, some species-specific primers were made to increase the specificity of the reactions and perform primer-walking (table 1). All amplifications were done with 5 mM  $Mg^{2+}$ , Dynazyme polymerase (Finnzymes), and relatively low annealing temperatures.

PCR products were TA-cloned into pGEM-T vector (Promega), and both strands were sequenced by cyclic dideoxy chain termination using a Vistra 725 sequencer with Texas red-labeled primers (Amersham Pharmacia Biotech) or manually using  $^{33}P$ -labeled dideoxy nucleotides (Amersham Pharmacia Biotech). Roughly 5% of the PCR products showed similarity to plastid SSU rDNA (BLAST, version 2.0; Altschul et al. 1997), and the rest of the sequences were discarded as bacterial contamination or nuclear SSU rDNA sequences. Overlapping sequence fragments were assembled, and a single  $\sim 1,400$ -bp region spanned by primer 1554GenR and 1GenF (table 1) was generated from the three dinoflagellates.

Plastid-specific and general primers (table 1) were used to amplify plastid SSU rDNA from *Pavlova gyrans* (Pavlovophyceae) and *Chrysochromulina polylepis* (Prymnesiophyceae) from regular DNA isolations (as described for nuclear SSU rDNA).

**Table 2**  
**Accession Numbers and Strain References for the Nuclear SSU rDNA (18S) Sequences Used in the Analysis**

Species	Accession No.	Strain
<i>Alexandrium fundyense</i>	U09048	— <sup>a</sup>
<i>Alexandrium ostenfeldii</i>	U27500	—
<i>Cachonina hallii</i>	AF033865	—
<i>Ceratium fusus</i>	AF022153	CCMP 154
<i>Ceratium tenue</i>	AF022192	—
<i>Chlamydomonas pulsatilla</i>	AB001038	CCAP 11/106
<i>Chrysochromulina polylepis</i>	AJ004868	PCC200
<i>Colpoda inflata</i>	M97908	—
<i>Cryptosporidium muris</i>	X64342	pCMU221
<i>Cryptosporidium parvum</i>	L16997	—
<i>Cyanophora paradox</i>	X68483	Kies
<i>Emiliania huxleyi</i>	M87327	—
<i>Fucus disticus</i>	AB011423	Evanescens
<i>Glaucocystis nostochinearum</i>	X70803	SAG 45.88
<i>Goniomonas truncata</i>	U03072	pF45,pF46,pF47
<i>Gonyaulax spinifera</i>	AF052190	GSTL1
<i>Gracilaria lemaneiformis</i>	M54986	—
<i>Gymnodinium breve</i>	AF72714	CCMP 718
<i>Gymnodinium catenatum</i>	AF022193	—
<i>Gymnodinium fuscum</i>	AF022194	—
<i>Gymnodinium galatheanum</i>	AF172712	KT-77B
<i>Gyrodinium aureolum</i>	AF172713	KT-77D
<i>Gyrodinium impudicum</i>	AF022197	—
<i>Klebsormidium flaccidum</i>	X75520	SAG 335-2b
<i>Mallomonas papillosa</i>	M55285	CCMP A3807
<i>Neospora caninum</i>	U17345	BPA1
<i>Ochromonas danica</i>	M32704	—
<i>Onychodromus quadricornutus</i>	X53485	—
<i>Oxytricha nova</i>	X03948	—
<i>Palmaria palmata</i>	Z14142	—
<i>Pavlova gyrans</i>	U40922	CCMP 607
<i>Pavlova salina</i>	L34669	Plymouth 486
<i>Pentapharsodinium tyrrhenicum</i>	AF022201	—
<i>Perkinsus</i> sp.	L07375	—
<i>Phaeocystis antarctica</i>	X77477	CCMP 1374
<i>Porphyra purpurea</i>	L26201	Avonport
<i>Prorocentrum mexicanum</i>	Y16232	PMOO04
<i>Prymnesium patelliferum</i>	L34670	Plymouth 527
<i>Pyrenomonas salina</i>	X54276	—
<i>Sarcosystis muris</i>	M64244	—
<i>Skeletonema costatum</i>	X85395	CCAP 1077/3
<i>Staurastrum</i> sp.	X74752	M752
<i>Stylonychia pustulata</i>	X03947	—
<i>Symbiodinium corcolorum</i>	L13717	350
<i>Symbiodinium pilosum</i>	M88518	—
<i>Thalassionema nitzschioides</i>	X77702	CCAP 1084/1
<i>Toxoplasma gondii</i>	M97703	RH

<sup>a</sup> No strain references available.

### Phylogenetic Analyses

The framework for our nuclear SSU rDNA alignment was downloaded from the rRNA WWW server (De Rijk, Van de Peer, and De Wachter 1998), and additional sequences were added using the software SeqPup (Gilbert 1996). Forty-seven species (table 2) and 1,709 unambiguously aligned characters were used in the analysis (alignment available on request). An initial topology was determined by neighbor joining (NJ) with LogDet distances using proportion of invariable sites (pinvar) estimated from an NJ tree using Kimura two-parameter distances (K2P). LogDet is thought to perform relatively well on data sets with A+T contents varying between the sequences (Lake 1994; Lockhart et

al. 1994; Swofford et al. 1996). Maximum-likelihood distances were determined using a gamma shape parameter ( $\Gamma$ , four categories)/pinvar/base frequencies/general time reversible (GTR) substitution matrix model, with parameters estimated simultaneously using the NJ tree. These distances were used to calculate a minimum-evolution (ME) tree with 20 heuristic searches using random addition of the sequences and tree bisection-reconnection (TBR) branch swapping. This model was significantly better than simpler nested models when compared according to the likelihood ratio test (Huelsenbeck and Rannala 1997). The data set was also bootstrapped with 100 replicates, using one heuristic search per replicate (random addition of the sequences) and the same model as the initial searches. All of the analyses were done using PAUP\*, version 4.0d64 (test version, D. L. Swofford).

The plastid sequences for the 16S SSU rDNA alignment were downloaded from GenBank and aligned using Pileup (GCG [Genetics Computer Group], Wisconsin Package, version 8.1-UNIX; Deveraux, Haeberli, and Smithies 1984). The plastid SSU rDNA sequence from *Heterocapsa triquetra* (Zhang, Green, and Cavalier-Smith 1999) was included in this alignment but had a very low sequence similarity when compared with the other plastid SSU rDNAs. To evaluate whether the highly divergent *H. triquetra* 16S SSU rDNA was likely to be useful for our analyses, average Jukes-Cantor (JC) distances between *H. triquetra* SSU rDNA and all other taxa in the 16S SSU rDNA alignment were estimated using Distance (GCG). GAP (GCG) was also used to measure the pairwise sequence similarity between *H. triquetra* SSU rDNA and representatives from all of the plastid groups in the alignment. Based on these analyses, the *H. triquetra* SSU rDNA sequence was excluded from our matrix. Our novel 16S SSU rDNA sequences were added and the alignment was modified according to the inferred secondary structure of the rRNA products (Neefs et al. 1993; Gutell 1994). The analysis was done using 72 species (table 3), and 1,798 unambiguously aligned characters (alignment available on request). Again, an NJ LogDet tree with pinvar estimated from an NJ/K2P tree was generated with PAUP\*, version 4.0d64, to get an initial estimate of a topology. The maximum-likelihood value for pinvar was used as an approximation of the true fraction of invariant sites for LogDet analyses. Heuristic searches (25 times) with random addition of sequences and TBR branch swapping were done in a LogDet distance analysis (ME). The data set was then bootstrapped with 100 replicates, using one heuristic search with random addition of the sequences per replicate and the same model as the initial searches.

To exclude other apparently problematic taxa, the euglenophytes and chlorarachniophytes were removed from the matrix, and a subset of the initial data set was used for maximum-likelihood analysis. Maximum-likelihood transition/transversion ratio values and base frequencies were simultaneously estimated from a new NJ LogDet tree (with pinvar estimated from an NJ/K2P tree) using PAUP\*, version 4.0d64. FastDNAm1 (version 1.1.1; Felsenstein 1981; Olsen 1994) was then used

with these parameters. Random addition of the sequences (fastDNAMLloop) with global rearrangements was performed until the same tree topology was found five times. The data set was bootstrapped 100 times using the program seqboot (PHYLIP; Felsenstein 1993), and five searches (fastDNAMLloop) were done on each data set using the same model as in the initial searches.

All of the PAUP\*, version 4.0d64, analyses were done at the Center for Information Technology Services (USIT, University of Oslo) using an SGI ONYX MPIS R10000 processor (195 MHz). The fastDNAML/PHYLIP work was done on (six parallel) IBM RS6000 SP POWER2 processors (120 MHz, part of the 32-node IBM cluster at USIT). SeqPup was used on various tabletop Macintosh computers.

## Results

### Cell Morphology and Nuclear Phylogeny

*Gyrodinium aureolum* and *G. breve* shared several distinctive morphological features when compared with *G. galatheanum*. *Gyrodinium aureolum* and *G. breve* both have highly vesiculated cytoplasm (Steidinger, Truby, and Dawes 1978) and nearly identical plastid morphologies. They have approximately 20 bean-shaped plastids that are easily separated as individual structures on compression of the cell and plastid DNA arranged as beaded bands along the ventral side of each plastid (fig. 1A; Kite and Dodge 1985). *Gymnodinium galatheanum*, on the other hand, has a compact nonvesiculated cytoplasm, the plastid(s) appear to be a single, lobate structure (fig. 1B; as indicated by Bjørnland and Tangen 1979; Kite and Dodge 1988), and the plastid DNA is arranged as scattered nucleoids (fig. 1B; Kite and Dodge 1988).

The 18S SSU rDNA sequences generated from the fucoxanthin-containing dinoflagellates were quite similar to the other dinoflagellate sequences already in GenBank (a partial sequence for *G. galatheanum* was already present; see Rowan and Powers 1992). They were easily aligned and consistently grouped within the dinoflagellate cluster throughout the phylogenetic analyses. Pinvar was estimated to be 0.40 from the NJ/K2P tree, and this value was in turn used to generate a LogDet/ME tree. In this LogDet/ME topology, all of the major groups of organisms could be recognized as monophyletic (tree not shown), and the parameters estimated were as follows: pinvar, 0.31;  $\Gamma$ , 0.68; base frequencies—A, 0.27; C, 0.17; G, 0.26; T, 0.29; and a GTR substitution matrix (AC, AG, AT, CG, CT, GT; 1.29, 3.04, 1.11, 1.06, 5.84, 1). Using maximum-likelihood distances in an ME analysis with these parameters, the same tree was found 9 out of 25 times with random addition of the sequences.

Because the origin of the fucoxanthin-containing plastids was uncertain, our analyses included a wide range of photosynthetic taxa, and most major groups of photosynthetic protists can be recognized in the tree (fig. 2), with 100% bootstrap support for monophyly of several groups. There is also high support for monophyly of the alveolates (98%), rhodophyta (95%), and chlo-

rophyta (95%; used as an outgroup). The fucoxanthin-containing dinoflagellates are all placed unequivocally within the dinoflagellate group. *Gyrodinium aureolum* and *G. breve* group together with 100% support, and they had a high degree of sequence similarity (>99% similarity using JC distances). *Gymnodinium galatheanum* comes out as a sister species, and our analyses indicate that the three species are closely related and probably belong to the same group within the dinoflagellates (71% bootstrap support).

### Plastid SSU rDNA

Distance analyses suggested that the *H. triquetra* plastid SSU rDNA sequence was too divergent to be useful in these analyses. The average JC distance between the taxa in the 16S SSU rDNA alignment generated by Pileup was estimated to be 26.92 (changes per 100 bp, *H. triquetra* excluded), whereas the average distance from *H. triquetra* 16S SSU rDNA to the other sequences was 157.82. On inspection of this alignment, no synapomorphic characters were observed for the fucoxanthin-containing dinoflagellates and *H. triquetra*, and the average degree of sequence similarity (estimated by GAP) between *H. triquetra* and selected representatives of the major plastid groups was 48.4%.

The plastid SSU rDNA sequences from the fucoxanthin-containing dinoflagellates had a relatively low sequence similarity when compared with other homologous sequences present in GenBank. Average JC distances between the fucoxanthin-containing dinoflagellates and the rest of the 16S SSU rDNA sequences in the modified alignment were 23.33 changes per 100 bp (ambiguously aligned characters excluded), whereas the average distance between the rest of the taxa was 16.80. *Gyrodinium aureolum* and *G. breve* shared at least two insertions, and *G. galatheanum* contained at least one insertion with no apparent homolog in any of the GenBank sequences or any of the other two fucoxanthin-containing dinoflagellates. The fucoxanthin-containing dinoflagellate sequences were also quite dissimilar to each other: *G. aureolum* and *G. breve* had a nucleotide identity of 85.65% (JC distances), and they were both only ~76% similar to *G. galatheanum*. Although highly divergent (long branches), the dinoflagellate sequences always grouped together with the haptophyte plastids in phylogenetic analyses using various distance, maximum-likelihood, and parsimony methods (data not shown).

To resolve the exact phylogenetic placement of the fucoxanthin-containing dinoflagellate plastids, an analytical approach similar to that described above was employed. The NJ LogDet tree used for parameter estimation was determined with pinvar set to 0.39, and the topology was generally compatible with other published trees (Van De Peer 1996; Daugbjerg and Andersen 1997; tree not shown), although some familiar analytical artifacts were observed (e.g., the euglenophytes grouped together with the heterokonts). This tree was used to estimate the maximum-likelihood value for the pinvar, and this was set to 0.34 for a LogDet analysis using

**Table 3**  
**Accession Numbers and Strain References for the Plastid SSU rDNA (16S) Sequences Used in the Analysis**

Species	Accession No.	Strain
<i>Anabaena</i> sp.....	X59559	PCC 7120
<i>Antithamnion</i> sp.....	X54299	— <sup>a</sup>
<i>Calothrix</i> D253.....	X99213	—
<i>Chara</i> sp.....	X75519	Huss-1993
<i>Chlamydomonas moewusii</i> .....	X15850	—
<i>Chlamydomonas pallidostigmatica</i> .....	L39865	—
<i>Chlamydomonas reinhardtii</i> .....	J01395	—
<i>Chlorarachnion reptans</i> .....	U03275	CCMP 238
<i>Chlorarachnion</i> sp.....	U21491	CCMP 240
<i>Chlorella ellipsoidea</i> .....	X12742	C87
<i>Chlorella kessleri</i> .....	D11346	211-11h
<i>Chlorella mirabilis</i> .....	X65100	748-I
<i>Chlorella protothecoides</i> .....	X65688	211-7a
<i>Chlorella saccharophila</i> .....	D11349	211-1d
<i>Chlorella sorokiniana</i> .....	X65689	211-8k
<i>Chlorella vulgaris</i> 1.....	X16579	211-11b
<i>C. vulgaris</i> 2.....	D11347	211-1e
<i>Chlorogloeopsis</i> sp.....	X68780	PCC7518
<i>Chondrus crispus</i> .....	Z29521	—
<i>Chrysochromulina polylepis</i> .....	AF172719	B152j
<i>Coleochaete orbicularis</i> .....	U24579	—
<i>Cyanidium caldarium</i> .....	X52985	14-1-1
<i>Cyanophora paradoxa</i> 1.....	U30821	Pringsheim LB555
<i>C. paradoxa</i> 2.....	X81840	Kies strain, SAG B 45.84
<i>Emiliania huxleyi</i> .....	X82156	PML 92D
<i>Euglena gracilis</i> .....	X70810	Z
Eukaryote clone 1.....	U32670	OM20, Cape Hattera
Eukaryote clone 2.....	U32671	OM21, Cape Hattera
<i>Glaucocystis nostochinearum</i> .....	X82496	SAG 45.88
<i>Glaucosphaera vacuolata</i> .....	X81903	SAG B 13.82
<i>Gloeochara wittrockiana</i> .....	X82495	—
<i>Gloeothece membranacea</i> .....	X78680	PCC6501
<i>Glycine max</i> .....	X07675	Maple arrow
<i>Guillardia theta</i> .....	AF041468	—
<i>Gymnodinium breve</i> .....	AF172718	CCM P718
<i>Gymnodinium galatheanum</i> .....	AF172716	KT-77B
<i>Gyrodinium aureolum</i> .....	AF172717	KT-77D
<i>Isochrysis</i> sp.....	X75518	SAG 927-2
<i>Klebsormidium flaccidum</i> .....	X75522	SAG 335-2b
<i>Mantoniella squamata</i> .....	X90641	CCAP 1965/1
<i>Marchantia polymorpha</i> .....	X04465	—
<i>Microcoleus</i> sp.....	X70770	PCC7420
<i>Microcystis aeruginosa</i> .....	D89032	NIES98
<i>Microcystis holsatica</i> .....	D89036	NIES43
<i>Microcystis viridis</i> .....	D89033	NIES102
<i>Microcystis wesenbergii</i> .....	D89034	NIES111
<i>Nanochlorum eucaryotum</i> .....	X76084	—
<i>Nodularia</i> sp.....	AJ224447	BCNOD9427
<i>Ochromonas danica</i> .....	X53183	SAG 933-7
<i>Ochrosphaera neapolitana</i> .....	X99077	CCMP593
<i>Ochrosphaera</i> sp.....	X65101	181
<i>Odontella sinensis</i> .....	Z67753	—
<i>Olisthodiscus luteus</i> .....	M82860	—
<i>Oscillatoria neglecta</i> .....	AB003168	M-82
<i>Oscillatoria rosea</i> .....	AB003164	M-220
<i>Oscillatoria</i> sp.....	AB003163	M-117
<i>Palmaria palmata</i> .....	Z18289	—
<i>Pavlova gyrans</i> .....	AF172715	—
<i>Phormidium minutum</i> .....	X62686	D5
<i>Phormidium mucicola</i> .....	AB003165	M-221
<i>Phormidium</i> sp.....	AB003169	M-99
<i>Pleurocapsa</i> sp.....	X78681	PCC7516
<i>Porphyra purpurea</i> .....	U38804	Avonport
<i>Prochloron</i> sp.....	X63141	—
<i>Prototheca wickerhamii</i> .....	X74309	263-11
<i>Pylaiella littoralis</i> .....	X14873	pPLiE4
<i>Pyrenomonas salina</i> .....	X55015	—
<i>Skeletonema costatum</i> .....	X82154	UBS-18
<i>Skeletonema pseudocostatum</i> .....	X82155	CSIRO culture CS-76

**Table 3**  
**Continued**

Species	Accession No.	Strain
<i>Spirogyra maxima</i> .....	U24596	—
<i>Spirulina subsalsa</i> .....	AB003166	M-223
<i>Trichodesmium</i> sp. ....	X70767	NIBB 1067

<sup>a</sup> No strain references available.

heuristic searches. The best tree was found 4 out of 20 times using this model, and in the tree generated, all of the major groups of plastids can be recognized, albeit with only moderate support for several groups (fig. 3). The fucoxanthin-containing dinoflagellates always grouped together with the haptophyte plastids, and this clade is supported by an 80% bootstrap value in the distance analyses presented. Consistent with the indels observed in the alignment, *G. aureolum* and *G. breve* are held together with 100% bootstrap support, while the position of *G. galatheanum* has more moderate support (68% bootstrap).

The basal branching pattern of the haptophytes was not strongly supported, and the branch separating *P. gyrans* from the rest of the haptophytes (including the fucoxanthin-containing dinoflagellates) had low bootstrap support (thus, the branch indicated by a black dot in fig. 3 did not get support in the 50% majority-rule consensus tree).

Maximum-likelihood analysis with the euglenophytes and chlorarachniophytes excluded provided the strongest support for the basal branching pattern of the haptophytes (including the fucoxanthin-containing dinoflagellates). Both euglenophytes and chlorarachniophytes have proved to be difficult groups in analyses of plastid SSU rDNA (Nelissen et al. 1995; Van De Peer 1996), and our analyses were no exception. However, the inclusion or exclusion of representatives of these groups did not affect the placement of the fucoxanthin-containing dinoflagellates with the haptophytes (data not shown). An NJ LogDet analysis (pinvar set to 0.39) was performed with chlorarachniophytes and euglenophytes excluded (tree not shown). The LogDet tree was fully compatible with the ME tree (fig. 3) and was used to estimate the parameters subsequently used in a maxi-

mum-likelihood analysis: transition/transversion ratio, 1.88; base frequencies—A, 0.27; C, 0.21; G, 0.24; T, 0.28. The tree generated by fastDNAMl using the F84 model (with no correction for site-to-site rate variation) was fully resolved (fig. 4). There was moderate (78%) support for monophyly of the haptophyte plastids (including the fucoxanthin-containing dinoflagellates), with *P. gyrans* as the most basal branching haptophyte species. Monophyly of the haptophyte plastids is consistent with analyses of *rbcL* (Medlin et al. 1997; Daugbjerg and Andersen 1997), and assuming that this is correct, there is 96% bootstrap support for the embedding of the fucoxanthin-containing dinoflagellates within the haptophyte clade.

## Discussion

### The Fucoxanthin-Containing Dinoflagellates Have Plastids of Tertiary Origin

Phylogenetic analyses of plastid SSU rDNA place the plastids of the fucoxanthin-containing dinoflagellates unequivocally among those of haptophytes. The close relationship between the fucoxanthin-containing dinoflagellate plastids and those of haptophytes is also supported by common lack of a girdle lamella and the presence of 19'hexanoyloxy-fucoxanthin in both groups (Steidinger, Truby, and Dawes 1978; Bjørnland and Tangen 1979; Kite and Dodge 1985, 1988). Because the plastids of haptophytes are themselves thought to be secondary in origin (Gibbs 1981; Cavalier-Smith 1993; Medlin et al. 1997), and if the fucoxanthin-containing dinoflagellates acquired their plastids from a haptophyte, then these dinoflagellates are the result of three sequential endosymbiotic events (i.e., they are tertiary plastids; fig. 5). Our analyses support this conclusion, indicating that the plastids have been sequestered from within the haptophyte group.

If a dinoflagellate engulfed a haptophyte or haptophyte-like alga by phagocytosis and retained its plastids, it would be expected to generate plastids surrounded by six membranes (four from the original haptophyte plastid, one from the haptophyte host, and one derived from the food vacuole of the dinoflagellate). The number of membranes surrounding the plastids in the fucoxanthin-containing dinoflagellates is somewhat uncertain but is certainly less than six, with reported numbers ranging from two to four (Kite and Dodge 1988; Dodge 1989). Membrane loss appears to be possible after establishing endosymbiosis (Gibbs 1981; Schnepf and Elbrächter 1988), which may explain this relatively small number of membranes. An alternative scenario could be

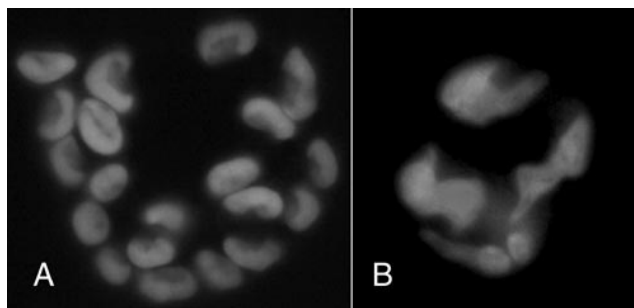


FIG. 1.—Plastid morphology of *Gymnodinium galatheanum* and *Gyrodinium aureolum* shown by blue-light-induced autofluorescence. A, The approximately 20 individual bean-shaped plastids show a band of plastid DNA along the ventral median in *G. aureolum*. B, The single, lobate plastid of *G. galatheanum*.

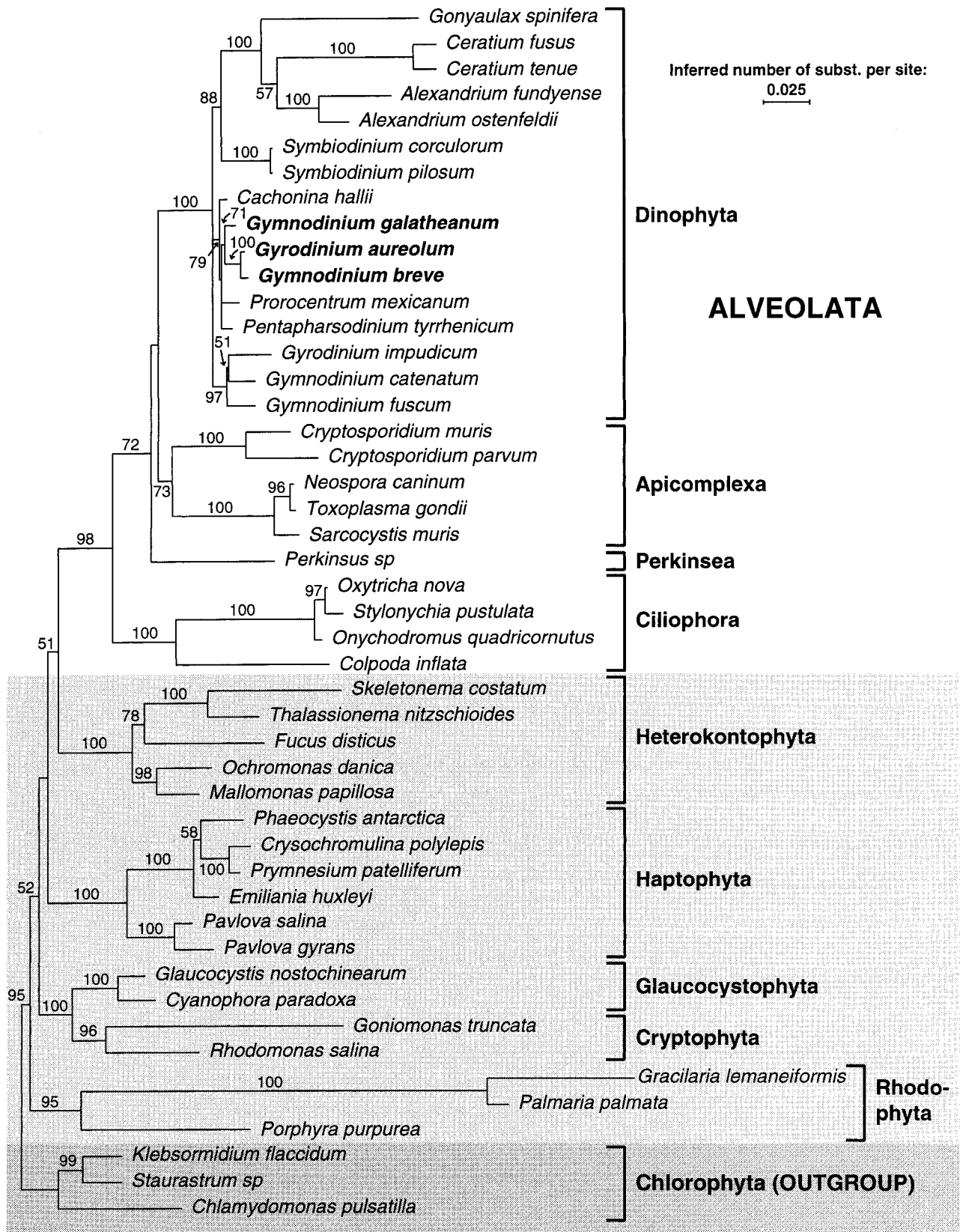


FIG. 2.—Distance tree (minimum evolution) using nuclear SSU rDNA (18S) and maximum-likelihood distances (PAUP\*, version 4.0d64). The tree topology was found 9 out of 25 times using heuristic searches with TBR branch swapping and random-addition sequences. Bootstrap values (100× heuristic searches with one random addition per replicate and TBR branch swapping) above 50% are indicated.



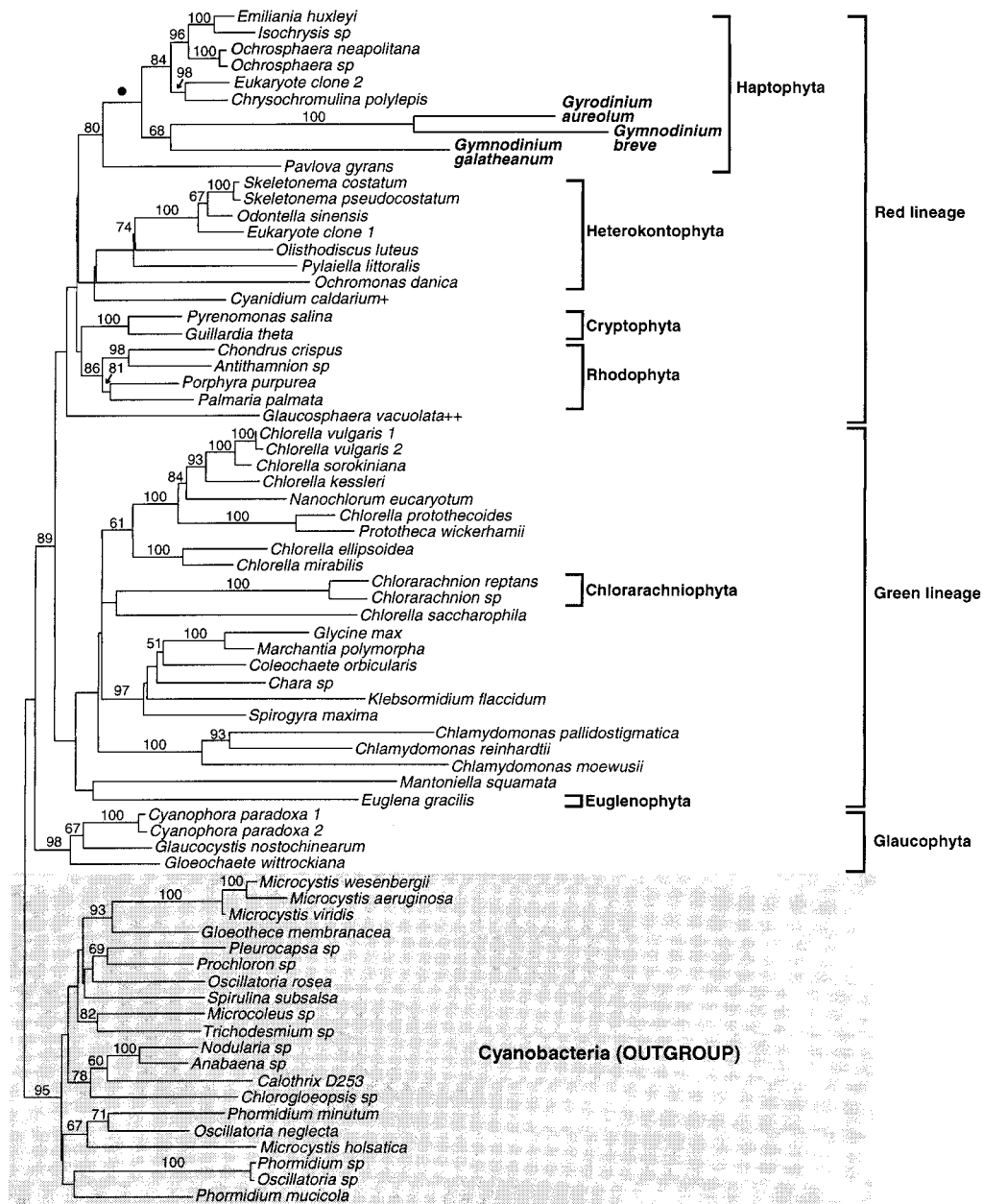


FIG. 3.—Distance tree (minimum evolution) using plastid SSU rDNA (16S) and LogDet distances (PAUP\*4.0d64). The tree topology was found 4 out of 20 times using heuristic searches with TBR branch swapping and random-addition sequences. Bootstrap values (100× with random addition and TBR branch swapping) above 50% are indicated. The branch indicated by a black dot was one of the features that did not get support in the 50% majority-rule consensus tree. + = synonymous with *Galdieria sulphuraria* and probably has rhodophyte plastids (Daugbjerg and Andersen 1997), but this has been difficult to show using plastid SSU rDNA (Nelissen et al. 1995). ++ = often classified as a glaucophyte, but has plastids that have been shown to belong within the red lineage (Helmchen, Bhattacharya, and Melkonian 1995).

that dinoflagellates have acquired their plastids through myzocytosis (Schnepf and Deichgräber 1984; Delwiche 1999), a process whereby a predatory dinoflagellate takes up the prey cell contents only, not its cell wall or plasmalemma. Myzocytotic uptake of prey cell cytoplasm leads to a food vacuole with a single membrane separating two cytoplasmic compartments (Schnepf and Deichgräber 1984). If the cytoplasm taken up by this process contains a plastid and perhaps some other vital components, the foundation of endosymbiosis may have been laid.

#### Cryptic Endosymbionts and Tertiary Endosymbiosis

Peridinin has long been recognized as the typical carotenoid for the dinoflagellates. Because the peridinin-pigmented dinoflagellates do not seem to be a monophyletic group (fig. 2), the distribution of peridinin within the dinoflagellates cannot be explained simply. One possibility is that a plastid with this unique pigment was present in the common ancestor of all dinoflagellate species (Bjørnland and Liaaen-Jensen 1989; Van den Hoek, Mann, and Jahns 1995; unpublished data). There seems

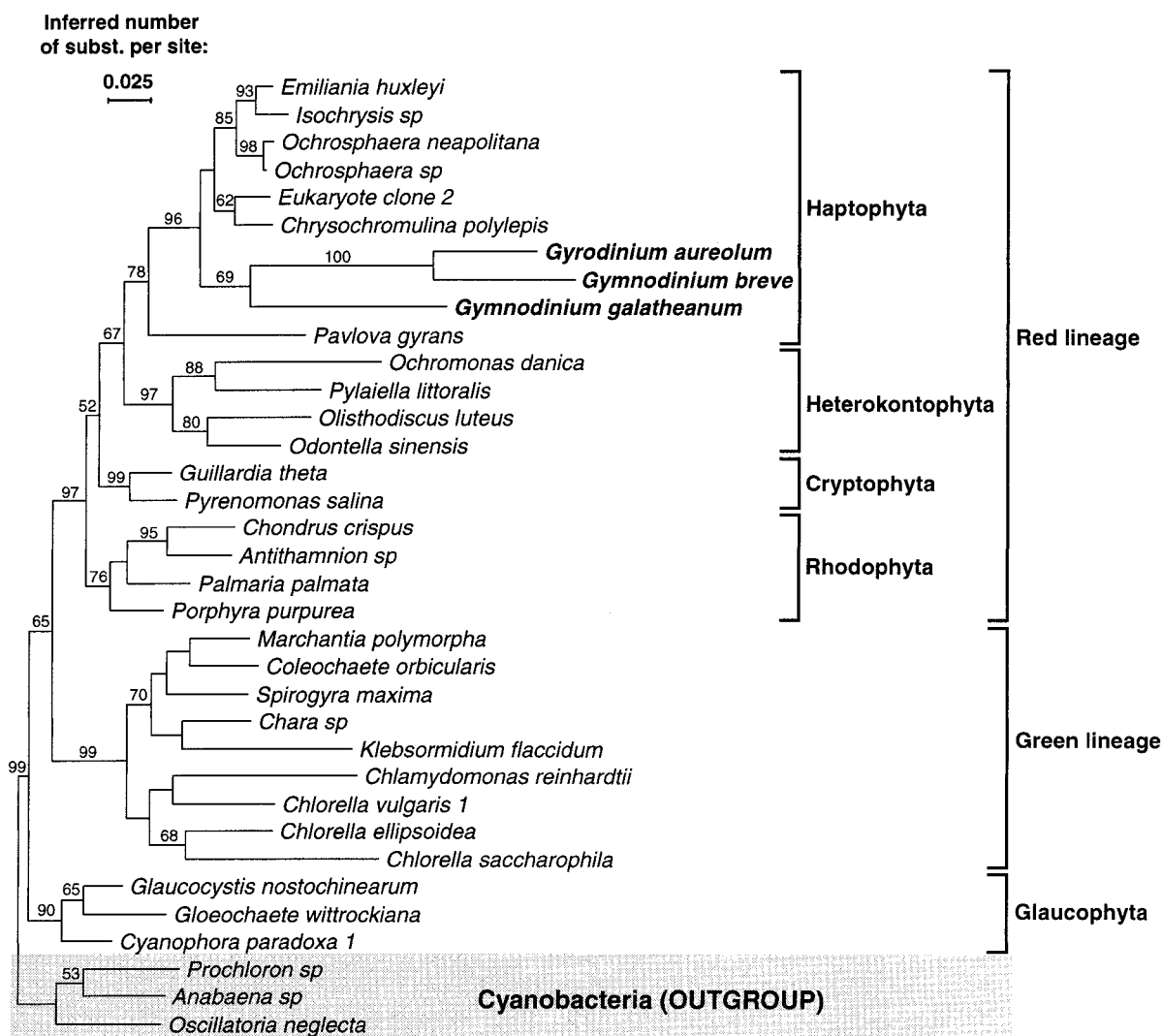


FIG. 4.—Maximum-likelihood tree using 16S plastid SSU rDNA (chlorarachniophytes and euglenophytes excluded). FastDNAmI was used (parameters estimated with PAUP\*, version 4.0d64) with random addition of the sequences and global rearrangements. Bootstrap values (100× with 5× random addition of the sequences and global rearrangements) above 50% are indicated.

to be no close relationship between the *H. triquetra* plastid SSU rDNA and the SSU rDNA sequences from the fucoxanthin-containing dinoflagellates, since no synapomorphic characters are observed between these two plastid types. Because of the very derived nature of the *H. triquetra* plastid SSU rDNA sequence (sequences with JC distances higher than 100 substitutions per 100 bp cannot be used reliably in distance analyses [Jin and Nei 1990], and the *H. triquetra* 16S SSU rDNA was considered too derived even for maximum-likelihood analyses), the exact phylogenetic placement of this sequence remains uncertain, although it seems highly unlikely that the peridinin-type plastid and the fucoxanthin-containing plastids have a common origin. It follows that an ancestor of the fucoxanthin-containing dinoflagellates would have at one time contained a peridinin-type plastid and that this lineage has gone through at least one “switch” in plastid type (“cryptic endosymbiosis”; Henze et al. 1995), perhaps including a period without plastids. Under this hypothesis, gene-

transfers from the cryptic endosymbionts (e.g., peridinin-containing plastids) might have provided the nucleus of the host with enough plastid-derived genes to facilitate such “plastid switches.”

#### Monophyletic or Polyphyletic Origin of the Dinoflagellate Fucoxanthin-Containing Plastids?

Do the endosymbioses represented by *G. galatheanum*, *G. aureolum*, and *G. breve* plastids represent a single event (i.e., uptake of one alga by a single dinoflagellate host) or two or more separate engulfment events of phylogenetically related haptophytes? There is high bootstrap support (100%) for monophyly of the *G. aureolum* and *G. breve* plastids; however, association of *G. galatheanum* with this group finds more moderate support (69% in the maximum-likelihood tree), and the three sequences did not form a monophyletic group under all analytical conditions (e.g., in maximum-likelihood analyses using PAUP\*, version 4.0d64, and the

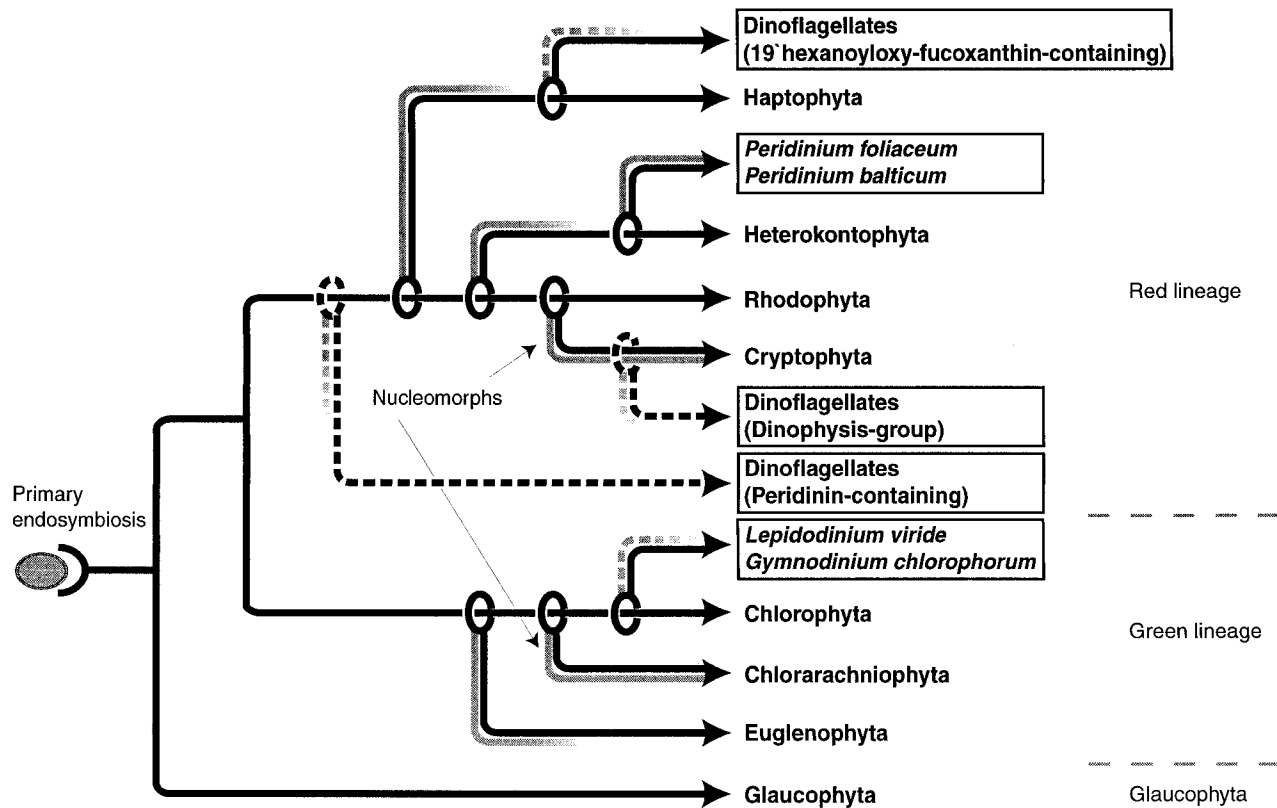


FIG. 5.—Schematic drawing of the endosymbiotic relationships between plastids with emphasis on dinoflagellates (shown in boxes). For simplicity, the figure assumes a monophyletic origin of all plastids, but this is a controversial issue (for discussion, see Palmer and Delwiche 1998). Three primary lineages of plastids can be recognized (chlorophyta, rhodophyta, and glaucophyta). Most of the data available indicate that glaucophyta has the most “primitive” plastids (Martin et al. 1998). Several secondary endosymbiosis events have also been indicated. Acquisition of endosymbiont nuclei (nucleomorphs) is shown by the gray lines. The order of these secondary events remains unresolved from our analyses; e.g., we cannot say whether haptophyte plastids originated before heterokont plastids or vice versa. The figure also assumes that the euglenophytes have secondary plastids, but this has been subject to discussion (Cavalier-Smith 1992). Phylogenetic analyses of genes from one peridinin-containing dinoflagellate (*Heterocapsa triquetra*) indicate a red algal origin of these plastids (Zhang, Green, and Cavalier-Smith 1999), but because of the highly derived nature of this plastid genome, the exact origin remains uncertain (dotted line). Other dotted lines indicate nucleomorph acquisition and reduction (fading lines) in other dinoflagellate species, but as with the placement of *Lepidodinium viride*, *Gymnodinium chlorophorum*, and *Dinophysis* species, there are no molecular data available for this. The plastids of apicomplexans have been omitted because of their uncertain origin (for discussion, see Delwiche 1999).

reduced data set; data not shown). In the trees in which the fucoxanthin-containing plastids did not form a monophyletic group, *G. aureolum* and *G. breve* were monophyletic and the sibling group to all haptophyte plastids except *P. gyrans*, while *G. galatheanum* grouped together with *Isochrysis* sp. and *Emiliania huxleyi*. Although there was overall low bootstrap support for this arrangement, the morphological differences between *G. galatheanum* and *G. aureolum/G. breve* plastids could also argue for two independent endosymbiosis events (see also Tangen and Bjørnland 1981; Kite and Dodge 1988). The plastid morphology described for *G. galatheanum* is not known from any haptophyte, and it is not unreasonable to believe it evolved after the plastids were resident in dinoflagellates. On the sequence level, *G. aureolum* and *G. breve* share at least one insertion in their plastid SSU rRNA genes, while *G. galatheanum* appears to have several unique insertions. Evidence in favor of a monophyletic origin of the fucoxanthin-containing dinoflagellate plastids is the presence of a rare carotenoid, gyroxanthin diester, found only in these species (Bjørnland et al. 1987).

#### Unusually High Evolutionary Rates in the Fucoxanthin-Containing Dinoflagellate Plastid SSU rDNA

The establishment of the fucoxanthin-containing plastid appears to be a relatively recent event in dinoflagellate evolution. All dinoflagellates with this pigmentation are closely related as measured by nuclear SSU rDNA (fig. 2), and they form a well-defined, monophyletic group in various phylogenetic analyses using a large number of nuclear SSU rDNA sequences (unpublished data). Why, then, are their plastids so derived both at the morphological level and at the molecular level (see figs. 1, 3, and 4)? For example, the *G. aureolum* and *G. breve* (99.47% sequence similarity of nuclear SSU rDNA using JC distances) plastid SSU rDNA sequences have a lower degree of sequence similarity than the rhodophyte *Porphyra purpurea* versus the chlorophyte *Chlorella mirabilis* plastid SSU rDNA (JC distances). It seems that these plastids have a very high evolutionary rate, but the precise reasons for this apparent acceleration are unknown.

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