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A Three-Gene Dinoflagellate Phylogeny Suggests Monophyly of Prorocentrales and a Basal Position for *Amphidinium* and *Heterocapsa*

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Abstract Many outstanding questions about dinoflagellate evolution can potentially be resolved by establishing a robust phylogeny. To do this, we generated a data set of mitochondrial cytochrome *b* (*cob*) and mitochondrial cytochrome *c* oxidase 1 (*cox1*) from a broad range of dinoflagellates. Maximum likelihood, maximum parsimony, and Bayesian methods were used to infer phylogenies from these genes separately and as a concatenated alignment with and without small subunit (SSU) rDNA sequences. These trees were largely congruent in topology with previously published phylogenies but revealed several unexpected results. *Prorocentrum* benthic and planktonic species previously placed in different clusters formed a monophyletic group in all trees, suggesting that the Prorocentrales is a monophyletic group. More strikingly, our analyses placed *Amphidinium* and *Heterocapsa* as early splits among dinoflagellates that diverged after the emergence of *O. marina*. This affiliation received strong bootstrap support, but these lineages exhibited relatively long branches. The approximately unbiased (AU-) test was used to assess this result using a three-gene (*cob* + *cox1* + SSU rDNA) DNA data set and the inferred tree. This analysis showed that forcing *Amphidinium* or *Heterocapsa* to relatively more derived positions in the phylogeny resulted in significantly lower likelihood scores, consistent with the phylogenies. The position of these lineages needs to be further verified.

Keywords *cob* · *cox*1 · Cytochrome *b* · Cytochrome *c* oxidase I · Dinoflagellates · Phylogeny · rDNA

Abbreviations

COB mitochondrial cytochrome b
cob gene coding for COB
COX1 mitochondrial cytochrome c oxidase 1
*cox*1 gene coding for COX1

Introduction

Dinoflagellates (subphylum Dinoflagellata, phylum Dinozoa) are a fascinating group of marine protists that have distinct cytological and biochemical features, a complex evolutionary history, and high divergence rates for many genes (for review, see Hackett et al. 2004). These characteristics make dinoflagellates an interesting but challenging model for phylogenetic and evolutionary studies. Although efforts to understand the evolutionary history of dinoflagellates and their plastids have significantly increased in recent years, many questions remain. Resolution of these questions will be aided by the provision of a resolved dinoflagellate host tree. The small subunit (SSU) ribosomal RNA gene (rDNA) has been used most frequently in advancing our understanding of dinoflagellate evolution (reviewed by Saunders et al. 1997; Saldarriaga et al. 2001), however, the low resolving power of this gene regarding

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lineage relationships has resulted in an incomplete understanding of the major splits among these taxa despite a taxonomically broad sampling of data (Saunders et al. 1997; Saldarriaga et al. 2004; Shalchian-Tabrizi et al. 2006a). Lineages of the so-called GPP (Gymnodiniales, Peridiniales, and Prorocentrales) complex are still poorly resolved. Among other issues, the Prorocentrales lineage has often been shown to be polyphyletic. In addition, the identity of basal dinoflagellate lineages remains unclear.

Recent studies have revealed the advantage of using multiple genes for phylogenetic analysis (e.g., Pryer et al. 2001; Mattern 2004; Yoon et al. 2004). Protein-coding genes have been employed successfully to address some of the phylogenetic questions, but the numbers of genes and taxa sampled for each gene are limited (Saldarriaga et al. 2004). Genes that have been explored include actin, tubulin, *rbc*L, *psb*A, and several other plastid genes (Yoon et al. 2002; Saldarriaga et al. 2003). Additional genes from a broader sampling of taxa are needed. Mitochondrial (mt) genes are potentially useful candidates for phylogeny reconstruction for several reasons: these sequences have a higher mutation rate than nuclear genes such as SSU rDNA (Avise 1994; Saccone et al. 2000; Garesse and Vallejo 2001), the organelle is of an ancient origin and shared among virtually all eukaryotes (Gray et al. 1999), and the encoded sequences tend to exhibit a more clock-like behavior than SSU rDNA (Saccone et al. 2000). Cytochrome *b* (*cob*) is one of the mitochondrial genes that are most widely used for phylogenetic and population genetic analyses (e.g., Conway et al. 2000; Taylor and Hellberg 2003). A combination of *cob* and nuclear genes has provided robust phylogenetic trees for alveolates and other organisms (Serizawa et al. 2000; Rathore et al. 2001), and its potential utility for inferring the dinoflagellate phylogeny has been demonstrated on the basis of analysis of a small number of species (Zhang et al. 2005). Cytochrome *b* gene sequences, combined with their mRNA editing characteristics, also appear promising for providing species-specific dinoflagellate molecular markers, as demonstrated recently for *Prorocentrum* spp. (Lin et al. 2006). Furthermore, the potential of cytochrome *c* oxidase I (*cox1*) for distinguishing closely related organisms constitutes the basis of the ongoing DNA barcoding studies (Hebert et al. 2004), and the utility of this gene for dinoflagellate phylogeny has been demonstrated in separating different clades of *Symbiodinium* (Takabayashi et al. 2004). Both *cob* and *cox1* have been verified to be encoded in the mitochondria (Zhang et al. 2007). However, the resolving power of mt genes for dinoflagellate phylogeny still awaits examination using a wider range of taxa in the analysis. Toward this goal, we sequenced *cob* and *cox1* from 24 and 33 species, respectively. We then used the existing and newly obtained *cob* and *cox1* as well as SSU rDNA sequences to reconstruct trees using different phylogenetic methods, and used the approximately unbiased (AU-) test to assess our results. Whereas the tree topologies we found are generally similar to those previously inferred, *Amphidinium* and *Heterocapsa* were unexpectedly found to be early-diverging lineages of dinoflagellates and *Prorocentrum* was shown to be a monophyletic lineage. These new trees also confirm the hypothesis that the presence of a theca and traits of toxin production and photosynthesis were gained and lost many times in dinoflagellate evolution.

Materials and Methods

Taxon Selection and Dinoflagellate Cultures

Dinoflagellate species were selected to maximize diversity within Prorocentrales and the number of basal lineages included in the analyses. The apicomplexans *Plasmodium yoelii* and *P. berghei* were used to root the trees because Apicomplexa are considered to be the closest extant relatives of dinoflagellates. Cultures of dinoflagellates and other algae used in this study were obtained from several sources (Table 1). Two taxa of Prorocentrales had historically been classified as both *Prorocentrum* and *Exuviaella* (for review see Gómez 2005): *Prorocentrum cassubicum* (= *Exuviaella cassubica*) and *P. lima* (=*E. lima*). As our results suggest (see below), we used *Prorocentrum* as the genus name throughout the paper. *Katodinium rotundata* is synonymous to *Heterocapsa rotundatum* (Hansen 1995). *Karlodinium micrum* has recently been renamed as *K. veneficum* (Bergholtz et al. 2005). The photosynthetic species were grown in f/2 medium, whereas heterotrophic dinoflagellates (*Oxyrrhis marina*, *Pfiesteria piscicida*, *Pfiesteria*-like CCMP1828, and *Pseudopfiesteria shumwayae*) were grown with algal prey (*Dunaliella tertiolecta* CCMP1320 or *Rhodomonas* sp. CCMP768). Seawater was adjusted to 28 PSU (practical salinity unit) for most species and to 15 PSU for *Rhodomonas* sp., *K. veneficum*, and heterotrophic taxa. Cultures were maintained at 15, 20, or 25 ± 1°C, according to the suggested optimal culturing temperature for each strain. Illumination was provided in a 12:12-h light-dark cycle with a photon flux of about 100 $\mu E \cdot m^{-2} \cdot s^{-1}$. Growth rate was monitored by microscopic cell counts using a Sedgwick-Rafter chamber.

Sample Collection, RNA Extraction, and cDNA Synthesis

For autotrophic dinoflagellates, samples were collected when cultures were in the exponential growth phase; for heterotrophic species, samples were collected after feeding was discontinued for over 2 days when very few (<2% of total cells) prey algae (*D. tertiolecta* or *Rhodomonas* sp.)

Table 1 The dinoflagellate and *Plasmodium* species included in the phylogenetic analyses

Taxon	Strain and source	Theca[a]	Toxicity detected[b]	Trophic mode[c]	SSU rDNA[d]	*Cob*	*Cox1*
Nondinoflagellate							
Plasmodium berghei					AJ243513*	AAD01526*	AAD01525*
Plasmodium yoelii					AF180727*	AAC25924*	AAC25923*
Dinoflagellates							
Adenoides eludens	CCMP1891[e]	–	N	P	AF274249*	EF036541	EF036565
Akashiwo sanguinea	LIS1[f]	–	N	P	AY456106**	EF036542	EF036566
Alexandrium affine	CCMP112[e]	++	Y	P	AY831409*	EF036543	EF377324
Alexandrium tamarense	CB307; D. M. Anderson	++	Y	P	AF022191[#]	DQ082987*	EF036567
Amphidinium carterae	CCMP1314[e]	–	Y	P	AF274251*	EF036544	EF036568
A. operculatum	CCMP123[e]	–	N	P	AY443011[#]	EF036545	EF036569
Ceratium longipes	CCMP1770[e]	++	N		DQ388462*	EF036546	EF036570
Ceratocorys horrida	CCMP157 [e]	++	N	P	DQ388456*	EF036547	EF036571
Coolia monotis	CCMP304 [e]	++	N	P	AJ415509[#]		EF036572
Crypthecodinium cohnii	WHd; M. Gray	++	N	N	M64245*	AF403221*	AF487783*
Gambierdiscus toxicus	CCMP401[e]	++	Y	P	DQ388463*	EF036550	EF036575
Gonyaulax cochlea	CCMP1592[e]	++	N	P	DQ388465*	EF036551	EF036576
Gymnodinium catenatum	CCMP1937[e]	–	Y	P	AF022193[#]	EF036552	
G. simplex	CCMP419[e]	–	N	P	DQ388466*	EF036553	EF036577
Heterocapsa triquetra	CCPM449[e]	++	N	P	AJ415514[#]	EF036554	EF036578
H. rotundata (=*Katodinium rotundatum*)	CCMP1542[e]	–	N	P	DQ388464*	EF036556	EF036582
Karlodinium veneficum (formerly K. micrum)	CCMP1975[e]	–	Y	M	EF036540	DQ082989*	EF036579
Karenia brevis	CCPM2229[e]	–	Y	P	AF274259[#]	EF036555	EF036580
Karenia mikimotoi	CCPM429[e]	–	Y	P	AF009131[#]		EF036581
Noctiluca scintillans	NS3; E. J. Buskey	–	N	N	DQ388461		EF036583
Oxyrrhis marina	CCMP1795[e]	–	N	N	AF482425[#]	EF036557	EF036584
Peridinium aciculiferum	PAER1	++	N	N	AY970653*	DQ094825*	
Pfiesteria piscicida	CCMP1831[e]	–	?	N	AF330620*	AF357518*	AF463413*
Pseudopfiesteria shumwayae	T4; P. Tester,	–	?	N	AF218805[#]	DQ082988*	EF036586
Pfiesteria-like	CCMP1828[e]	–	N	N	AY590476*	EF036558	EF036585
Prorocentrum dentatum	CCMP1517[e]	+	N	P	DQ336057*	DQ336059*	
P. donghaiense	S. Lü	+	N	P	DQ336054*	DQ336056*	EF036587
P. cassubicum (=*Exuviaella cassubica*)	LB1596[g]	+	N	P	DQ388460*	EF036548	EF036573
P. lima (=*E. lima*)	CCMP1966[e]	+	Y	P	EF377326	EF036559	EF377325
P. micans	CCMP1589[e]	+	N	P	AY585526**	AY745238*	EF036588
P. minimum	CCMP696[e]	+	Y	P	DQ336072*	AY030285*	AF463415*
P. minimum	JA9801; P. Glibert	+	?	P	DQ336063*	DQ336065*	
P. minimum	PTPM; P. Tester	+	?	P	DQ336069*	DQ336071*	
P. nanum	LB1008[g]	+	N	P	DQ388459*	EF036549	EF036574
Protoceratium reticulatum	CCMP1721[e]	+	N	P	AF274273[#]	EF036560	EF036589
Pyrodinium bahamense	Azanza-Corrales	+	Y	P	AY456115*	AY456114**	
Pyrocystis lunula	J.W. Hastings	++	N	P	AF274274[#]	EF036561	EF036590
P. noctiluca	CCMP732[e]	++	N	P	AF022156*	EF036562	EF036591
Scrippsiella sp.	LIS[f]	++	?	P	AY743960*	AY743962*	EF036592
S. sweeneyae	CCCM280[h]	++	Y	P	AF274276*	EF036563	EF036593
Symbiodinium goreaui	CCMP2466[e]	–	N	P	EF036539	EF036564	

Table 1 continued

Taxon	Strain and source	Theca[a]	Toxicity detected[b]	Trophic mode[c]	SSU rDNA[d]	*Cob*	*Cox1*
Symbiodinium microadriaticum	CCMP830[e]	–	N	P	AY456111*	DQ082985*	EF036594
Symbiodinium sp.	CCMP832[e]	–	N	P	AY456113*	DQ082986*	EF036595

[a] (–) No strong theca; (+) two-valve theca; (++) multiplate theca

[b] Recognized (Y), no known (N), or questionable (?) ability to produce toxins

[c] Photosynthetic (P), mixotrophic (M), or nonphotosynthetic (N)

[d] *Sequences from GenBank; #sequences from GenBank originally obtained from other strains of the same species as used in this study; **sequences from Zhang et al. (2005); unmarked, sequences obtained in this study

[e] Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, Maine

[f] Isolated from Long Island Sound in April 2003, morphologically similar to *Akashiwo sanguinea* based on the description by Steidinger and Tengen (1997) and SSU rDNA sequence (identical to AF276818). The LIS isolate of *Scrippsiella* sp. is most closely related to *S. trochoidea* based on morphology (Steidinger and Tengen 1997) and SSU rDNA identity (1738 of 1754 bp identical to AY421792)

[g] The Culture Collection of Algae (UTEX), The University of Texas at Austin, Texas

[h] Canadian Center for the Culture of Microorganisms, University of British Columbia, Vancouver, BC, Canada

could be detected by microscopic examination. The cells were harvested by centrifugation at 3000*g* at 4°C for 20 min, cell pellets were resuspended in Trizol and subjected to RNA extraction, and cDNA was synthesized essentially following Lin et al. (2002).

PCR, Cloning, and Sequencing

To PCR-amplify *cob* and *cox1* from dinoflagellates, several sets of primers were designed from the conserved regions of these genes in dinoflagellates (Table 2). PCR reactions were carried out with a single incubation for 1 min at 95°C, followed by 40 cycles of 20 s at 94°C, 30 s at 48 to 58°C, and 40 s at 72°C. For several dinoflagellates, SSU rDNA was also amplified using the universal primers or a universal primer paired with a dinoflagellate-specific primer (Zhang et al. 2005) (Table 2).

The PCR products were purified using DNA Clean & Concentrator (Zymo Research, Orange, CA) and directly sequenced on both strands using BigDye reagents and an ABI Prizm automated sequencer (Perkin Elmer, Branchburg, NJ). In a few cases, the PCR products were cloned into a T-vector and sequenced (Zhang et al 2006). To identify potential PCR-related polymorphisms, plasmids were isolated from 5 to 10 colonies, then sequenced over both strands, and these sequences were compared to each other.

Phylogenetic Analysis

To maintain codon integrity, DNA sequences were aligned using REVTRANS (http://www.cbs.dtu.dk/services/RevTrans/) with the default values. We analyzed dinoflagellate mitochondrial cytochrome b (COB) and mitochondrial cytochrome c oxidase 1 (COX1) proteins (303 and 446 amino acids [aa], respectively) that were deduced from the corresponding cDNA sequences, as individual data sets as well as a concatenated data set, then combined the cDNA sequences to produce a *cob* + *cox1* two-gene data set (1963 nucleotides [nt]) and with SSU rDNA (1714 nt) to generate a *cob* + *cox1* + SSU rDNA three-gene (3667 nt) data set. The apicomplexans *Plasmodium yoelii* and *P. berghei* were used to root the protein trees, whereas the early diverging dinoflagellate *Oxyrrhis marina* was used to root the two-gene and three-gene DNA trees. The *O. marina* rooting was necessary due to the high divergence of the apicomplexan SSU rDNA sequences that destabilized the dinoflagellate tree (results not shown).

Using the COB + COX1 concatenated data set, we first tested the congruence of the data partitions using the partition homogeneity test (ILD test in PAUP*, 1000 replicates). This analysis showed an absence of significant incongruence between these protein data partitions (p = 0.136). For the phylogenetic analyses, we used ProtTest V1.3 (Abascal et al. 2005) to identify the best-fit model for the COB, COX1, and COB + COX1 data sets with "Fast" optimization and a BIONJ tree. The ProtTest parameter values were then used in maximum likelihood (ML) analyses with the PHYML V2.4.3 computer program and tree optimization. The results of 100 PHYML bootstrap analyses (PHB) were used to assess the robustness of monophyletic clades in these trees. We also used Bayesian inference (MrBayes V3.0b4 [Huelsenbeck and Ronquist 2001]) with each protein data set. The ProtTest best-fit evolutionary model for each data set was used in these analyses with Metropolis-coupled Markov chain Monte Carlo from a random starting tree. We did analyses for each

Table 2 Primers used in the present study

Primer name	Sequence (5′–3′)	Application
Dinocob1F[a]	ATGAAATCTCATTTACAWWCATATCCTTGTCC	Dinoflagellate *cob* forward
Dinocob1R[a]	TCTCTTGAGGKAATTGWKMACCTATCCA	Dinoflagellate *cob* reverse
Dinocob2F	ATWAATTYTTTYTGGAATHtBGGTTT	Dinoflagellate *cob* forward
Dinocob3F	GAATTACTATTRTTATHCARATHNTWACWGG	Dinoflagellate *cob* forward
Dinocob4F	AGCATTTATGGGTTATGTNTTACCTTT	Dinoflagellate *cob* forward
Dinocob5F	ATCTCTTCTTTATCACATCCAGATAATG	Dinoflagellate *cob* forward
Dinocob2R	CGAGCATAAGATAKAAACWTCTCTTGAGG	Dinoflagellate *cob* reverse
Dinocob3R	AGCTTCTANDGMATTATCTGGATG	Dinoflagellate *cob* reverse
Dinocob4R	AGATAATARATTWGTAATDACAGT	Dinoflagellate *cob* reverse
Dinocob5R	ATTWGTAATDACWGTWGCWCCCCA	Dinoflagellate *cob* reverse
Dinocox1F[b]	AAAAATTGTAATCATAAACGCTTAGG	Dinoflagellate *cox*1 forward
Dinocox1R[b]	TGTTGAGCCACCTATAGTAAACATTA	Dinoflagellate *cox*1 reverse
Dinocox1F2	GAATTATATCTCCTGAAAACCAGAACTTC	Dinoflagellate *cox*1 forward
Dinocox1F3	TTATGATCTTCTTYTTWRTWATGCC	Dinoflagellate *cox*1 forward
Dinocox1F4	TTTGGAGGTGGWWCWGGNTGGAC	Dinoflagellate *cox*1 forward
Dinocox1F5	TGGTTGGACATTATATCCTCCATTATC	Dinoflagellate *cox*1 forward
Dinocox1F6	GGTTCTTTGGACATCCTGAAGTTTA	Dinoflagellate *cox*1 forward
Amp-heterocox1F1	GGTGGATTTGGTAATTAYTTCTYACC	*Amphidinium-Heterocapsa cox*1 forward
Dinocox1R2	AGTTATTCCTGATCCAATAGATGACAG	Dinoflagellate *cox*1 reverse
Dinocox1R3	CTGATCCAATAGATGACAGAAAATTCC	Dinoflagellate *cox*1 reverse
Dinocox1R4	TGGAAATGWGCWAYWAYATAATAWGTRTCATG	Dinoflagellate *cox*1 reverse
Katocox1R1	ATAGCAAAGGAAAGTAGAGTAACAC	*Katodinium cox*1 reverse
18ScomF1[a]	GCTTGTCTCAAAGATTAAGCCATGC	Universal SSU rDNA forward
18ScomR1[a]	CACCTACGGAAACCTTGTTACGAC	Universal SSU rDNA reverse
Dino18SF1[a]	AAGGGTTGTGTTTATTAGNTACAGAAC	Dinoflagellate SSU rDNA forward
Dino18SR1[a]	GAGCCAGATRCWCACCCAG	Dinoflagellate SSU rDNA reverse

[a] From Zhang et al. (2005)

[b] From Lin et al. (2002)

data set with the "covarion" option on and off in MrBayes to test for potential rate variation across the tree. The average likelihood of the post burn-in trees was compared under the standard versus the covarion model to see if adding the covarion option markedly increased the tree likelihoods. To increase the probability of chain convergence Bayesian run lengths were set at 1 million generations, with trees sampled each 100 cycles. Four chains were run simultaneously, of which three were heated and one was cold, with the initial 500,000 cycles (5000 trees) discarded as the burn-in. A consensus tree was made with the remaining 5000 trees to determine the posterior probabilities (BPP) at the different nodes in the PHYML trees. Finally, for the COB + COX1 data sets, we also did an unweighted maximum parsimony (MP) bootstrap analysis (MPB; 500 replications) using heuristic searches and TBR branch-swapping to find the shortest trees (using PAUP*V4.0b10 [Swofford 1998]). The number of random-addition replicates was set to 10 for each bootstrap tree search and best-scoring trees were held at each step.

For the two-gene (*cob* + *cox1*) DNA data set, the ILD (1000 replicates) showed significant incongruence between these data partitions ($p = 0.003$). This same result was found with the rDNA + *cob* ($p = 0.001$) and rDNA + *cox1* ($p = 0.001$) data partitions. However, we chose to combine the data because of substantial controversy regarding the utility of the ILD test (e.g., Barker and Lutzoni 2001; Hipp et al. 2004). The two-gene and three-gene trees were inferred using PAUP* and the site-specific GTR model (ssGTR [Rodriguez et al. 1990]) with different evolutionary rates for each amino acid codon position and for the rDNA data. Bootstrap analyses were done using PHYML (100 replicates) with the GTR + I + Γ model over all nucleotide positions. Bayesian posterior probabilities for the ssGTR tree were calculated using MrBayes and the site-specific GTR + I + Γ model over the three and four data partitions, respectively. These analyses were run as described above. We also did unweighted MP bootstrap analyses with the two-gene and three-gene data sets as described above.

Testing the Tree Topology

To assess the positions of different dinoflagellates in the three-gene tree, we generated a backbone phylogeny that was identical to the "best" ssGTR topology. Using this 30-taxon tree, we removed either *O. marina*, *Amphidinium* spp., *H. rotundata*, and *H. triquetra* or the Suessiales. These taxa were then added individually (using MacClade V4.05 [Maddison and Maddison 2002]) to each branch in the tree to generate a set of topologies that addressed all possible positions for each taxon in the tree. The site-by-site likelihoods for these trees were calculated using the 30-taxon 3-gene data set and baseml implemented in PAML V3.13 (Yang 1997) with the GTR + Γ model (the alpha value for the gamma distribution was identified using PHYML) and the default settings. The AU-test was implemented using CONSEL V0.1f (Shimodaira and Hasegawa 2001) to assign probabilities to the different trees in each test.

Results

General Phylogenetic Relationships

The best-fit models identified by ProtTest for the COB and COX1 protein data sets using the Akaike Information Criterion were cpREV + I (0.100) + Γ (1.433) + F (as observed) and cpREV + I (0.126) + Γ (1.049) + F (as observed), respectively. Using these parameters and the PHYML ML method, the COB and COX1 phylogenetic trees (Figs. 1A and B, respectively) were overall quite similar for supported groups (i.e., those with high bootstrap values for ML and Bayesian posterior probabilities). In both data sets, *O. marina* was positioned as the earliest-diverging dinoflagellate. Prorocentrales formed a monophyletic group with strong bootstrap support, as did the group of *Symbiodinium* species in our analysis. In contrast, the orders Peridiniales, Gymnodiniales, and Gonyaulacales were not united as monophyletic groups. Surprisingly, *Amphidinium* spp. and *Heterocapsa* spp. were placed in a basal position with long branches, diverging after *O. marina* in both trees. Due to failure to isolate *cob* from *N. scintillans*, this species was represented only in the COX1 tree, where it was placed after the basal lineages *Amphidinium* and *Heterocapsa*. The exact position of *N. scintillans* was, however, unclear due to lack of bootstrap support (Fig. 1B). Other small differences between the COB and the COX1 trees occurred in the positions of Suessiales and *Akashiwo sanguinea*.

The COB + COX1 concatenated amino acid data set was analyzed using the best-fit model cpREV + I (0.116) + Γ (1.197) + F (as observed) to compare to the branching pattern observed from the two individual genes trees (Fig. 2). In this phylogeny, *O. marina, Amphidinium* spp., and *Heterocapsa* spp. remained in ancestral positions as in the COB and COX1 trees and the Prorocentrales formed a monophyletic cluster. The fucoxanthin containing

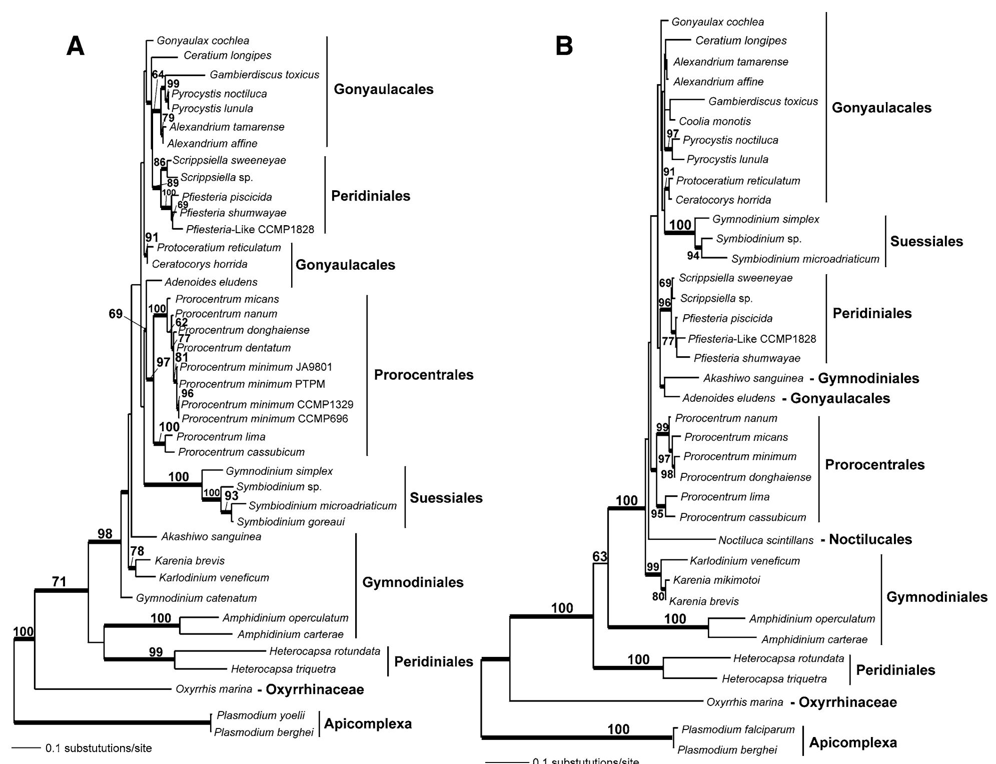

Fig. 1 Protein maximum likelihood phylogenetic trees of dinoflagellates inferred from COB (**A**) and COX1 (**B**). Bootstrap values resulting from a PHYML analysis are shown above the branches; only values >60% are shown. In B, the value at the base of Prorocentrales clade is 55%. Thicker branches denote a Bayesian posterior probability >0.95. Branch lengths are proportional to the number of substitutions per site (see scale bar). The new COB and COX1 sequences are available in GenBank; the accession numbers for those of dinoflagellates are shown in Table 1

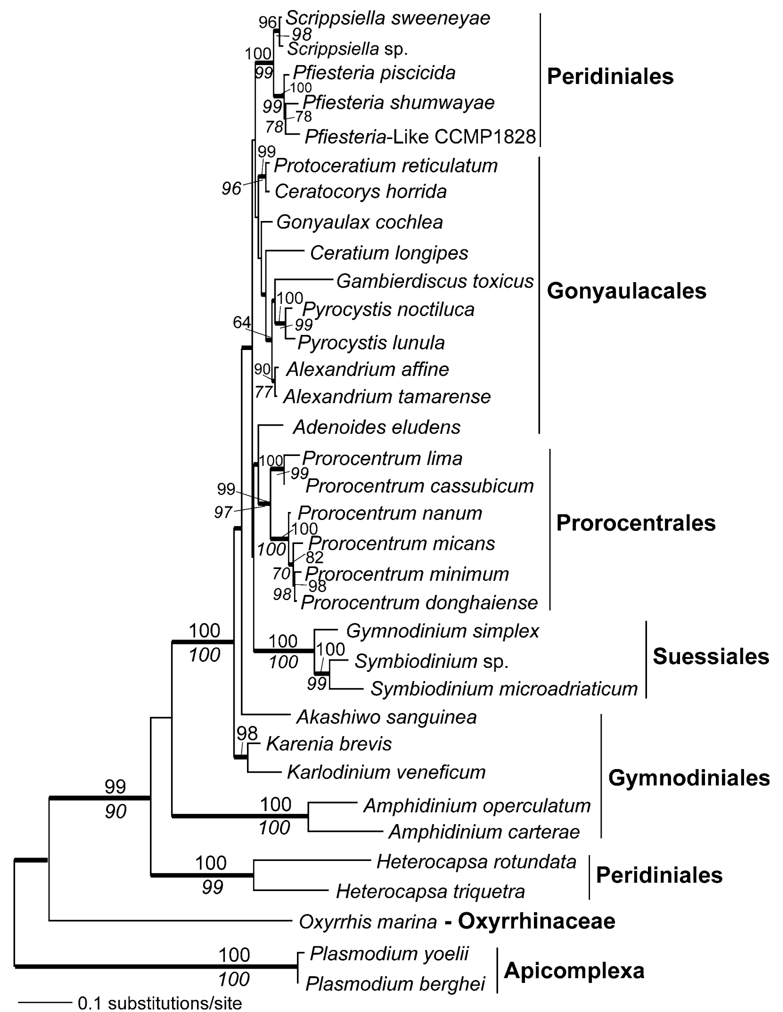

Fig. 2 Protein maximum likelihood phylogenetic tree of dinoflagellates inferred from a concatenated data set of COB + COX1. Bootstrap values resulting from PHYML and unweighted maximum parsimony analyses are shown above and below the branches, respectively; only values >60% are shown. Thicker branches denote a Bayesian posterior probability >0.95. Branch lengths are proportional to the number of substitutions per site (see scale bar)

Gymnodiniales and *A. sanguinea* did not form a monophyletic group in this and separate COB and COX1 trees. In the *cob* + *cox1* and *cob* + *cox1* + SSU rDNA two-gene and three-gene trees (Fig. 3), however, there was substantially more support for nodes in the tree showing, for example, the monophyly of the Gymnodiniales and *A. sanguinea* clade (two-gene, PHB = 67%) and for all Gonyaulacales (two-gene, PHB = 95%, BPP = 1.0; three-gene, PHB = 100%, MPB = 100%, BPP = 1.0) except *Adenoides eludens*, which grouped with Prorocentrales (two-gene, PHB = 67%, MPB = 69%, BPP = 0.99; three-gene, BPP = 1.0). The branching order of *Amphidinium* spp., *H. rotundata*, *H. triquetra*, and *Symbiodinium* spp. was generally consistent with the results of the protein analyses supporting an early divergence of these taxa as two independent lineages.

Approximate Unbiased Test

We tested several hypotheses with the AU test. The first was the early divergence of *O. marina* as suggested in our mitochondrial protein trees. All alternative positions of this taxon (i.e., relative to that shown in Fig. 3B) in the three-gene, 30-taxon tree were rejected at $p < 0.05$. The position of *Amphidinium* spp. received strong support as an early divergence (Fig. 3), although the tree of highest likelihood positioned this taxon as diverging after the *Heterocapsa* spp. clade ($p = 0.974$; see below). All other trees, including that shown in Fig. 3B ($p = 0.024$), were rejected in our analysis. The AU test was also used to assess support for the surprising finding of an early divergence of *Heterocapsa* spp. in our trees. The tree receiving the highest support in this analysis placed this clade as an independent divergence before the split of *Amphidinium* spp. ($p = 0.638$). All other topologies were rejected by the AU-test. And finally, we tested the early divergence of the Suessiales among the relatively derived dinoflagellates in our analysis. The AU-test overwhelmingly supported the position of this clade shown in Fig. 3 ($p = 0.999$). Therefore, our analyses of the three-gene data are consistent with an early divergence of *O. marina*, *Amphidinium* spp., *Heterocapsa* spp., and the Suessiales and do not support the

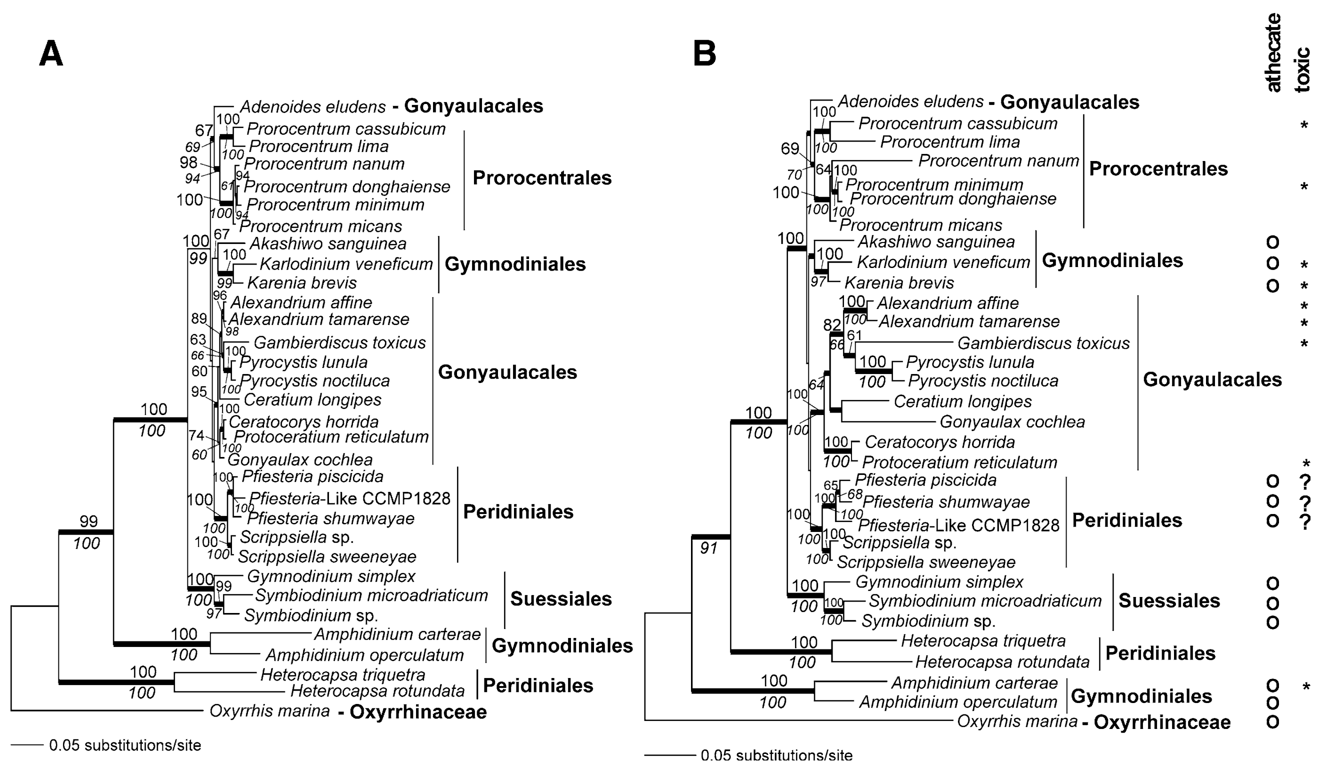

Fig. 3 Maximum likelihood phylogenetic trees of dinoflagellates inferred form the two-gene (**A**; *cob* + *cox1*) and three-gene (**B**; *cob* + *cox1* + SSU rDNA) DNA data sets. Bootstrap values resulting from PHYML and unweighted maximum parsimony analyses are shown above and below the branches, respectively; only values >60% are shown. Thicker branches denote a Bayesian posterior probability >0.95. Branch lengths are proportional to the number of substitutions per site (see scale bars). The symbols on the right in B indicate lack of strong thecate (○) and recognized ability to produce toxins (*)

monophyly of the Peridiniales and Gymnodiniales in our tree. Forcing the monophyly of *H. rotundata*/*H. triquetra* with the Peridiniales was rejected at $p < 0.01$, as was uniting *Amphidinium* spp. with the *Akashiwo* sp. + fucoxanthin dinoflagellate clade.

Discussion

In this study, we isolated mitochondrial *cob* and *cox1* from a total of 33 dinoflagellate species or strains. Together with *cob* and *cox1* from our previous studies (Lin et al. 2002, 2006; Zhang et al. 2005), the sequences generated in this study constitute the first broad-taxon *cob* and *cox1* data sets, which will be useful for future phylogenetic studies of dinoflagellates. The existing dinoflagellate molecular data have been limited to the SSU rDNA accompanied by a recent increase in smaller data sets of cytoskeletal proteins (actin, tubulin), heat shock proteins, and plastid genes such as *psb*A. The latter, however, suffers potentially from a biased amino codon usage or mutational saturation (Inagaki et al. 2004; Shalchian-Tabrizi et al. 2006b). The *cob* and *cox1* data set is second in taxon coverage to the SSU rDNA data set and is, therefore, potentially useful as a source of protein coding genes for dinoflagellate phylogeny reconstruction. For dinoflagellates, the nuclear-encoded protein genes such as actin and tubulin are rich in paralogues (Zhang and Lin, unpublished data) and their application to phylogenetic reconstruction requires caution. For *cob* and *cox1* we have found only one functional mRNA sequence for each gene in the dinoflagellates investigated (Zhang et al. 2005; this study), suggesting that they are likely to be good candidates for phylogenetic analysis. However, in dinoflagellates, there is widespread mRNA editing (Lin et al. 2002; Zhang and Lin 2005); therefore, mixing genomic DNA and cDNA sequences in a phylogenetic analysis is not recommended. Moreover, the existence of numerous *cob* and *cox1* pseudogenes in the mitochondrial genome of some dinoflagellate lineages (Zhang and Lin, unpublished data) renders it difficult to retrieve the functional gene sequence in those lineages. Therefore, we obtained *cob* and *cox1* cDNA instead of genomic sequences in this study, which made it easier to attain the gene sequences and allowed protein phylogenetic analyses based on the deduced COB and COX1 sequences. Even so, because dinoflagellate *cob* and *cox1* are highly

AT-rich (Lin et al. 2002; this study), it is generally difficult to design primers for PCR amplification. In some of the dinoflagellate lineages, such as *Gyrodinium*, *Gymnodinium*, *Lingulodinium*, and *Noctiluca*, expressed pseudogenes of *cob* and *cox1* were found (Chaput et al 2002; Zhang and Lin, unpublished data), making it a challenge to isolate the functional *cob* and *cox1* sequences from these species. As a result, many PCR experiments with different primer sets (Table 2) and annealing temperatures were made before *cob* and *cox1* could be retrieved. For *N. scintillans*, only *cox1* was successfully isolated; this species was excluded from two-gene and three-gene analysis. Nevertheless, the results of phylogenetic analyses based on the sequences successfully retrieved allow us to gain insights into the overall dinoflagellate tree and, in particular, into basal splits in this group.

The Phylogeny

The two-gene and three-gene phylogenies are in agreement with previous trees inferred from other genes with respect to the major branches of dinoflagellates. For instance, the clades Gonyaulacales (*Alexandrium*, *Pyrocystis*, *Protoceratium*), Suessiales, *Pfiesteria* (and related lineages), and the fucoxanthin-containing Gymnodiniales (*Karenia*, *Karlodinium*) were all found as monophyletic groups with strong bootstrap support. Of these clades, the fucoxanthin cluster (on DNA trees) had the peridinin-containing *Akashiwo* as an early divergence as previously shown (Zhang et al. 2005). It is noteworthy that *P. shumwayae* consistently allied with the *Pfiesteria*-like CCMP1828 in all our analyses except in one case, in which *P. shumwayae* clustered with *P. piscicida* with weak bootstrap support (Fig. 3B). This observation is in favor of the recently proposed separation of *P. shumwayae* from the genus *Pfiesteria* (Litaker et al. 2005), which was subsequently reverted (Marshall et al. 2006). Further analysis including more *Pfiesteria*-related taxa and more genes is needed to address the issue.

Basal Position of *Oxyrrhis marina*

The position of *O. marina* has been debated. This organism has been regarded as a dinoflagellate based on its general morphology including flagellar structure (e.g., Dodge 1985; Sournia 1986) but has been explicitly excluded from the dinoflagellate phylum (Fensome et al. 1993) because of cytological characteristics distinct from those in "true" dinoflagellates. In contrast to typical dinoflagellates, the mitotic spindle in *O. marina* is intranuclear, the nucleus is not dinokaryotic, and there is no girdle and sulcus. No molecular phylogenetic analysis has been conducted for this lineage until recently. Saldarriaga et al. (2003) found that SSU rDNA sequence in *O. marina* is highly divergent and branches within the gonyaulacoid clade, and actin, α-tubulin, and β-tubulin genes placed this species at the base of dinoflagellates. *Oxyrrhis marina* is clearly placed between *Plasmodium* and all other dinoflagellates in our present study. A recent study reveals that *O. marina* shares with typical dinoflagellates the trait of spliced leader RNA *trans*-splicing and the sequence of the leader RNA that is *trans*-spliced to the 5' end of the nuclear-encoded mRNAs (Zhang et al. 2007). All of these insights together support the idea that *O. marina* is an early diverging lineage within the dinoflagellate phylum.

Monophyly of Prorocentrales

Prorocentralean taxa have undergone several revisions at the species and genus level due to inconsistencies between morphological and molecular data (Grzebyk et al. 1998). The genus *Prorocentrum* was initially described by Ehrenberg (1834), who designated *P. micans* as the type species. The genus was later revised, with those species having a discernible apical spine being retained in the genus *Prorocentrum* and those lacking apical spines placed into the sister genus *Exuviaella* (Cienkowski 1881). Later, based on observations that the apical spine did not allow unambiguous distinction of the two genera, Dodge (1975) united the two genera in *Prorocentrum*. In the last two decades, many new species have been described from various environments, some of which could not be classified into the systematic scheme defined by Dodge (Loeblich et al. 1979; Faust 1991). More recently, molecular phylogenetic data have spawned more questions about the monophyly of the genus *Prorocentrum*. A LSU rDNA sequence analysis revealed significant genetic difference between the benthic *P. lima* and several planktonic species (Zardoya et al. 1995). Later, McLachlan et al. (1997) summarized several former observations and resurrected the old genus *Exuviaella* from *Prorocentrum*, moving three benthic species—*P. lima, P. maculosum*, and *P. hoffmannianum*—to the genus of *Exuviaella*. Grzebyk et al. (1998) reanalyzed several species from these two genera based on SSU rDNA data set and showed that these species formed two separate clades: one represented by the "core" taxa such as *P. minimum* and *P. micans*, and the other by *P. lima* and *P. maculosum*. Since then, molecular phylogenetic analyses based on both SSU and LSU rDNA have shown that *P. lima* and other benthic *Prorocentrum* species form a clade distinct from the core lineages (Saldarriaga et al. 2001, 2004), with few exceptions (Litaker et al. 1999; LUS tree in Saldarriaga et al. 2004). In the present study, all five

trees, inferred from COB, COX1, COB-COX1 (amino acid sequences), *cob-cox1*, and *cob-cox1*-SSU (nucleotide sequences) data sets, showed that all the *Prorocentrum* species examined in this study formed a strongly supported clade. In this clade, *P. nanum* clustered with *P. minimum/P. micans* with strong support (except for the moderate support in the three-gene tree). *P. cassubicum* is united with *P. lima*, and both are separated from the cluster of *P. minimum/P. micans/P. nanum*. Consistent with the SSU rDNA tree of Litaker et al. (1999) and the LSU tree in Saldarriaga et al. (2004), this result suggests the need for further investigation to determine whether these lineages should be assigned to two separate genera.

Early Divergence of *Amphidinium* and *Heterocapsa*

The most significant discrepancy between the present and the previous studies lies in the placement of *Amphidinium* and *Heterocapsa*. The basal position of these lineages revealed in this study has previously not been noted. Both COB and COX1 trees as well as the two-gene and three-gene phylogenies placed these lineages in an early-diverging position, immediately following *O. marina*. It is possible that COB and COX1 in these taxa have mutated more rapidly than in other related lineages and hence these taxa were "attracted" to the ancestor of dinoflagellates, thus misleading both the phylogenetic analyses and the AU test. A rapid mutation rate is suggested by the relatively long branches of these taxa (Figs. 1–3). The phylogenetic position of *Amphidinium* and *Heterocapsa* is therefore uncertain. Recently, *Amphidinium* was redescribed based on its unique epicone morphology. Morphological characteristics and LSU rDNA sequences place *Amphidinium* in an early diverging position (Flø-Jorgensen et al. 2004), whereas a more recent analysis by these authors using a different evolutionary model placed *Amphidinium* in a more derived position (Murray et al. 2005). It is notable that in these studies *Amphidinium* also had long branches. The classification of *Heterocapsa* as a Peridiniales taxon has not been questioned. However, this genus has occasionally occupied a basal position within dinoflagellates (e.g., in the rDNA trees in Saldarriaga et al. 2004). Our AU-test (again, tempered by the long branch issue with this taxon) indicates that placing this lineage in advanced positions results in significantly "worse" likelihood scores. The apparent basal position of *Heterocapsa* is not entirely inconsistent with morphological and paleontological data. Sulcal tabulation in this genus is somewhat atypical and can be interpreted as primitive relative to that in the rest of the Peridiniales and the Gonyaulacales (see discussion in Saldarriaga et al. 2004). The earliest fossils from the family Heterocapsaceae are in the early Jurassic, prior to the explosion radiation of all other peridinialean and gonyaulacalean forms in the Mesozoic (Fensome et al. 1999). Therefore, unless other morphological, life history, and molecular evidence argues otherwise, *Heterocapsa* may be a basal lineage. If verified, this provides additional evidence for the polyphyly of Peridiniales.

Evolution of Theca, Toxin-Producing Ability, and Photosynthesis

Results from the present study do not present any evidence that thecal plate evolution follows the pattern predicted by the Plate Increase, Plate Reduction, and Plate Fragmentation hypotheses (Bujak and Williams 1981). In none of our phylogenies does the thecal plate pattern show a general increase, decrease, or fragmentation trend. Rather, naked taxa are found in various different positions in the phylogenetic tree (Fig. 3B). Because Prorocentrales appears to be monophyletic, the thecal plate condition of two major plates with several small apical plates must have arisen only once. However, because Gonyaulacales and Peridiniales appear to be polyphyletic, as shown in this and previous studies, the number of thecal plates did not seem to evolve unidirectionally (Table 1). Nevertheless, the ancestral position of *Oxyrrhis* and *Amphidinium*, both lacking armored thecal plates, constitutes evidence that dinoflagellate ancestor may be athecate (Saldarriaga et al. 2004).

Ancestors of dinoflagellates are believed to have been photosynthetic, and the current heterotrophic lineages are presumably derived via plastid loss. Support for this hypothesis can come from the identity of the ancestral lineage of dinoflagellates (Saldarriaga et al. 2001). If *O. marina* ultimately proves to be a basal but true dinoflagellate lineage, the ancestor of dinoflagellates may be a heterotroph or have lost the plastid secondarily. Recent discoveries of novel small-sized dinoflagellate lineages in the deep dark ocean (López-García et al. 2001) and coastal regions (e.g., Lin et al. 2006) that are heterotrophic or phylogenetically related to parasitic lineages also favors the possibility that the phylum of dinoflagellates may have experienced a nonphotosynthetic period. This would contradict a key feature of the chromaveolate hypothesis in which the ancestor of chromists and alveolates is postulated to share a common secondary red algal secondary endosymbiont (Cavalier-Smith 1998; Yoon et al. 2002; Harper and Keeling 2003). Final proof of the plastid loss hypothesis will await the detection of remnant genes that once encoded plastid targeted proteins in the basal, putatively plastid-lacking, lineages. One important piece of this puzzle that was recently published is the finding of a plant-type ferredoxin redox system in the early-diverging

parasitic dinoflagellate *Perkinsus marinus*, as well as a putative plastid in this species (Stelter et al. 2007). This "pre-dinoflagellate" diverges prior to *O. marina* (Saldarriaga et al. 2003; H.S. Yoon, L.A. Katz, and D. Bhattacharya, unpublished data) and apparently contains a remnant chromalveolate plastid that had escaped discovery in previous studies.

The capacity to produce toxins (Fig. 3B) is also scattered on the phylogenetic tree, indicating that there is no clear trend in the evolution of this trait. This result suggests that toxin production has appeared and disappeared multiple times during dinoflagellate evolution. All of the results discussed above suggest that caution is needed when traits such as toxin production and presence or absence of the plastid or thecal plates are used as the basis of dinoflagellate systematics.

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