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Dinoflagellate Nuclear SSU rRNA Phylogeny Suggests Multiple Plastid Losses and Replacements

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Abstract. Dinoflagellates are a trophically diverse group of protists with photosynthetic and non-photosynthetic members that appears to incorporate and lose endosymbionts relatively easily. To trace the gain and loss of plastids in dinoflagellates, we have sequenced the nuclear small subunit rRNA gene of 28 photosynthetic and four non-photosynthetic species, and produced phylogenetic trees with a total of 81 dinoflagellate sequences. Patterns of plastid gain, loss, and replacement were plotted onto this phylogeny. With the exception of the apparently early-diverging Syndiniales and Noctilucales, all non-photosynthetic dinoflagellates are very likely to have had photosynthetic ancestors with peridinin-containing plastids. The same is true for all dinoflagellates with plastids other than the peridinin-containing plastid: their ancestors have replaced one type of plastid for another, in some cases most likely through a non-photosynthetic intermediate. Eight independent instances of plastid loss and three of replacement can be inferred from existing data, but as more non-photosynthetic lineages are characterized these numbers will surely grow.

Key words: Plastid — Dinoflagellates — Small subunit rRNA — Phylogeny — Endosymbiosis

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Introduction

There is now no serious doubt that mitochondria and plastids are descendants of free-living prokaryotic cells (Gray and Spencer 1996). The primary endosymbioses that incorporated these cells into eukaryotic organisms are, however, exceedingly rare events: mitochondria were probably incorporated only once in the history of life (Roger 1999), and the same is probably true for plastids (Delwiche 1999; Cavalier-Smith 2000). Vertical descendants of plastids obtained through primary endosymbiosis are now found in many photosynthetic organisms (glaucophytes, red and green algae, and land plants), but the plastids of other algae have a more complicated history. In euglenoids, chlorarachniophytes, sporozoans (apicomplexans), dinoflagellates, and chromists (heterokonts, cryptomonads, and haptophytes), plastids were acquired by secondary endosymbioses: the uptake and retention of photosynthetic protists by heterotrophic eukaryotes (Taylor 1974; McFadden and Gilson 1995). Although more frequent than primary endosymbiosis, this process is also very rare (Delwiche 1999; Cavalier-Smith 2000), probably because it involves the generation of a protein-import machinery and topogenic import sequences on all the genes transferred from the endosymbiont into the nucleus, which necessitates large numbers of mutations (Cavalier-Smith and Lee 1985).

Organellar losses could be more common, but they are very difficult to document: loss of function does not imply the loss of the organelle itself, and it is often very difficult to determine whether an organelle is absent or

only degenerated to a point where it is unrecognizable. Loss of photosynthesis has certainly been more frequent than complete loss of plastids, and many secondarily non-photosynthetic eukaryotes (e.g. the euglenoid *Astasia*, sporozoans, and some higher plants) have retained plastids for functions different than photosynthesis, for example, starch biosynthesis and storage, fatty acid biosynthesis, etc. (Siemeister and Hachtel 1989; Depamphilis and Palmer 1990; Wilson 1993). In other cases, electron microscopy has failed to identify a plastid in organisms with a clear photosynthetic ancestry. This is the case in *Khawkinea* (Euglenozoa, Linton et al. 1999), and in several heterokonts such as some pedinellids (e.g. *Ciliophrys*, *Pteridomonas*, and *Actinomonas*, Cavalier-Smith et al. 1995) and *Oikomonas* (clearly related to chrysophytes, Cavalier-Smith et al. 1996). In all of these cases, true plastid losses are likely to have occurred. However, the group that may have experienced the largest number of plastid losses (and possibly also the largest number of new gains) is the dinoflagellates, a group of alveolate protists with an exceptionally varied trophic behavior (Taylor 1980, 1987; Schnepf and Elbraechter 1992, 1999; Stoecker 1999).

Roughly half of the known dinoflagellates are photosynthetic (Taylor 1987). Typical dinoflagellate plastids are surrounded by three membranes and contain closely appressed thylakoids in groups of three, chlorophylls a and c_2, and a number of carotenoids including peridinin (e.g. Schnepf and Elbraechter 1999). The genome of at least some of these peridinin-containing plastids exists as single-gene mini-circles, an organization unique to dinoflagellates (Zhang et al. 1999). From the position of peridinin-containing dinoflagellates in published 18S rRNA trees, it appears that these organisms acquired their plastids only once, relatively early in their evolutionary history (Saunders et al. 1997).

Other, atypical plastids also exist in dinoflagellates. *Gymnodinium breve*, *Gymnodinium mikimotoi*, and *Gyrodinium galatheanum* (recently renamed as *Karenia brevis*, *Karenia mikimotoi*, and *Karlodinium micrum*, Daugbjerg et al. 2000) have 19′-hexanoyloxyfucoxanthin-containing plastids derived from haptophytes (Tengs et al. 2000), while *Lepidodinium viride* and *Gymnodinium chlorophorum* have plastids with prasinophyte pigments (Watanabe and Sasa 1991; Schnepf and Elbraechter 1999). *Kryptoperidinium foliaceum* and *Durinskia baltica* (as *Peridinium foliaceum* and *P. balticum* in Chesnick et al. 1997) have fucoxanthin-containing diatoms as cytoplasmic endosymbionts. The order Dinophysiales includes colorless heterotrophic species as well as photosynthetic forms (Taylor 1980) that contain cryptomonad-like plastids (Schnepf and Elbraechter 1988) with phycobilins in the thylakoid lumen. Photosynthetic (and non-photosynthetic) members of the order have been impossible to culture, and so the suspicion exists that their photosynthetic organelles may be kleptochloroplasts (functional but non-reproductive plastids that are regularly taken up from photosynthetic prey, an occasional occurrence in heterotrophic dinoflagellates, e.g. Stoecker 1999) and not fully reproductive plastids. However, the plastids of Dinophysiales are remarkably homogeneous, a feature that weakens the kleptochloroplast argument. A very different type of plastid appears to exist in *Dinophysis* (*Phalacroma*) *rapa* (Schnepf and Elbraechter 1999), but there is little information about it. As a whole, dinoflagellates appear to have an unusual ability to take in endosymbionts.

The history of plastid gain, loss, and replacement in dinoflagellates is poorly understood, partly because dinoflagellate phylogeny itself is unclear. Traditionally, two morphological sets of characters have been used to chart their phylogeny: the presence of a dinokaryon (the uniquely modified nucleus of most dinoflagellates, e.g. Rizzo 1987), and the arrangement of the cortical alveolae (amphiesmal vesicles) in the group. Together, these two characters have given many indications of dinoflagellate evolution, but some difficulties remain, particularly with regard to the phylogeny of athecate groups and the relationships of the different dinoflagellate orders to one another (Taylor 1980; Fensome et al. 1993, 1999; Daugbjerg et al. 2000). Saunders et al. (1997) produced the first large-scale molecular study of dinoflagellate phylogeny (31 complete small subunit sequences, 41 partial ones) to address some of those issues, and argued for an early origin of the peridinin-containing plastid. However, their study contained only two non-photosynthetic species, so questions related to plastid losses could not be addressed satisfactorily.

Since then, the small subunit sequences for several non-photosynthetic dinoflagellates have become available (Gunderson et al. 1999; Litaker et al. 1999). We used those as well as 32 new 18S rRNA dinoflagellate sequences (four from non-photosynthetic species) to construct a more comprehensive phylogenetic tree of dinoflagellates on which to plot the gains and losses of plastids. Our results indicate at least eight independent plastid losses in the evolution of dinoflagellates (very probably more), and at least three instances of plastid replacement.

Materials and Methods

Organisms, DNA Extraction, Amplification, and Sequencing

Most photosynthetic dinoflagellate species were obtained from non-axenic culture collections (Table 1), but *Pyrodinium bahamense* was provided by Tony Wagey from cultures isolated in Manila Bay, Philippines. The organisms were cultured according to culture collection protocols, and DNA extracted using the DNeasy Plant DNA Purification Kit (Qiagen). Heterotrophic dinoflagellates were collected from nature: *Haplozoon axiothellae* was obtained from the gut of its

Table 1. List of strains examined in this study and GenBank Accession Numbers for their nuclear SSU rRNA sequences

Taxon	Strain Number	GenBank Accession Number
Adenoides eludens (Herdman) Balech	CCCM 683	AF274249
Amphidinium asymmetricum Kofoid and Swezy	CCCM 067	AF274250
Amphidinium carterae Hulburt	CCMP 1314	AF274251
Amphidinium corpulentum Kofoid and Swezy	UTEX LB 1562	AF274252
Amphidinium herdmanii Kofoid and Swezy	CCCM 532	AF274253
Amphidinium longum Lohmann[2]	none	AF274254
Amphidinium massartii Biecheler	CCCM 439	AF274255
Amphidinium semilunatum Herdman[2]	none	AF274256
Glenodiniopsis steinii[3] (Lemmermann) Woloszynska (as *Glenodiniopsis uliginosa*)	NIES 463	AF274257
Gonyaulax cochlea Meunier	CCMP 1592	AF274258
Gymnodinium breve Davis[4] = *Karenia brevis* (Davis) Hansen & Moestrup	CCMP 718	AF274259
Gymnodinium sp.[2]	none	AF274260
Gyrodinium dorsum Kofoid and Swezy	UTEX LB 2334	AF274261
Gyrodinium galatheanum (Braarud) Taylor[1,4] = *Karlodinium micrum* (Leadbeater & Dodge) Larsen	CCCM 555	AF274262
Gyrodinium uncatenum Hulburt	CCCM 533	AF274263
Haplozoon axiothellae Siebert[2]	none	AF274264
Heterocapsa niei (Loeblich) Morrill & Loeblich III[1]	CCMP 447	AF274265
Heterocapsa pygmaea Loeblich III, Schmidt and Sherley	CCCM 681	AF274266
Heterocapsa rotundata (Lohmann) Hansen	CCCM 680	AF274267
Kryptoperidinium foliaceum (Stein) Lindemann[1]	UTEX LB 1688	AF274268
Lingulodinium polyedrum (Stein) Dodge	CCCM 202	AF274269
Pentapharsodinium sp. Indelicato & Loeblich III (as *Scrippsiella faeroense*)	CCMP 771	AF274270
Peridinium umbonatum Stein[3] (as *Peridinium inconspicuum*)	UTEX LB 2255	AF274271
Peridinium willei Huitfeld-Kaas[3]	NIES 304	AF274272
Peridinium willei Huitfeld-Kaas[3] (as *Peridinium volzii*)	NIES 365	AF274280
Protoceratium reticulatum (Claparède & Lachmann) Bütschli	CCCM 535	AF274273
Pyrocystis lunula (Schütt) Schütt	CCCM 517	AF274274
Pyrodinium bahamense Plate	none	AF274275
Scrippsiella sweeneyae Balech ex Loeblich III	CCCM 280	AF274276
Scrippsiella trochoidea (Stein) Loeblich III	CCCM 602	AF274277
Thoracosphaera heimii (Lohmann) Kamptner[1]	CCCM 670	AF274278
Undescribed species (as *Gymnodinium varians*)	CCMP 421	AF274279

[1] Partial small subunit sequences existed before the present work.
[2] Heterotrophic species.
[3] For freshwater species we used the nomenclature of Popovsky and Pfiester 1990.
[4] Names recently changed (Daugbjerg et al. 2000).

host, the maldanid polychaete *Axiothella rubrocincta*, collected in Argyle Lagoon, San Juan Island, Washington, USA; *Amphidinium longum* and *Gymnodinium* sp. were provided by Suzanne Strom (University of Western Washington) from cultures isolated from Puget Sound, Washington, USA, and *Amphidinium semilunatum* was isolated by Mona Hoppenrath (Wattenmeerstation Sylt) from the intertidal sand flats of the island of Sylt, Germany. In these cases, 40–250 cells (or ca. 50 colonies of *Haplozoon*) were micropipetted from their environment and washed repeatedly. Isolated cells were centrifuged and stored at room temperature in the lysis buffer of the purification kit indicated above.

Whenever possible, the 18S (nuclear SSU) rRNA gene was amplified as a single fragment using a polymerase chain reaction with two eukaryotic universal SSU primers (5′-CGAATTCAACCTGGTTGATCCTGCCAGT-3′ and 5′-CCGGATCCTGATCCTTCTGCAGGTTCACCTAC-3′). However, in many cases two overlapping fragments had to be produced using internal primers designed to match existing eukaryotic SSU sequences (4F: 5′-CGGAATTCCAGTC-3′ and 11R: 5′-GGATCACAGCTG-3′). PCR products were either sequenced directly or cloned into pCR-2.1 vector using the TOPO TA cloning kit (Invitrogen). Sequencing reactions were completed with both of the original PCR primers as well as 2–3 additional primers in each direction. When using cloned fragments, 2–4 clones were sequenced to detect and clarify possible ambiguities.

Phylogenetic Analysis

New sequences and all dinoflagellate sequences available in public databases were added to the alignment of Van de Peer et al. (1998), and this alignment was modified manually using GDE v. 2.2 (Smith et al. 1994). The final multiple alignment contained 81 dinoflagellate species, plus *Perkinsus*, *Parvilucifera*, and several ciliate and sporozoan sequences that were used as outgroups. Only unambiguously-aligned sections of the molecule were used in the phylogenetic analysis. For trees using ciliates and sporozoans as outgroups, 1640 characters of the alignment were considered, while 1765 characters could be used in trees restricted to dinoflagellates and *Perkinsus*.

Distances were calculated from 91 alveolate species with PUZZLE 4.0.1. (Strimmer and von Haeseler 1996) using the HKY substitution frequency matrix. Nucleotide frequencies and transition/transversion ratios were estimated from the data, and site-to-site variation was modeled on a gamma distribution with invariable sites plus eight variable rate categories and the shape parameter estimated from the data. Distance trees were constructed using BioNJ (Gascuel 1997), Weighbor (Bruno et al. 2000) and Fitch-Margoliash (Felsenstein 1993). LogDet distance trees were inferred using PAUP 4.0 (Swofford 1999) using default settings. Unweighted parsimony trees were built using DNAPARS (Felsenstein 1993) with five jumbles. One hundred boot-

strap data sets were made using SEQBOOT and trees inferred as described for parsimony and corrected distances, where distances were calculated using puzzleboot (by M. Holder and A. Roger) with the gamma shape parameter, nucleotide frequencies, and transition/transversion ratio from the initial tree enforced on the 100 replicates. To confirm the position of selected taxa (mostly non-photosynthetic species or dinoflagellates with atypical plastids), alternative tree topologies were constructed, and compared by the Kishino-Hasegawa test using PUZZLE 4.0.1 and the settings used for the tree construction (Kishino and Hasegawa 1989).

Large maximum likelihood trees corrected for rate heterogeneity proved to be impossible to infer in a reasonable amount of time. We compromised in two ways: by correcting for rate heterogeneity in smaller trees (40 species in total), and by inferring larger trees without correcting for rate heterogeneity (83 species were chosen by omitting only the obviously redundant taxa). The smaller trees were inferred under a HKY model incorporating a discrete gamma distribution to correct for rate heterogeneity (invariable sites and eight variable rate categories; shape parameter, nucleotide frequencies, and transition/transversion ratio estimated from the data, five jumbles, PAUP 4.0, Swofford 1999). The larger trees were calculated using fastDNAml (F84 model, Olsen et al. 1994). Initially 20 fastDNAml trees were calculated for a more restricted set of 70 taxa (nine outgroup and four ingroup taxa were removed, no major groups were excluded) using four separate transition/transversion ratios (1.5, 1.65, 1.8, and 2.13, the latter suggested by PUZZLE analysis) and at least two jumbles for each. As a ratio of 1.8 gave on average trees with the highest log likelihood, this value was used for the 83 taxa trees (five jumbles).

Results and Discussion

Dinoflagellate Small Subunit rRNA Phylogeny

The SSU rRNA phylogeny of dinoflagellates is generally poorly supported, but it is sufficiently well resolved to suggest several important conclusions regarding the evolution of plastids in this group. In general, any consistently supported features of rRNA trees based on different methods agreed with one another and with previously published data, but other characteristics of the phylogeny differed greatly. Features characteristic of most trees (e.g. Figs. 1, 2, and 3) include the monophyly of dinoflagellates (in the LogDet tree *Amoebophrya* grouped with *Perkinsus*) and the early divergence of *Amoebophrya* and *Noctiluca* (not always in that order and sometimes as a clade, e.g. in many of the 70-taxa ML trees; the Weighbor and Fitch trees put *Amoebophrya* further up in the tree). Also found in most trees (although not in parsimony) was the monophyly of the order *Gonyaulacales* (*Amphidinium asymmetricum* was included in the group in the Fitch tree and in the corrected ML, Fig. 3). Other smaller groups that were found consistently include a *Gymnodinium* sensu stricto (i.e. *G. fuscum, G. catenatum, Gyrodinium impudicum*)/*Lepidodinium* clade, a *Pfiesteria*/*Amyloodinium* clade, and a Suessialean clade that always included *Polarella, Symbiodinium,* and several species of '*Gymnodinium*'. The genera *Symbiodinium, Heterocapsa, Scrippsiella, Pentapharsodinium, Pyrocystis, Ceratium,* and *Alexandrium* were consistently monophyletic with high bootstrap values. Conversely, *Gymnodinium, Gyrodinium, Amphidinium,* and *Prorocentrum* always appeared to be polyphyletic; alternative trees with the first three genera constrained to be monophyletic were always rejected at the 5% confidence level by the Kishino-Hasegawa test. This was not true for *Prorocentrum,* where constrained monophyly was not rejected at that same confidence level. Distance, parsimony and some likelihood trees also often showed a poorly supported group including the 19′-hexanoyloxyfucoxanthin-containing dinoflagellates (*Gymnodinium breve, G. mikimotoi,* and *Gyrodinium galatheanum*), together with two heterotrophic species (*Amphidinium semilunatum* and *Gymnodinium* sp.), and *Amphidinium herdmanii,* a peridinin-containing, sand-dwelling dinoflagellate (in maximum likelihood trees the heterotrophic *Amphidinium semilunatum* was often excluded from the group). While all these groups were consistently found in different analyses, the relationships between them were not consistent, and varied considerably when different methods were used.

A very conspicuous, general characteristic of all SSU rRNA trees of dinoflagellates is an extreme asymmetry in evolutionary rates. Species of the order Gonyaulacales generally have long branches compared with other dinoflagellates (in the case of *Gonyaulax cochlea* this is extreme), as do *Amoebophrya, Haplozoon,* and some species of *Amphidinium.* On the other hand, many of the species that Saunders et al. (1997) grouped in their GPP complex (consisting mostly of Gymnodiniales, Peridiniales, and Prorocentrales) have extremely short branches. For instance, the distance (as calculated by PUZZLE with the parameters noted above) between *Perkinsus marinus* and *Gonyaulax cochlea* is 3.4 times that between *Perkinsus* and *Pentapharsodinium tyrrhenicum,* a very short-branched species.

In our maximum likelihood and gamma-corrected distance trees the Gonyaulacales are nested within the other peridinin-containing dinoflagellates, and do not appear to be their sisters as previously published trees suggested (Saunders et al. 1997). Although this derived position of the Gonyaulacales does not have strong bootstrap support, their earlier, more basal position is likely to have been an artifact of their much longer branches and the more limited taxonomic representation and methods of analysis previously used. The taxonomic implications of the overall tree structure and the apparent polyphyly of several genera will be discussed in a subsequent paper.

Plastid Loss

With the exception of *Amoebophyra* and *Noctiluca,* all non-photosynthetic dinoflagellates in the trees (*Haplozoon, Amyloodinium, Pfiesteria, Crypthecodinium, Amphidinium semilunatum, A. longum,* and *Gymnodinium* sp.) were generally scattered among the photosynthetic

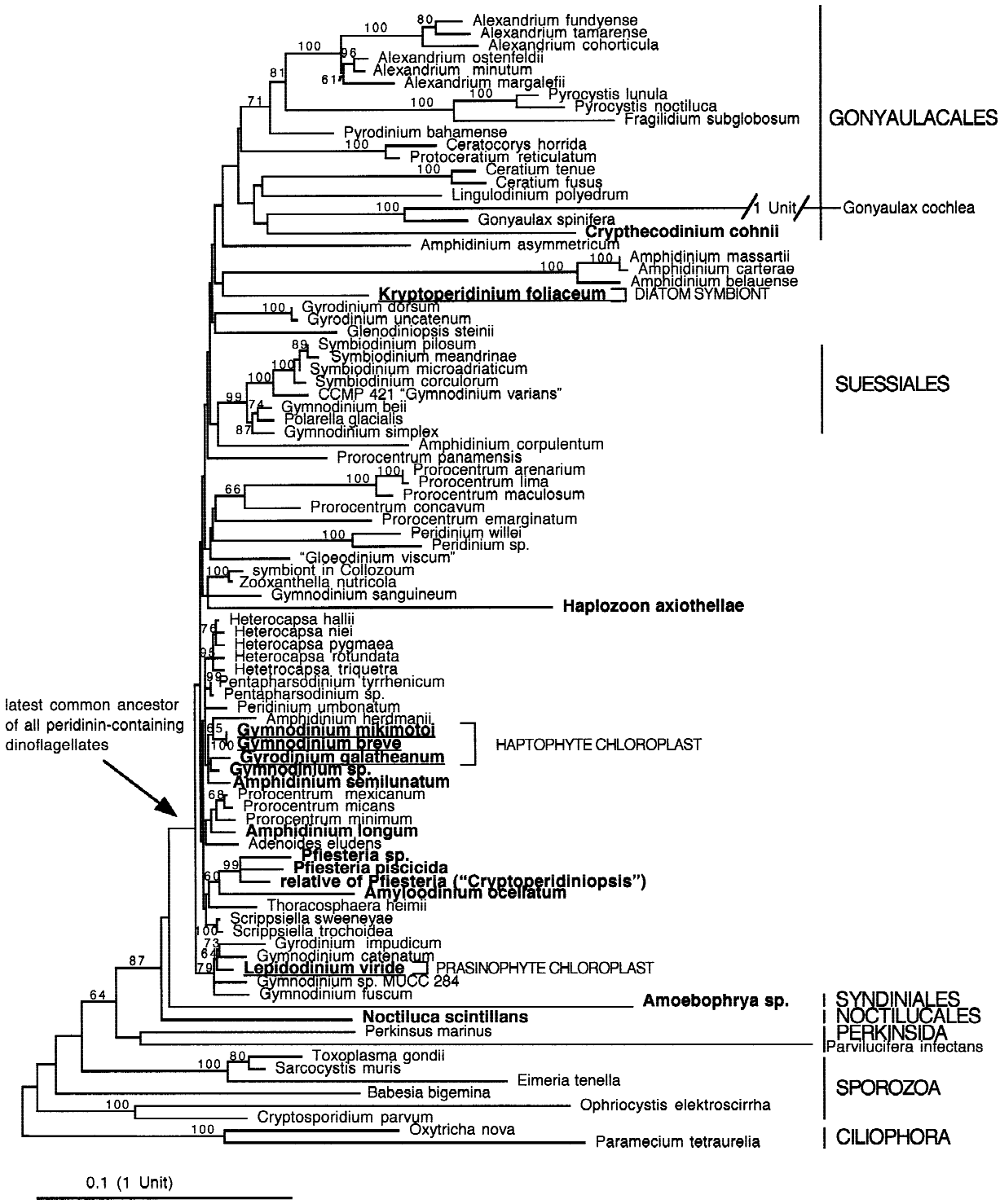

Fig. 1. Phylogenetic tree constructed by neighbor-joining from a gamma-weighted distance matrix of complete SSU rRNA sequences from 91 alveolates (dinoflagellates, perkinsids, sporozoans, and ciliates). Bootstrap values are shown above the internodes when higher than 60%. Transition/transversion ratio: 2.18. Dinoflagellate species lacking functional peridinin plastids are in bold; photosynthetic species with aberrant plastids are underlined. Putative origins of aberrant plastids are given. Problematic names of organisms are given in quotes; they should be regarded as provisional. *Gymnodinium, Gyrodinium,* and *Amphidinium* (as well as the order Gymnodiniales as a whole) are obviously polyphyletic and scatter among Peridiniales and Prorocentrales.

lineages (exceptions are *Haplozoon axiothellae* in a few uncorrected ML trees and in the Fitch tree, and *Amphidinium semilunatum* in many ML trees, e.g. Figs. 2, 3) and unrelated to one another. In Kishino-Hasegawa tests, alternative trees where each individual non-photosynthetic species or group was placed between *Amoebophrya*/*Noctiluca* and the rest of the dinoflagellates were generally not rejected at the 5% confidence

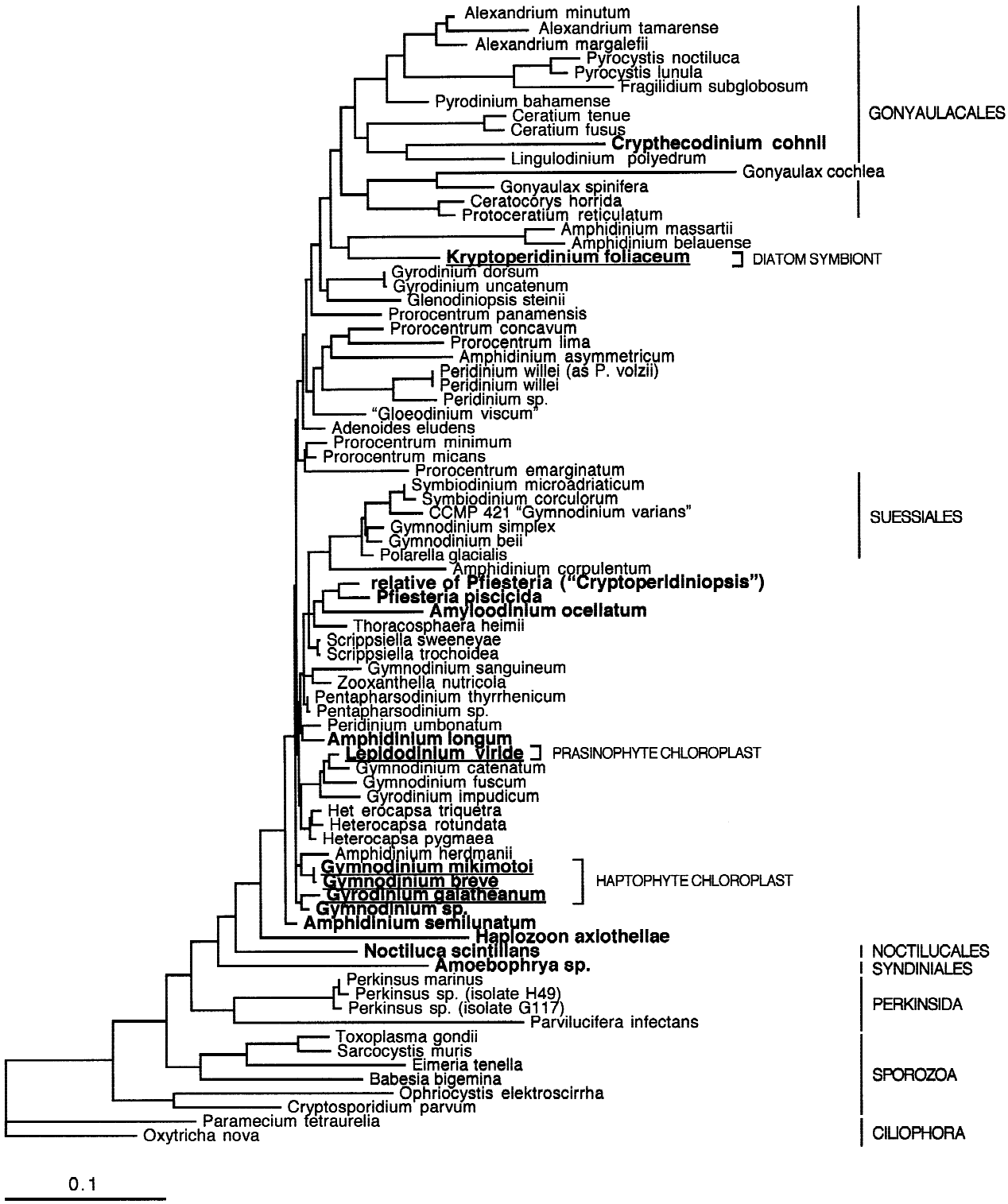

Fig. 2. Maximum likelihood phylogenetic tree constructed from SSU rRNA sequences from 83 alveolates (dinoflagellates, perkinsids, sporozoans, and ciliates). Transition/transversion ratio: 1.8, log likelihood = −35430.100; other trees found with slightly lower log likelihoods differed only in minor details. Dinoflagellate species lacking functional peridinin plastids are in bold, photosynthetic species with aberrant plastids are underlined. Putative origins of aberrant plastids are given. Problematic names of organisms are given in quotes; they should be regarded as provisional.

level (the exception being *A. longum*). However, Kishino-Hasegawa tests did resoundingly reject alternative trees where all non-photosynthetic dinoflagellates are grouped together (with or without *Amoebophrya* and *Noctiluca*), irrespective of their position in the trees. Because a close relationship between all non-photosynthetic dinoflagellates is rejected by the phylogenies and the Kishino-Hasegawa tests, at least some non-

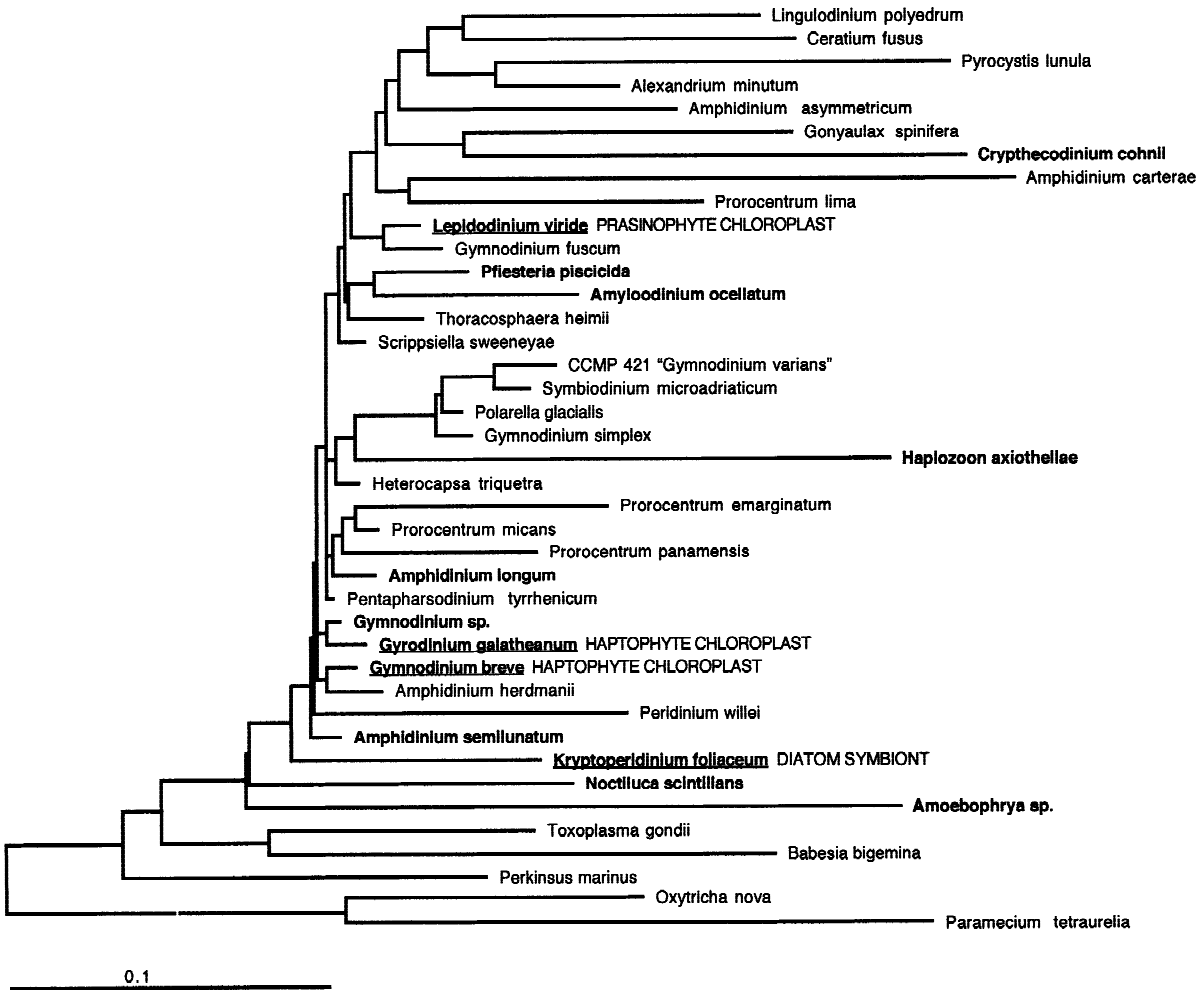

Fig. 3. Maximum likelihood phylogenetic tree constructed from 40 alveolate SSU rRNA sequences and corrected for rate heterogeneity. Site to site rate variation modelled on a gamma distribution with eight categories, shape parameter estimated from the data (0.26). Transition/transversion ratio: 2.03, log likelihood: −15436.54878. Dinoflagellate species lacking functional peridinin plastids are in bold; photosynthetic species with aberrant plastids are underlined. Putative origins of aberrant plastids are given. Problematic names of organisms are given in quotes; they should be regarded as provisional.

photosynthetic dinoflagellates must have originated after the latest possible common ancestor of all peridinin-containing dinoflagellates, making plastid losses within the group a virtual certainty.

While SSU rRNA phylogeny does support plastid loss in *Haplozoon, Amyloodinium, Pfiesteria, Crypthecodinium, Amphidinium semilunatum, A. longum,* and *Gymnodinium* sp., it is not sufficiently firmly resolved to be compelling in the absence of additional data. Fortunately, for many of these taxa there are clear morphological signs of their evolutionary origin. For example, *Crypthecodinium cohnii* has a gonyaulacoid tabulation (pattern of cortical armor plates), although somewhat atypical (Fensome et al. 1993). In some molecular studies, this species was seen to branch conspicuously early (e.g. Litaker et al. 1999), but in the majority of our trees, *Crypthecodinium* appears to be clearly related to the Gonyaulacales, a placement consistent with its tabulation. The only trees that did not clearly place *Crypthecodinium* in its cytologically supported position within the Gonyaulacales were the unweighted parsimony trees, which would be most likely to have been artifactually influenced by the unusually long branch of *Crypthecodinium.* We thus argue that this species is secondarily heterotrophic and that its early position in previous trees was an artifact of its long branch coupled with sparse taxon sampling.

Amphidinium semilunatum, Amphidinium longum, and *Gymnodinium* sp. are all athecate dinoflagellates. Traditionally, all exclusively dinokaryotic naked dinoflagellates have been classified in the order Gymnodiniales, a taxon that is very probably polyphyletic (Taylor 1980; Fensome et al. 1993). In spite of the fact that in SSU phylogenetic trees the Gymnodiniales never form a monophyletic group, all members of the order do branch after *Amoebophrya* and *Noctiluca,* usually scattered among thecate forms. This scattering suggests repeated instances of *thecal* loss within dinoflagellates, and also that the non-photosynthetic members of the order probably had photosynthetic ancestors. Admittedly, the posi-

tions of *Gymnodinium* sp. and especially *Amphidinium semilunatum* within the photosynthetic dinoflagellates are not very stable, but there are no morphological reasons to consider them to be particularly early-diverging. The case for plastid loss in *A. longum* is much stronger, since alternative trees with this species diverging before the latest possible common ancestor of peridinin-containing dinoflagellates were rejected by Kishino-Hasegawa tests.

Haplozoon axiothellae is a very unusual, non-photosynthetic, multicellular, parasitic dinoflagellate, and its phylogenetic position within the group has never been clear. Traditionally, *Haplozoon* and *Amyloodinium* have both been considered to be members of the order Blastodiniales, a group of parasitic dinoflagellates that is defined by the presence of non-dinokaryotic nuclei in certain stages of their life cycles (Fensome et al. 1993). Our phylogenetic trees do not support a relationship between these two genera: *Amyloodinium* consistently forms a group with *Pfiesteria* and its close relatives, and this group never included *Haplozoon.* Conversely, no position of *Haplozoon* is strongly supported by SSU phylogeny, and this organism can be placed essentially anywhere within dinoflagellates without causing the resulting tree to be rejected by the Kishino-Hasegawa test. *Haplozoon axiothellae* does appear to have several characters that differentiate it from other Blastodiniales *sensu* Fensome et al. (1993). Notably, it may well be completely dinokaryotic: the multicellular trophont has been shown to have a dinokaryon (Siebert and West 1974), and, although the nucleus of the motile stages has never been investigated, they probably also have one (in organisms with both dinokaryotic and non-dinokaryotic phases the motile phases are always dinokaryotic: Cachon and Cachon 1987). Altogether, it seems most likely that *Haplozoon* is not a blastodinialean, and probably descended from photosynthetic ancestors. The position of the branch that includes *Amyloodinium* and *Pfiesteria* is also uncertain, but since those two genera have motile stages with unquestionably peridinialean tabulation (Landsberg et al. 1994; Steidinger et al. 1996; Fensome et al. 1999) we also believe them to be secondarily heterotrophic, as all our trees weakly suggest.

Plastid Replacement

Several groups of dinoflagellates contain plastids that differ in pigmentation from the typical peridinin plastids. Our trees contain three dinoflagellate taxa with true aberrant plastids: *Lepidodinium viride, Kryptoperidinium foliaceum,* and the 19′-hexanoyloxyfucoxanthin group. All of these typically branch after the latest possible common ancestor of peridinin-containing dinoflagellates (exceptions are many ML trees where either the 19′-hexanoyloxyfucoxanthin group or *Kryptoperidinium foliaceum* fall between *Amoebophrya*/*Noctiluca* and the rest of the dinoflagellates, e.g. Figs. 2, 3). Alternative trees with all aberrantly-pigmented dinoflagellates or *Lepidodinium* alone placed in basal positions were rejected by Kishino-Hasegawa tests at the 5% confidence levels; trees with *Kryptoperidinium* or the 19′-hexanoyloxyfucoxanthin group in those positions were not. Nevertheless, morphological features in the aberrantly-pigmented dinoflagellates make it unlikely that they arose prior to the peridinin-containing plastid: *Lepidodinium* is very similar to several peridinin-containing members of the genus *Gymnodinium* (*Gymnodinium* sensu stricto in Daugbjerg et al. 2000), and *Kryptoperidinium foliaceum* has a peridinialean tabulation, albeit somewhat atypical. The case for the 19′-hexanoyloxyfucoxanthin group is weaker, since there are no obvious morphological features linking them to another dinoflagellate taxon. However, in our trees the (weakly supported) group that contains them also includes a peridinin-containing species (*Amphidinium herdmanii*). We thus argue that all dinoflagellates with aberrant plastids had peridinin-containing ancestors, and that they all replaced one type of plastid for another.

The degree to which new plastids are integrated varies greatly. The replacement process can be thought to be “in progress” in *Kryptoperidinium foliaceum* (as well as in *Durinskia baltica*, not yet on the tree), both organisms with a raphid pennate diatom endosymbiont (Chesnick et al. 1997). In both cases, as well as in *Peridinium quinquecorne* (Horiguchi and Pienaar 1991) the endosymbiont appears to be relatively complete, having a nucleus, mitochondria and other organelles but lacking a cell wall or obvious mitotic spindle (Dodge 1983). They also carry a probable remnant of the old peridinin-containing plastid in the form of an eyespot surrounded by three membranes (Jeffrey and Vesk 1976; Horiguchi and Pienaar 1991; Schnepf and Elbraechter 1999). In the other two replacement instances discussed here, the plastids themselves are all that remains of the endosymbiont: *Lepidodinium viride* (as well as *Gymnodinium chlorophorum*, not on the tree) contains green plastids of probable prasinophyte origin with chlorophyll a and b (Schnepf and Elbraechter 1999), and the 19′-hexanoyloxyfucoxanthin-containing species carry plastids derived from haptophytes (Tengs et al. 2000).

We found two species of heterotrophic dinoflagellates that tend to branch at the base of the 19′-hexanoyloxyfucoxanthin group: *Amphidinium semilunatum* and *Gymnodinium* sp., although this is only weakly supported by bootstrap analysis and alternative positions are not rejected in KH tests. Saunders et al. (1997) also found a non-photosynthetic species (*Polykrykos schwartzii*) as a sister to *G. mikimotoi* (100% bootstrap support, unpublished SSU sequence). If these positions are correct, then haptophyte-containing dinoflagellates may have had non-photosynthetic ancestors. This would imply a replacement of peridinin-containing plastids by

haptophyte-derived plastids through non-photosynthetic intermediate stages, a situation very different from the replacement process in *Kryptoperidinium* and *Durinskia* if their eyespot is indeed a remnant of the old plastid.

Other than a partial sequence from *Dinophysis acuminata* that branches within the GPP complex (Saunders et al. 1997), no data from Dinophysiales have been used in published dinoflagellate SSU trees. If this position is correct, then Dinophysiales must have had a peridinin-containing ancestor and must also have lost that plastid at some point in their evolutionary history.

Origin of the Peridinin-Containing Plastid

Traditionally, dinoflagellates have been viewed as essentially heterotrophic organisms with members that gained photosynthetic abilities through one or more endosymbiotic events (Dodge 1975; Taylor 1980, 1999). One reason for this is the trophic behaviour of the group: despite the photosynthetic nature of many dinoflagellates, very few species are strict autotrophs and most need organic compounds to grow (Schnepf and Elbraechter 1992). In addition to this, non-dinokaryotic groups (i.e. the order Syndiniales, most often viewed as the earliest offshoot of the group because of their nuclear similarity to other eukaryotes) are always heterotrophic. However, since the discovery of plastids in sporozoans, the sister group of dinoflagellates (review in McFadden and Waller 1997), the view that dinoflagellates were ancestrally non-photosynthetic has come under attack (Palmer 1992; Cavalier-Smith 1999).

Recent work has shown relationships between red algal plastids and the plastids of both sporozoans (McFadden and Waller 1997; Stoebe and Kowallik 1999) and dinoflagellates (Zhang et al. 2000), suggesting a red algal origin for the plastids of both groups. Moreover, plastid gene sequences from dinoflagellates and sporozoans have been argued to show a close phylogenetic relationship, although plastid-encoded sequences from both groups are so divergent that long-branch artifacts could not be ruled out (Zhang et al. 2000). Most recently, plastid-targeted homologues of glyceraldehyde-3-phosphate dehydrogenase from both dinoflagellates and sporozoa have been shown to have originated by a common gene duplication event, suggesting very strongly that the ancestor of both groups already contained the plastid (Fast et al. 2001).

Our new data do not answer the question as to whether the common ancestor of sporozoans and dinoflagellates was photosynthetic or heterotrophic, but imply that photosynthetic dinoflagellates all diverge from each other after *Amoebophrya* and *Noctiluca*, suggesting a placement for the *latest* possible common ancestor of all peridinin-containing dinoflagellates (Figs. 1, 2). If the dinoflagellate and sporozoan plastids arose independently, then these apparently early diverging dinoflagellates cannot be said to have lost plastids. If, however, the dinoflagellate and sporozoan plastids do share a common origin (Cavalier-Smith 1999; Fast et al. 2001), then even these deep lineages lost their plastids, pushing the number of plastid losses still further to include *Amoebophrya* and *Noctiluca*, as well as *Perkinsus* and all other non-photosynthetic alveolates that branch between sporozoans and dinoflagellates.

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