Evolutionary relationship between dinoflagellates bearing obligate diatom endosymbionts: insight into tertiary endosymbiosis

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The marine dinoflagellates *Peridinium balticum* and *Peridinium foliaceum* are known for bearing diatom endosymbionts instead of peridinin-containing plastids. While evidence clearly indicates that their endosymbionts are closely related, the relationship between the host dinoflagellate cells is not settled. To examine the relationship of the two dinoflagellates, the DNA sequences of nuclear small-subunit rRNA genes (SSU rDNA) from *Peridinium balticum*, *Peridinium foliaceum* and one other peridinin-containing species, *Peridinium bipes*, were amplified, cloned and sequenced. While phylogenetic analyses under simple models of nucleotide substitution weakly support the monophyly of *Peridinium balticum* and *Peridinium foliaceum*, analyses under more sophisticated models significantly increased the statistical support for this relationship. Combining these results with the similarity between the two endosymbionts, it is concluded that (i) the two hosts have the closest sister relationship among dinoflagellates tested, (ii) the hypothesis that the diatom endosymbiosis occurred prior to the separation of the host cells is most likely to explain their evolutionary histories, and (iii) phylogenetic inferences under complex nucleotide evolution models seem to be able to compensate significant rate variation in the two SSU rDNA.

Keywords: *Peridinium*, plastid, SSU rDNA, among-site rate variation, nucleotide substitution model

INTRODUCTION

It is well established that plastids (or chloroplasts) in eukaryotes are derived either directly or indirectly from highly reduced endosymbiotic cyanobacteria. However, the evolution of extant photosynthetic eukaryotes is quite complex (reviewed by Gray, 1992; Gibbs, 1993; Delwiche & Palmer, 1997; Bhattacharya & Medlin, 1998; Cavalier-Smith, 1999; Delwiche, 1999). Plastids surrounded by double membranes, so

called ‘primary’ plastids, are found in green algae, land plants, red algae and glaucocystophytes. These primary plastids are believed to be evolved via direct eukaryote–cyanobacterium endosymbiosis. It remains unclear whether plastids in the four lineages are direct descendants of a single endosymbiotic event. However, recent evidence supports the monophyly of green algae (plus land plants) and red algae, with weaker evidence pointing also to a sister relationship with glaucocystophytes (Moreira *et al.*, 2000).

‘Secondary’ plastids, be they triple or quadruple membrane bound, are found in as widely divergent eukaryotic lineages as euglenoids, apicomplexan parasites, chromist algae (heterokonts, haptophytes, cryptomonads and chlorarachniophytes) and dinoflagellates. This type of plastid is presumed to be derived from a photosynthetic eukaryote bearing primary

Abbreviations: Dist, distance; indels, insertions/deletions; ML, maximum-likelihood; ML-Dist, maximum-likelihood distance; MP, maximum-parsimony; SSU rDNA, small-subunit rRNA gene; –lnL, log likelihood; P_{inv} , proportion of invariable sites.

The GenBank accession numbers for the *Peridinium balticum*, *Peridinium foliaceum* and *Peridinium bipes* SSU rDNA sequences are AF231803, AF231804 and AF231805, respectively.

plastids that was engulfed by a heterotrophic eukaryote through phagocytosis (or myzocytosis). Chlorarachniophytes and cryptomonads provide direct evidence for this hypothesis (reviewed by Gilson & McFadden, 1997); remnants of endosymbiotic nuclei (nucleomorphs) and plastids are found in the periplastidal cytoplasm that is separated from the host cytosol by two membranes. These cell structures have been interpreted as intermediates in the reduction of the engulfed photosynthetic eukaryotes into secondary plastids without nucleomorphs. Recent molecular phylogenetic studies have demonstrated that eukaryotes bearing secondary plastids usually have heterotrophic relatives, and they are most probably the result of the independent acquisition of their plastids after the separation of the photosynthetic lineage from their heterotrophic sisters (Van de Peer *et al.*, 1996; Inagaki *et al.*, 1997, 1998; Van der Auwera *et al.*, 1998).

In addition, several dinoflagellates maintain chromist algae, which themselves have secondary plastids, as endosymbionts (Delwiche, 1999). Such 'tertiary' endosymbioses are not as well studied, but the marine dinoflagellates *Peridinium balticum* and *Peridinium foliaceum* are well-established cases. The endosymbionts of *Peridinium balticum* and *Peridinium foliaceum* are remarkably similar in their structural features and pigment composition (reviewed by Chesnick *et al.*, 1997). The endosymbionts are separated from the host cytosol by a single membrane, presumably having lost their cell wall (Dodge, 1971; Tomas *et al.*, 1973), and the endosymbiont of *Peridinium balticum* undergoes amitotic cell division (Tippit & Pickett-Heaps, 1976). These observations strongly suggest that the algae are not the transient association of the host cells with an autonomous photosynthetic eukaryote. Given this, it is surprising that the endosymbionts appeared to retain their mitochondria as well as eukaryotic nucleus and plastids (Dodge, 1971; Tomas *et al.*, 1973).

Based on the photosynthetic pigmentation and recent phylogenetic study of the nuclear small-subunit rRNA (SSU rDNA) of the two endosymbionts, it was clearly shown that the two endosymbionts are closely related, and descendants of pennate diatoms (family Bacillariaceae) (Dodge, 1971; Tomas *et al.*, 1973; Chesnick *et al.*, 1997). The phylogenetic analysis inferred from a plastid gene, *rbcL* (encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase), also indicated that the endosymbiont of *Peridinium foliaceum* has a diatom origin (Chesnick *et al.*, 1996). Curiously, despite the ultrastructural similarities of the two hosts, *Peridinium balticum* and *Peridinium foliaceum*, their isozyme profiles are significantly different (Whitten & Hayhome, 1986). These conflicting data about their relationship make it difficult to determine whether the obligate diatom endosymbiosis occurred before or after the diversification of the host lineages.

To examine the phylogenetic positions of *Peridinium balticum* and *Peridinium foliaceum* among dinoflagel-

lates, we amplified, cloned and sequenced their (nuclear) SSU rDNA sequences, as well as the sequence of an additional peridinoid species bearing a standard (peridinin-containing) dinoflagellate plastid, *Peridinium bipes*. Our exhaustive phylogenetic analyses inferred from SSU rDNA sequences succeeded in recovering a closest sister relationship between *Peridinium balticum* and *Peridinium foliaceum*, and we conclude that the ancestor of the two dinoflagellates engulfed a pennate diatom.

METHODS

Amplification, cloning and sequencing of SSU rDNA. *Peridinium balticum* (CS-38) and *Peridinium foliaceum* (LB1688) were purchased from the CSIRO division of Marine Research, Australia (Castray Esplanade, Hobart, Tasmania 7000, Australia). *Peridinium bipes* (NIES 364) was purchased from the National Institute for Environmental Studies, Japan (16-2 Onogawa, Tsukuba, Ibaragi 305-0053, Japan). Cells were cultured with the medium recommended by the suppliers with 50 mg gentamicin ml⁻¹ to prevent bacterial growth. One gram of frozen cells was ground into a fine powder using a bead-mill and resuspended in one volume of CTAB extraction buffer [2% (w/v) cetyltrimethylammonium bromide (CTAB), 100 mM Tris/HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl]. Nucleic acids were extracted twice with chloroform following incubation for 1 h at 65 °C, and then precipitated by addition of an equal volume of CTAB precipitation buffer [1% (w/v) CTAB, 50 mM Tris/HCl (pH 8.0), 10 mM EDTA (pH 8.0)]. The resultant pellet was resuspended in high-salt buffer [10 mM Tris/HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), 1 M NaCl]. After 2-propanol precipitation, nucleic acids were dissolved in distilled water and used for PCR.

A pair of synthesized primers, 5'-TACCTGGTTGATCC TGCCAGTA-3' and 5'-CCATCCGCAGGTTACCTCA-3', was used to amplify nearly the entire SSU rDNA. PCR was carried out as follows: denaturation at 94 °C for 15 s, annealing at 60 °C for 1 min and extension at 72 °C for 2 min for 30 cycles. The amplified fragments were subsequently ligated into the pT7Blue T-vector (Novagen) and transformed into *Escherichia coli* JM109. DNA sequences were determined for both strands by cycle sequencing with dye-terminators using a DNA sequencer model 377 (Applied Biosystems).

Phylogenetic analyses. The three *Peridinium* rDNA sequences were manually aligned along with the previously published sequences of 49 dinoflagellates and two apicomplexan parasites (*Perkinsus* sp., L07375; *Toxoplasma gondii*, X65508) using MACCLADE version 4.0b12 (Maddison & Maddison, 2000). For alignment editing, we referred to the secondary structure of SSU rRNA molecule deposited in the rRNA WWW Server at the University of Antwerp (<http://rrna.uia.ac.be/index.html>). After polishing of the alignment by eye and exclusion of all ambiguous sites, it included 54 taxa with 1723 sites. Preliminary phylogenetic analyses were performed on this dataset under maximum-parsimony (MP) optimality criteria. Subsequently we generated an additional two datasets for the later analyses.

A second alignment comprising 40 taxa and 1723 sites was generated by removing redundant taxa (i.e. taxa in the clades with bootstrap scores of 100 in MP analyses). This dataset was tested for optimal fit of various models of nucleotide

evolution using MODELTEST version 3.0b3 (Posada & Crandall, 1998). The proportion of invariable sites (P_{INV}), a discrete γ distribution (four categories) and base frequencies were similarly estimated from the dataset. A maximum-likelihood distance (ML-Dist) bootstrap analysis (100 resampling) under an optimal model of nucleotide substitution incorporating the P_{INV} and a discrete γ distribution with the empirical base frequencies was performed. For each replicate, starting trees were constructed by 100 random additions.

The third dataset of 22 taxa and 1718 sites was subjected to analysis using MODELTEST (Posada & Crandall, 1998), and analysed using maximum-likelihood (ML) and distance (Dist) methods under two optimal models recommended as well as three simple models. Bootstrap analyses (100 resampling) using the Dist method under the five models were performed. ML bootstrap analyses under the two optimal models (100 resamplings) were operated without branch-swapping (the 'Fast' stepwise-addition option in PAUP* version 4.0b3; Swofford, 1998) to save computational time. For all phylogenetic analyses in this study, PAUP* version 4.0b3 was used (Swofford, 1998).

RESULTS

The *Peridinium* SSU rDNA sequences

The amplified regions of SSU rDNA for *Peridinium bipes*, *Peridinium balticum* and *Peridinium foliaceum* were 1796, 1796 and 1795 bp, respectively. G+C contents are 48, 47 and 46 mol%, respectively. The partial sequence of the previously reported SSU rDNA for *Peridinium foliaceum* (M88517) was identical to the sequence determined in this study. The number of nucleotide substitutions and single nucleotide insertions/deletions (indels) between the *Peridinium balticum* and *Peridinium foliaceum* genes were 145 and 4, respectively (Table 1), while the endosymbiont SSU rDNAs have only 1% difference (18 substitutions out of 1788 sites; Chesnick *et al.*, 1997). No difference between the putative secondary structures of the *Peridinium balticum* and *Peridinium foliaceum* rDNAs was detected (data not shown). Based on their structures, 103 out of 145 substitutions and two of the indels corresponded to non-conserved regions. Comparing

the four *Peridinium* SSU rDNA, we measured the number of unique substitutions among 1730 sites in each sequence (Table 1). Interestingly, the *Peridinium foliaceum* gene appeared to have 73 unique substitutions, almost twice that observed in the other three taxa (Table 1). These observations strongly imply that the *Peridinium foliaceum* SSU rDNA evolves almost twice as fast as the *Peridinium balticum* gene.

Phylogenetic analyses

In preliminary MP analyses, *Peridinium balticum* and *Peridinium foliaceum* were positioned as sister taxa (bootstrap score, 35; data not shown). Corresponding to the outstanding number of the unique substitutions found in the *Peridinium foliaceum* gene (Table 1), the *Peridinium foliaceum* branch was twice as long as that of *Peridinium balticum* in the most parsimonious trees (data not shown, but the same results were observed in Fig. 1). Recently it has been shown that phylogenetic inference without accounting for among-site rate variation can lead to artefactual results (e.g. Silberman *et al.*, 1999; Stiller & Hall, 1999). Because the difference in substitution rate between the SSU rDNA sequences of interest may mask phylogenetic signal, it is possible that our MP analyses may not be able to robustly reconstruct relationships.

A subdataset (40 taxa/1723 sites), which does not include redundant taxa, was analysed using MODELTEST (Posada & Crandall, 1998). Through calculation of log-likelihood ($-\ln L$) scores, this program found that a TrN model of nucleotide evolution (Tamura & Nei, 1993) incorporating the proportion of invariable sites and a discrete γ distribution (four categories) (TrN + P_{INV} + γ) was significantly better than other models examined. The dataset was therefore analysed using a ML-Dist method under this model (Fig. 1a). For the most part, the resultant tree corresponds to the general classification and previous investigations of dinoflagellate phylogeny based on SSU rDNA sequences (e.g. Grzebyk *et al.*, 1998; Montresor *et al.*, 1999). Our analysis showed weak affinity for the *Peridinium balticum*–*Peridinium foliaceum* clade and strong support for the *Peridinium bipes*–*Peridinium* sp. relationship (bootstrap scores, 54 and 100, respectively; Fig. 1a).

Considering computational time, for this set of analyses, a smaller dataset (20 taxa/1718 sites) was generated. MODELTEST (Posada & Crandall, 1998) found that TrN (Tamura & Nei, 1993) and general-time-reversible (GTR) models (Rodríguez *et al.*, 1990) incorporating the P_{INV} and a discrete γ distribution best describe the dataset ($-\ln L$ scores estimated under the two models are almost the same, 10782). The $-\ln L$ scores under the simple models, JC (Jukes & Cantor, 1969), F81 (Felsenstein, 1981) and K2P (Kimura, 1980), that incorporated neither P_{INV} and a discrete γ distribution, were poor ($-\ln L = 12146$, 12126 and 11811, respectively). Phylogenies inferred from the third dataset matched the overall topology of those in the first two

Table 1. Nucleotide substitution matrix of the four *Peridinium* SSU rDNA and number of unique substitutions

Pfol, *Peridinium foliaceum*; Pbal, *Peridinium balticum*; Pbip, *Peridinium bipes*; Psp., *Peridinium* sp. Number of single nucleotide insertions/deletions are indicated in parentheses.

	Pbal	Pbip	Psp.	Unique substitutions*
Pfol	145 (4)	223 (0)	233 (1)	73
Pbal		175 (2)	141 (5)	34
Pbip			108 (1)	28
Psp.				36

* Includes unique insertions/deletions.

Fig. 1. Phylogenetic trees inferred from dinoflagellate SSU rDNA sequences. (a) The maximum-likelihood distance (ML-Dist) tree (40 taxa/1723 sites) reconstructed under a TrN nucleotide substitution model (Tamura & Nei, 1993) incorporating the proportion of invariable sites (P_{INV}) and a discrete γ distribution (four categories) (TrN + P_{INV} + γ). For the bootstrap analyses, 100 replicates of the original dataset were used, with starting trees for each replicate constructed by 100 random addition. Only bootstrap scores above 50% are indicated. (b) The best maximum-likelihood (ML) tree inferred from the dataset (20 taxa/1718 sites) under a TrN + P_{INV} + γ model. The bootstrap scores indicated in roman and *italic* were calculated by ML-Dist and ML methods under a TrN + P_{INV} + γ model (100 resamplings), respectively. Only bootstrap score above 50% are indicated. For the *Peridinium balticum*–*Peridinium foliaceum* clade, bootstrap scores obtained from ML-Dist and ML analyses under a general-time-reversible (GTR) model (Rodríguez *et al.*, 1990) incorporating the P_{INV} and a discrete γ distribution (four categories) are also indicated. ML bootstrap analyses were conducted with no branch-swapping using the 'Fast' stepwise-addition option in PAUP* version 4.0b3 (Swofford, 1998). Lineages bearing tertiary plastid are highlighted by arrows. The *Gymnodinium breve*, *Gymnodinium galatheanum* and *Gymnodinium aureolum* SSU rDNA are not included in these analyses.

(Fig. 1b). For the *Peridinium balticum*–*P. foliaceum* clade, bootstrap scores of 62 and 78 were obtained using ML-Dist methods under TrN + P_{INV} + γ and GTR + P_{INV} + γ models, respectively. The ML bootstrap analyses under those two optimal models gave bootstrap scores of 77 and 74 for the same clade (Fig. 1b; Table 2). All of the top 100 ML trees reconstructed under the optimal models included the *Peridinium*

balticum–*Peridinium foliaceum* clade (data not shown). However, neither Dist or ML analyses under the simple models, JC, F81 and K2P, supported this clade (Table 2). While the *Peridinium bipes*–*Peridinium* sp. clade was solid irrespective of the methods or models (Table 2), we failed to detect any connection between the *Peridinium balticum*–*Peridinium foliaceum* and *Peridinium bipes*–*Peridinium* sp. clades (Fig. 1).

Table 2. Impact of nucleotide substitution and among-site rate variation models on dinoflagellate phylogeny

Symbols/abbreviations: +, the best ML tree includes the topology of interest; –, the bootstrap consensus tree or best ML tree does not have the topology of interest; ND, bootstrap analysis using ML method under JC, F81 or K2P model was not done. ML bootstrap analyses were conducted with no branch-swapping (PAUP* version 4.0b3; Swofford, 1998).

Criteria		Model				
		JC	F81	K2P	TrN + $P_{INV} + \gamma$	GTR + $P_{INV} + \gamma$
<i>Peridinium balticum</i> – <i>Peridinium foliaceum</i> clade	Dist (bootstrap)	–	–	–	62	78
	ML (bootstrap)	ND	ND	ND	77	74
	best ML tree	–	–	–	+	+
<i>Peridinium bipes</i> – <i>Peridinium</i> sp. clade	Dist (bootstrap)	100	100	100	100	100
	ML (bootstrap)	ND	ND	ND	100	100
	best ML tree	+	+	+	+	+
Basal branching of <i>Gonyaulales</i> and <i>Nocticales</i>	Dist (bootstrap)	79	74	74	–	–
	ML (bootstrap)	ND	ND	ND	–	–
	best ML tree	+	+	+	–	–

DISCUSSION

Impact of nucleotide evolution models on dinoflagellate SSU rDNA phylogeny

Our SSU rDNA analyses reconstruct major dinoflagellate clades, i.e. the clades comprised of the species belonging to the families Gonyaulacales, the *Symbiodinium* and *Gymnodinium* species, the two *Gymnodinium* species and *Lepidodinium viride* (Fig. 1), in agreement with other previous studies (e.g. Grzebyk *et al.*, 1998; Montresor *et al.*, 1999). In phylogenetic inferences under simple nucleotide substitution models without accounting for among-site rate variation, species belonging to the families Gonyaulacales and Nocticales, such as *Alexandrium minutum*, *Alexandrium margalefeii*, *Ceratium tenue*, *Crypthecodinium cohnii*, *Amphidinium belauense* and *Noctiluca scintillans*, were found as basal branches among dinoflagellates (Table 2). However, in our phylogenies reconstructed under the complex models, which best described our SSU rDNA dataset, there is no statistical support for relative branching order between the robust clades that we obtained, nor for the deeply diverging branches in our trees (Fig. 1; Table 2). Considering their long branch lengths, we suspect the positions of some of the basal diverging dinoflagellate sequences, in particular the family Gonyaulacales, may be artefactual, resulting from ‘long branch attraction’. Our analyses suggest that the complex models seem less susceptible to artificial resolution due to long branch attraction as shown by Silberman *et al.* (1999).

‘Single-endosymbiosis’ versus ‘separate-endosymbiosis’

Despite both morphological and molecular evidence strongly indicating a close relationship between the diatom endosymbionts of *Peridinium balticum* and *Peridinium foliaceum*, the available data on the dino-

flagellate hosts themselves are not sufficient to resolve their relationships (reviewed by Chesnick *et al.*, 1997). This relationship is the key to the history of plastid acquisition in these two *Peridinium* species. So far, isozyme profile (Whitten & Hayhome, 1986) is consistent with the ‘separate-endosymbiosis’ hypothesis – that the two dinoflagellates captured the same or a similar diatom species independently. However, no molecular sequence data have been available for both *Peridinium balticum* and *Peridinium foliaceum* host cells, and statistical examinations of their relationship were therefore impossible. If a phylogenetic affinity between the two hosts was statistically supported, the ‘single-endosymbiosis’ hypothesis – that the ancestor of two dinoflagellates engulfed a diatom would be favoured over the ‘separate-endosymbiosis’ hypothesis.

Preliminary MP analyses lent no support to either acquisition scenarios of the diatom endosymbionts in *Peridinium balticum* and *Peridinium foliaceum*. However, as these analyses did not account for rate heterogeneity among sites, more stringent analyses with biologically relevant models for nucleotide evolution were performed. Analyses using ML-Dist and ML methods under the complex models of nucleotide evolution significantly improved the statistical support for the *Peridinium balticum*–*Peridinium foliaceum* clade (Table 2). These results are in contrast to inferences under simple models without consideration of among-site rate variation (Table 2). Difficulty in resolving the relationship between *Peridinium balticum* and *Peridinium foliaceum* is most probably caused by the variation of nucleotide substitution rates between the two SSU rDNA, and thus biologically relevant nucleotide evolution models should be adopted to deal with this problematic dataset. Since these models were found to best describe the dataset, we conclude that *Peridinium balticum* and *Peridinium foliaceum* are the closest relatives among the species examined. This is in

agreement with morphological comparisons of relevant cell structure (reviewed by Chesnick *et al.*, 1997).

Large indels in SSU rDNA are presumed to accelerate the substitution rate over the entire gene in order to preserve overall rRNA structure (Stiller & Hall, 1999). Unfortunately, comparison between the putative secondary structures of the *Peridinium balticum* and *Peridinium foliaceum* SSU rDNA suggests no such indels (data not shown). We can see no obvious molecular or biological rationale for the rate acceleration of the *Peridinium foliaceum* SSU rDNA. Further sequencing and comparison of the *Peridinium balticum* and *Peridinium foliaceum* protein-coding genes are necessary to examine whether the rate acceleration extends to the entire nuclear genome of *Peridinium foliaceum*. If so, this might reconcile their isozyme profiles (Whitten & Hayhome, 1986) that seem contradictory to the conclusions reached by morphological studies and our molecular analyses. Assembled, these data lead us to prefer the scenario that a common ancestor of the two dinoflagellates engulfed a pennate diatom.

Nevertheless, the relationship between *Peridinium balticum* and *Peridinium foliaceum* still needs to be tested by SSU rDNA phylogeny with a broader taxon sampling. If some species with peridinin-containing plastids directly clusters with either *Peridinium balticum* or *Peridinium foliaceum* in a future study, the 'single-endosymbiosis' scenario will be disfavoured.

Frequency of tertiary endosymbiosis in dinoflagellate evolution

When and how many times tertiary endosymbioses have occurred during dinoflagellate evolution is an intriguing question. The relative positions of the tertiary plastid-containing dinoflagellates might give some indication of the number of times these events have occurred. Unfortunately, the relative position of the *Peridinium balticum*–*Peridinium foliaceum* clade in the dinoflagellate phylogeny is totally unresolved; this clade showed no affinity to other species including *Peridinium* sp. and *Peridinium bipes* (Fig. 1). Kishino–Hasegawa tests rejected neither the monophyly of the four *Peridinium* species nor the basal separation of the *Peridinium balticum*–*Peridinium foliaceum* clade (data not shown). However, the triple-enveloped eyespots in the cytosols of *Peridinium balticum* and *Peridinium foliaceum* have been described, and presumed to be vestiges of plastids surrounded by three membranes (Horiguchi & Pienaar, 1991). Therefore, the ancestor of the two dinoflagellates may have replaced the original plastid with a diatom endosymbiont, arguing against the diatom endosymbiosis as the basal condition for the dinoflagellate clade.

Recently, the three marine dinoflagellates *Dinorthis paradoxa*, *Gymnodinium quadrilobatum* and *Peridinium quinquecorne* have been described as bearing diatom endosymbionts (Horiguchi & Pienaar, 1991, 1994; Horiguchi & Chihara, 1993). Unfortunately,

whether these diatoms are permanent residents is unclear. Cavalier-Smith & Lee (1985) make the distinction between transient endosymbiont and permanent resident (organelle) based on extent of host-endosymbiont integration. Organelles transfer some of their genes to their host's nucleus, and possess the molecular machinery to translocate the proteins encoded by such genes. Searching the nuclear genomes of diatom-bearing dinoflagellates for genes that are unambiguously derived from the diatom endosymbiont might clarify the question of whether the endosymbionts deserve true organelle status. The relationships among the dinoflagellates bearing diatom endosymbionts, including *Peridinium balticum* and *Peridinium foliaceum*, and their relative positions in dinoflagellate phylogeny are important in estimating the frequency of tertiary endosymbiosis between diatoms and dinoflagellates.

The extant plastid in the dinoflagellate *Lapidodinium viride* is most probably acquired by plastid replacement via tertiary endosymbiosis (reviewed by Delwiche, 1999). This dinoflagellate possesses a green-pigmented plastid surrounded by four membranes. In our analyses, *Lapidodinium* robustly clustered with *Gymnodinium catenatum* and *Gymnodinium fuscum*, which have peridinin-containing plastids (Fig. 1). Therefore, plastid replacement occurred after the separation of *Lapidodinium* and the two *Gymnodinium* species. Other tertiary plastids, assumed to be originated from haptophytes, have been reported in *Gymnodinium breve*, *Gymnodinium galatheanum* and *Gymnodinium aureolum* (Delwiche, 1999). Then we presume that tertiary endosymbioses (and plastid-replacement) have occurred at least three times during dinoflagellate evolution (highlighted by arrows in Fig. 1; *Gymnodinium breve*, *Gymnodinium galatheanum* and *Gymnodinium aureolum* are not included).

Dinoflagellates are easily maintained in the laboratory, are experimentally tractable, and have had a complex and varied history of plastid acquisition. They therefore make excellent models for the investigation of tertiary endosymbiosis. In order to best study this fascinating problem, however, a robust phylogeny of dinoflagellates based both on biologically relevant models of evolution for SSU rDNA as well as analyses from protein-coding genes are crucial.

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