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Mitochondrial *cob* and *cox1* Genes and Editing of the Corresponding mRNAs in *Dinophysis acuminata* from Narragansett Bay, with Special Reference to the Phylogenetic Position of the Genus *Dinophysis*[▽]

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***Dinophysis acuminata* cells were isolated from Narragansett Bay water samples in June 2005 using flow cytometry. Dinoflagellate-specific PCR primers were used to isolate small-subunit rRNA (18S rRNA), mitochondrial cytochrome *b* (*cob*), and cytochrome *c* oxidase I (*cox1*) genes and the encoded cDNAs. Maximum-likelihood analysis of a concatenated data set of ribosomal DNA and cDNA sequences of *cob* and *cox1* showed that *D. acuminata* was sister to Gonyaulacoids, but without strong bootstrap support. The approximately unbiased test could not reject alternative positions of *D. acuminata*. To gain better resolution, mRNA editing of *cob* and *cox1* was inferred for *D. acuminata* and 13 other dinoflagellate species. The location and type of editing as well as the distribution pattern in *D. acuminata* were generally similar to those in other dinoflagellates except for two edited sites that are unique to this species. Bayesian analyses of a matrix that recorded the location and type of editing, and of a matrix that included the protein sequences of COB and COX1 with the editing data yielded tree topologies similar to the three-gene tree but again failed to resolve the phylogenetic position of *D. acuminata*. However, the density of edited sites in the *D. acuminata* mitochondrial genes, consistent with phylogenetic trees, indicated that *Dinophysis* is a derived dinoflagellate lineage, diverging after other lineages such as *Oxyrrhis*, *Amphidinium*, and *Symbiodinium*. We demonstrate that dinoflagellate-specific PCR coupled with flow cytometry can be a useful tool to analyze genes and their transcripts from a natural dinoflagellate population.**

Many members of the dinoflagellate genus *Dinophysis* produce potent polyether toxins that can accumulate in filter-feeding bivalves, leading to a syndrome known as diarrhetic shellfish poisoning in humans consuming tainted shellfish. These harmful algal bloom species are important not only for their potential impact on public health but also from an ecological point of view because of their dual role as primary and secondary producers in complex microbial food webs. Unlike the majority of photosynthetic dinoflagellates, many *Dinophysis* species harbor plastids of cryptophyte origin (13), and mounting evidence points to a temporary acquisition of plastids (i.e., kleptoplastidy) in this mixotrophic life style (16, 25, 28). It is of interest to understand the evolutionary history of toxin production, its possible association with plastid acquisition and function (18, 47), and the multiple trophic roles and the phylogenetic affiliation of *Dinophysis*. Given the limited knowledge of this genus and the fact that until recently most *Dinophysis* species were unculturable under laboratory conditions (28), molecular analyses, particularly those that can be applied to sorted or unsorted natural populations, provide an important window into the evolution of this group. Thus far, only limited work has been done in this regard with *Dinophysis*. Existing work has generally focused on the ribosomal DNA (rDNA) locus (>200 entries in GenBank) because of its high copy number in the cell that allows ready isolation of the gene from a small number of cells or even from a single cell (24). Few protein-encoding genes (e.g., the photosystem II D1 protein [*psbA*] and Rubisco large-subunit genes) have been studied.

Given the challenge of obtaining pure *Dinophysis* samples from natural microbial assemblages for DNA and RNA extraction, isolation of these cells can be made more efficient with the use of flow cytometry (6). Furthermore, dinoflagellate-specific primers can significantly reduce the amplification of gene homologs in other organisms still present in the sorted cell samples. In this study, we combined these two methods to maximize the chance of obtaining gene sequences from the target species. We chose to analyze three genes: the small-subunit (SSU) rRNA (18S rDNA), mitochondrial cytochrome *b* (*cob*), and cytochrome *c* oxidase I (*cox1*). The 18S rDNA gene was included because a large data set is available for this conserved gene. Mitochondrial genes were selected because of their higher mutation rates and a more clock-like behavior relative to 18S rDNA (see references 10 and 45 and references therein). In addition, mRNA editing characteristics of mitochondrial genes can potentially act as species-specific molecular markers (20). Thus, taking advantage of dinoflagellate-specific gene primers developed in recent years (19, 42), we sequenced these three genes and cDNAs of *cob* and *cox1* from *Dinophysis acuminata* collected in 2005 from a field population from Narragansett Bay, RI. We then characterized mRNA editing of *cob* and *cox1* for this and other dinoflagellate species. Our main goal was to develop an approach that would

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TABLE 1. Dinoflagellate species included in the phylogeny and mitochondrial *cob* and *cox1* editing analyses

Taxon	Abbreviation used in figures	Strain and/or source[a]	Trophic mode[e]	Accession no.[i]				
				SSU rDNA	*cob* cDNA	*cob* gDNA	*cox1* cDNA	*cox1* gDNA
Adenoides eludens		CCMP1891	P	AF274249	EF036541		EF036565	
Akashiwo sanguinea	Asa	LIS1[b]	P	AY456106	EF036542	AY456105	EF036566	EU126138[g]
Alexandrium affine		CCMP112	P	AY831409	EF036543		EF377324	
Alexandrium tamarense	Ata	CB307; D. M. Anderson	P	AF022191[f]	DQ082987	AY456116	EF036567	EU126139[g]
Amphidinium carterae	Aca	CCMP1314	P	AF274251	EF036544	EU126130	EF036568	EU126140
Amphidinium operculatum	Aop	CCMP123	P	AY443011[f]	EF036545	EU126131	EF036569	EU126141
Ceratium longipes		CCMP1770	P	DQ388462	EF036546		EF036570	
Ceratocorys horrida		CCMP157	P	DQ388456	EF036547		EF036571	
Coolia monotis		CCMP304	P	AJ415509[f]			EF036572	
Crypthecodinium cohnii	Cco	WHd; M. Gray	H	M64245	AF403221	AF403220	AF487783	AF186994
Dinophysis acuminata	Dac	Narragansett Bay sample	P	EU130569[g]	EU130567[g]	EU130568[g]	EU130565[g]	EU130566[g]
Gambierdiscus toxicus		CCMP401	P	DQ388463	EF036550		EF036575	
Gonyaulax cochlea		CCMP1592	P	DQ388465	EF036551		EF036576	
Gymnodinium catenatum		CCMP1937	P	AF022193[f]	EF036552			
Gymnodinium simplex		CCMP419	P	DQ388466	EF036553		EF036577	
Heterocapsa triquetra	Htr	CCPM449	P	AJ415514[f]	EF036554	EU126132	EF036578	EU126142
Heterocapsa rotundata (= *Katodinium rotundatum*)	Hro	CCMP1542	P	DQ388464	EF036556	EU126133	EF036582	EU126143
Karenia brevis	Kbr	CCPM2229	P	AF274259[f]	EF036555	AY456104[h]	EF036580	EU126144[g]
Karenia mikimotoi		CCPM429	P	AF009131[f]			EF036581	
Karlodinium veneficum (formerly *K. micrum*)	Kve	CCMP1975	M	EF036540	DQ082989	AY345908	EF036579	AF463416[g]
Noctiluca scintillans	Nsc	NS3; E. J. Buskey	H	DQ388461			EF036583	EU126145
Oxyrrhis marina	Oma	CCMP1795	H	AF482425[f]	EF036557	EU126134	EF036584	EU126146
Peridinium aciculiferum		PAER1	P	AY970653	DQ094825			
Pfiesteria piscicida	Ppi	CCMP1831	H	AF330620	AF357518	AF357519	AF463413	AF463412
Pfiesteria-like	Ppi-like	CCMP1828	H	AY590476	EF036558	AY456119	EF036585	EU126147[g]
Prorocentrum cassubicum (= *Exuviaella cassubica*)	Pca	LB1596[c]	P	DQ388460	EF036548	EU126135[g]	EF036573	
Prorocentrum dentatum	Pde	CCMP1517	P	DQ336057	DQ336059	DQ336058		
Prorocentrum donghaiense	Pdo	S. Lü	P	DQ336054	DQ336056	DQ336055	EF036587	
Prorocentrum lima (= *Exuviaella lima*)		CCMP1966	P	EF377326	EF036559		EF377325	
Prorocentrum micans	Pmic	CCMP1589	P	AY585526	AY745238	AY585525	EF036588	EU126148[g]
Prorocentrum minimum strain 696	Pmin	CCMP696	P	DQ336072	AY030285	AY030286	AF463415	AF463414
Prorocentrum minimum strain EXUV	PmEXUV	CCMP1329	P	DQ336060	DQ336062	DQ336061		
Prorocentrum minimum strain JA01	Pm01	JA01; P. Glibert	P	DQ336063	DQ336065	DQ336064		
Prorocentrum minimum strain PTPM	PmPTPM	PTPM; P. Tester	P	DQ336069	DQ336071	DQ336070		
Prorocentrum nanum (= *Exuviaella pusilla*)	Pna	LB1008[c]	P	DQ388459	EF036549	EU126136	EF036574	
Protoceratium reticulatum	Pre	CCMP1721	P	AF274273[f]	EF036560	EU126137[g]	EF036589	EU126149[g]
Pseudopfiesteria shumwayae	Psh	T4; P. Tester,	H	AF218805[f]	DQ082988	AF502593	EF036586	EU130570[g]
Pyrocystis lunula		J. W. Hastings	P	AF274274[f]	EF036561		EF036590	
Pyrocystis noctiluca		CCMP732	P	AF022156	EF036562		EF036591	
Scrippsiella sp.	Scrsp	LIS[b]	P	AY743960	AY743962	AY743961	EF036592	EU130571[g]
Scrippsiella sweeneyae		CCCM280[d]	P	AF274276	EF036563		EF036593	
Symbiodinium goreaui	Sgo	CCMP2466	P	EF036539	EF036564	EU130574[g]		
Symbiodinium microadriaticum	Smi	CCMP830	P	AY456111	DQ082985	AY456110	EF036594	EU130573[g]
Symbiodinium sp.	Symsp	CCMP832	P	AY456113	DQ082986	AY456112	EF036595	EU130572[g]

[a] CCMP strains are from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME.
[b] Isolated from Long Island Sound in April 2003, morphologically similar to *Akashiwo sanguinea* based on the description in reference 37 and SSU rDNA sequence (identical to AF276818). The LIS isolate of *Scrippsiella* sp. is most closely related to *S. trochoidea* based on morphology (37) and SSU rDNA identity (1,738 bp out of 1,754 bp identical to AY421792).
[c] The Culture Collection of Algae (UTEX), The University of Texas at Austin, TX.
[d] Canadian Center for the Culture of Microorganisms, University Boulevard, Vancouver, BC, Canada.
[e] P, photoautotrophic; H, heterotrophic; M, mixotrophic.
[f] Sequence from GenBank originally obtained from other strains of the same species as used in this study.
[g] Sequences and editing analyzed in the present study.
[h] Editing analyzed in the present study.
[i] Unless indicated otherwise, the taxon was reported previously (21, 43). gDNA, genomic DNA.

TABLE 2. Distribution of number and type of editing in *cob* and *cox1* mRNAs in dinoflagellates

Edit	No. (%) in mRNA from the indicated species[a]																		
	cob						*cox1*[d]												
	Dac	Kbr	Pca	Pna	Ppi-like	Pre	Dac	Kbr	Kve	Pmic	Pmin	Ppi-like	Pre	Psh	Scrsp	Smi	Sysp
Total[b]	31 (3.35)	40 (4.30)	32 (3.45)	33 (3.54)	32 (3.52)	30 (3.22)	30 (2.27)	30 (2.27)	39 (2.95)	32 (2.42)	34 (2.58)	27 (2.05)	27 (2.05)	25 (1.89)	28 (2.12)	26 (1.97)	28 (2.12)
A→G	7 (22.6)	11 (27.5)	12 (37.5)	13 (39.4)	11 (34.4)	11 (36.7)	15 (50.0)	16 (53.3)	20 (51.3)	15 (46.9)	18 (52.9)	13 (48.1)	12 (44.4)	12 (48.0)	14 (50.0)	18 (69.2)	15 (53.9)
G→A	1 (3.2)	1 (2.5)	0 (0)	2 (6.1)	1 (3.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4.0)	0 (0)	0 (0)	0 (0)
C→U	11 (35.5)	11 (27.5)	10 (31.3)	9 (27.3)	8 (25.0)	8 (26.7)	5 (16.7)	3 (10.0)	5 (12.8)	3 (9.4)	2 (5.9)	3 (11.1)	1 (3.7)	2 (8.0)	4 (14.3)	2 (7.7)	3 (10.7)
U→C	9 (29.0)	14 (35.0)	8 (25.0)	7 (21.2)	8 (25.0)	8 (26.7)	5 (16.7)	6 (20.0)	9 (23.1)	8 (25.0)	8 (23.5)	6 (22.2)	7 (25.9)	6 (24.0)	6 (21.4)	3 (11.5)	9 (28.6)
U→G	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
G→U	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
U→A[c]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A→C	—	—	—	—	—	—	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.8)	1 (3.6)
G→C	3 (9.7)	3 (7.5)	2 (6.3)	2 (6.1)	4 (12.5)	3 (10.0)	5 (16.7)	5 (16.7)	5 (12.8)	6 (18.8)	6 (17.6)	5 (18.5)	7 (25.9)	4 (16.0)	4 (14.3)	2 (7.7)	1 (3.6)
A, U→G, C	16 (51.6)	25 (62.5)		20 (60.6)	19 (59.4)	19 (63.3)	20 (66.7)	22 (73.3)	29 (74.4)	23 (71.9)	26 (76.5)	19 (70.4)	19 (70.4)	18 (72.0)	20 (71.4)	21 (80.8)	24 (82.8)

[a] Types of editing that are not detected in any species for an mRNA are denoted by dashes. Abbreviations of species names are as listed in Table 1.
[b] Editing density.
[c] One edit of this type has been documented in Ppi (21), but it is outside the region of sequence considered in this study (i.e., nt 88 to 1012 of *Pfiesteria piscicida cob* mRNA, GenBank accession no. AF357518 [for *Amphidinium operculatum*, the corresponding region is nt 126 to 1012]).
[d] The region of sequence considered in this study is equivalent to *Cryptheocodinium cohnii cox1* genomic clone coding region nt 85 to 1404, GenBank accession no. AF186994 (for *Amphidinium carterae* and *Amphidinium operculatum*, the corresponding regions are nt 215 to 1404 and nt 274 to 1404, respectively).

lead to an efficient genetic analysis of environmental samples for ecological and phylogenetic purposes and allow for the study of gene expression in a natural dinoflagellate population.

MATERIALS AND METHODS

Sample collection. A population of *D. acuminata* was collected between the surface and a depth of 0.5 m from Greenwich Cove, RI (41°39′30″N, 71°27′00″W) with a 10-μm mesh plankton net on 20 June 2005, 5 hours after sunrise. The water temperature at the 0.5-m depth was 20.5°C, and the salinity was 25.7 practical salinity units (PSU). To obtain an estimate of the original population density, a whole water sample was collected using a 2-m-long tube to integrate the entire water column and later quantitatively concentrated by reverse filtration and counted with a Sedgwick-Rafter chamber. The plankton sample was further enriched in *D. acuminata* by collecting the reddish "clouds" of swimming cells that formed a swarm near the surface of the sample. *D. acuminata* was identified as described by Steidinger and Tangen (37). Individual cells of this species were isolated the next day from the live enriched plankton sample using a BD FACSVantage flow cytometer cell sorter at the Northeast Fisheries Science Center Milford Laboratory, Connecticut, with adjustment for fluorescence, size, and scatter characteristics of the *D. acuminata* population. About 1,200 cells were sorted and preserved in 2% Lugol's solution for later DNA extraction (42), whereas 1,100 cells were sorted and kept alive for RNA extraction. Two additional sortings of ca. 1,000 cells each were inoculated in f/2-Si medium (11) to monitor potential growth of contaminant cells for 2 months. Within 2 hours, isolated cells were brought to the University of Connecticut Avery Point campus for molecular analyses. Cells for RNA extraction were harvested by centrifugation at 3,000 × *g* at 4°C for 20 min, resuspended in Trizol, and kept at −80°C until RNA extraction (21).

Dinoflagellate cultures and sample collection. The dinoflagellate taxa used in this study are listed in Table 1. All photosynthetic species were grown in f/2-Si medium except *D. acuminata* collected from Narragansett Bay and the heterotrophic species grown with an algal prey (*Rhodomonas* sp. strain CCMP768) (46). Salinity was adjusted to 28 PSU for most species and to 15 PSU for *Rhodomonas*, *Karlodinium veneficum*, and heterotrophic taxa (46). Cultures were maintained at 20 ± 1°C under a 12 h:2 h light-dark cycle with a photon flux density of ca. 100 μmol photons · m^{-2} · s^{-1}. The growth rate was monitored by microscopic cell counts using a Sedgwick-Rafter chamber.

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 could be detected by microscopic examination. The cells were harvested and kept in Trizol as described above for field-collected samples.

Cloning and sequencing of 18S rDNA, *cob*, and *cox1*. DNA and total RNA were extracted and cDNA synthesized as previously described (21). The resulting cDNAs and genomic DNA were subjected to PCR amplification with dinoflagellate specific primer sets 18ScomF1 (5′-GCTTGTCTCAAAGATTAAGCCATGC-3′)-Dino18SR1 (5′-GAGCCAGATRCDCACCCA-3′) and Dino18SF1 (5′-AAGGGTTGTGTTYATTAGNTACARAAC-3′)-18ScomR1 (5′-CACCTACGGAAACCTTGTTACGAC-3′) for 18S rDNA (17), Dinocob1F (5′-ATGAAATCTCATTTACAWWCATATCCTTGTCC-3′)-Dinocob1R (5′-TCTCTTGAGGKAATTGWKMACCTATCCA-3′) for *cob*, and Dinocox1F (5′-AAAAATTGTAATCATAAACGCTTAGG-3′)-Dinocox1R (5′-TGTTGAGCCACCTATAGTAAACATTA-3′) for *cox1* (21, 44). PCRs comprised 35 cycles of 25 s at 94°C, 30 s at 52°C, and 40 s at 72°C, followed by 10 min at 72°C. PCR products were directly sequenced as reported by Zhang and Lin (43).

Inference of RNA editing and analysis of editing characteristics. Genomic DNA and corresponding cDNA sequences of *cob* and *cox1* were aligned using CLUSTAL W (1.8) (http://clustalw.ddbj.nig.ac.jp/top-e.html). Differences between the genomic and cDNA sequences were identified as an indication of mRNA editing. Editing density, or the percent nucleotides of the gene sequence that are edited, was determined for each species. Editing densities were compared between groups of taxa that were clustered on phylogenetic trees. Furthermore, types of nucleotide and amino acid changes as a result of editing were analyzed. The percent edited sites that were mapped to the same location (location of editing [LOE]) between two species and that underwent the same type of nucleotide substitution (type of editing [TOE]) between the two species were also calculated. In addition, the frequency of editing at each position of the codon was analyzed.

Phylogenetic analysis. The DNA sequences encoding COB and COX1 were aligned using REVTRANS (http://www.cbs.dtu.dk/services/RevTrans/) with the default values. The *cob* and *cox1* cDNA sequences were combined to produce a

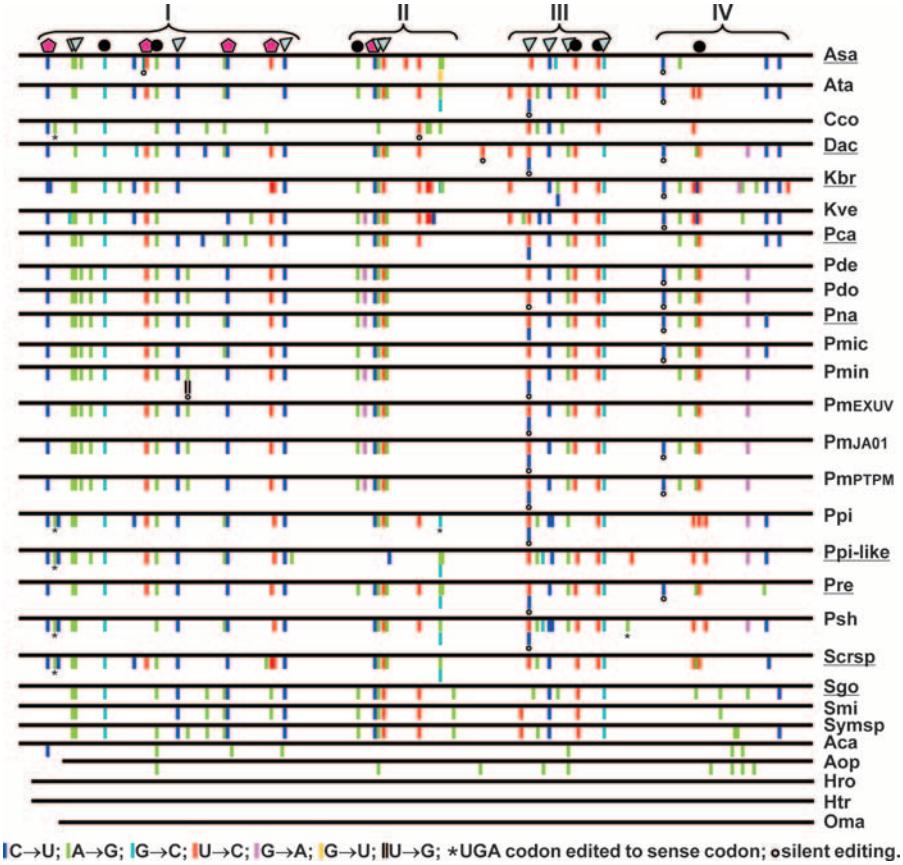

FIG. 1. Schematic diagram of the distribution of editing events in mitochondrial cytochrome *b* (*cob*) in dinoflagellates. The sites at which editing increases similarity between dinoflagellate and nondinoflagellate species are designated by either a closed circle (editing to an amino acid identical to the consensus) or a pentagon (editing to an amino acid chemically similar to the consensus). A triangle denotes dinoflagellate-specific editing. Brackets with Roman numerals indicate discrete clusters of editing events. Species abbreviations are as listed in Table 1, and those underlined are taxa whose gene sequences were analyzed in this study.

two-gene data set (1,963 nucleotides [nt]) and then with SSU rDNA (1,714 nt) to generate a *cob-cox1*-SSU rDNA three-gene (3,677-nt) data set. The apicomplexans *Plasmodium yoelii* and *Plasmodium berghei* were used to root the COB and COX1 protein tree, whereas the early diverging dinoflagellate *Oxyrrhis marina* was used to root the three-gene DNA tree (46). In the previous study (45), the congruence of COB, COX1, and rDNA data from dinoflagellates was tested (with the *D. acuminata* data not included) using the partition homogeneity test (ILD test in PAUP*; 1,000 replicates). An absence of significant incongruence between the COB and COX1 protein alignments ($P = 0.136$) and significant incongruence between the DNA data from these two partitions ($P = 0.003$) were noted. However, the single protein and DNA trees did not differ substantially from each other in these analyses, suggesting that gene concatenation would not mask a clear topological conflict (for details, see Fig. 1 and 3 in reference 45), but rather increased overall bootstrap support in the phylogenies. In this study we repeated the analysis for each gene pair in the three-gene alignment. These ILD test results again showed that $P = 0.001$, 0.001, and 0.003 for the rDNA-*cob*, rDNA-*cox1*, and *cob-cox1* data, respectively (see also reference 45). Although these were significant *P* values, we chose to combine these data because of our previous work (45) and because there was considerable debate about the usefulness of the ILD test (4, 14). We also did a preliminary maximum-parsimony (MP) analysis with each data partition to determine whether the position of *D. acuminata* was resolved in any of the single-gene trees with an MP bootstrap support value of ≥70%. This analysis revealed that this taxon emerged as an independent lineage in all three gene trees, leading us to concatenate the data in the hope of achieving greater phylogenetic resolution.

For the three-gene DNA data set, the "best" tree was inferred using PAUP* and the site-specific GTR model (ssGTR) (31) with different evolutionary rates for each amino acid codon position and for the rDNA data. Bootstrap analyses were done using PHYML (200 replicates) with the GTR + I + Γ model over all nucleotide positions. Bayesian posterior probabilities for the ssGTR tree were calculated using MrBayes and the ssGTR +I + Γ model over the four data partitions. These analyses were run as described by Zhang et al. (45). Unweighted MP bootstrap analyses were also performed with the three-gene data set (2,000 replications) using heuristic searches and TBR branch-swapping to find the shortest trees (38). 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 COB-COX1 data set, ProtTest V1.3 (1) was used to identify the best-fit model with "Fast" optimization and a BIONJ tree. The ProtTest parameter values were then used in maximum-likelihood analyses with the RAxML (VI-HPC, v2.2.1) computer program using the hill-climbing algorithm (36). The results of a PHYML V2.4.3 (12) bootstrap analysis (200 replicates) with tree optimization were used to assess the robustness of monophyletic clades in the RAxML tree. The protein data set was also analyzed using Bayesian inference (MrBayes V3.0b4) (15). The ProtTest best-fit evolutionary model was used in this analysis with Metropolis-coupled Markov chain Monte Carlo from a random starting tree. Four chains were run simultaneously, of which three were heated and one was cold, and the nrun = 2 command was used to monitor tree standard deviations. To increase the probability of chain convergence, trees were sampled after the standard deviations of the two runs were <0.05 to calculate the posterior probabilities (i.e., after 56,200 generations). The remaining phylogenies were discarded as burn-in. For the COB-COX1 data set, an unweighted MP bootstrap analysis was also performed as described above.

Testing the tree topology. To assess the position of *D. acuminata* in the three-gene DNA tree, we generated a backbone phylogeny that was identical to the "best" ssGTR topology but excluded this species. Using this backbone tree, *D. acuminata* was then added individually using MacClade V4.05 to each branch in the tree to generate a set of topologies that addressed all possible positions for this taxon (23). The site-by-site likelihoods for the trees in this analysis were

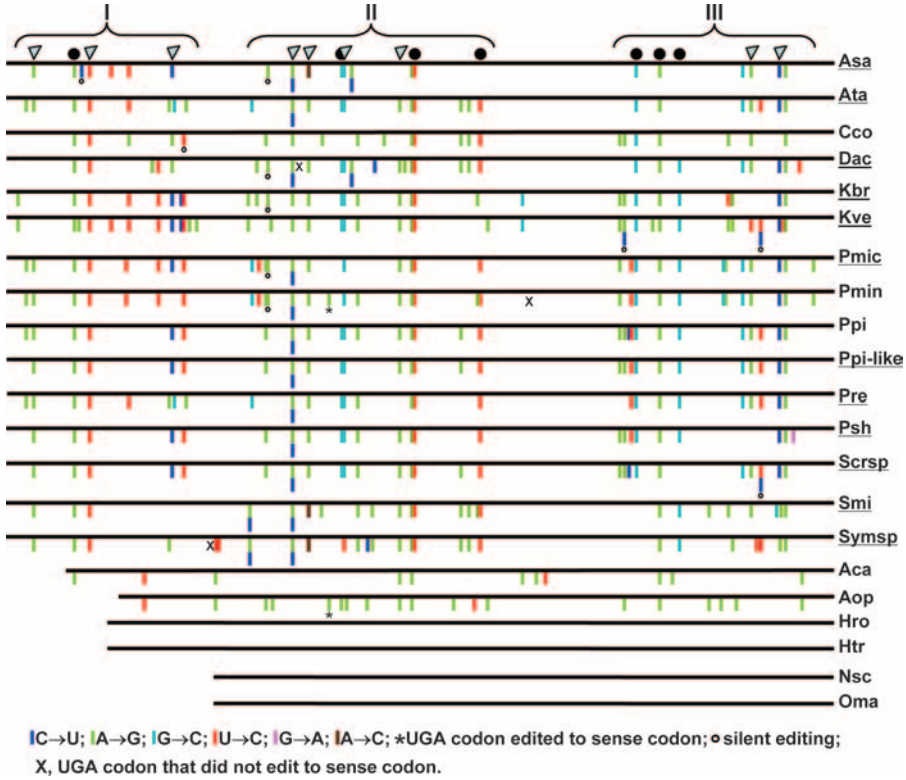

FIG. 2. Schematic diagram of distribution of editing events in mitochondrial cytochrome *c* oxidase subunit I (*cox1*) in dinoflagellates. Symbols are as in Fig. 1. Brackets with Roman numerals indicate discrete clusters of editing events. Species abbreviations are as listed in Table 1, and those underlined are taxa whose gene sequences were analyzed in this study.

calculated using the three-gene data set and TREEPUZZLE (V5.2) (33) with the GTR + I + Γ evolutionary model (the alpha value for the gamma distribution was identified using RAxML) and the default settings. The approximately unbiased (AU) test was implemented using CONSEL V0.1i (35) to identify the pool of probable trees in this test and to assign their probabilities. The phylogenetic relationship of LOE and TOE was analyzed for 17 dinoflagellates. Each edited site was designated as a character with presence (1) or absence (0) of editing in each species as its state. For the TOE-based MP analysis, each of the six TOEs that was observed was treated as a character, each of which was assigned a state value of 1 or 0 for presence or absence of that editing type in each species. The six characters for each species were recorded and concatenated into a single alignment for unweighted MP bootstrap analysis as described above. In addition, Bayesian analysis was conducted for the LOE and TOE concatenated data set in an alignment that also included the COB and COX1 protein sequences. We used an "unlinked" analysis in which independent model parameter estimates were calculated for each data partition, i.e., independent gamma parameter estimate for the editing data and independent invariants-gamma model parameter estimates for the protein data (i.e., cpREV + I + Γ as chosen by ProtTest V 1.3 [1]). The Bayesian analysis was done as described above, and the trees were sampled after the standard deviations of the two runs were <0.01 to calculate the posterior probabilities (i.e., after 59,000 generations).

RESULTS

***Dinophysis* cells isolated by flow cytometry.** On the day of collection, all *D. acuminata* cells observed using epifluorescence microscopy displayed a typical phycoerythrin pigmentation localized to digitated chloroplasts. The original population density was 52,500 cells liter^{-1}; we noticed a few cells (fewer than 0.5%) parasitized by the perkinsozoan *Parvilucifera infectans*. On 21 June, once the settings on the flow cytometer were deemed satisfactory, all samples were sorted into tubes and inspected for cell viability and purity by microscopy (phase contrast; magnification, ×200) within 2 hours. After 2 months, microscopic examination of the sorted additional samples showed that some *D. acuminata* cells were still alive; no contaminant phytoplankters were detected.

18S rDNA, *cob*, and *cox1* sequences. The use of two primer sets of the 18S rDNA yielded a longer gene fragment than possible with a single primer set, resulting in a 1,741-bp fragment. Genomic sequences of *cob* (926 bp) and *cox1* (1,338 bp) and corresponding cDNA sequences (*cob*, 926 bp; *cox1*, 1,338 bp) were also obtained. The 18S rDNA was identical to that reported for *D. acuminata* (accession no. AJ506972) and the *cox1* sequence obtained here was similar to those of dinoflagellates reported previously (21, 43). These sequences were colinear with homologs in other dinoflagellates, with no indels detected. When translated to amino acid sequences, the critical histidines conserved in COB (4 His; ligands for the heme *b* group in apocytochrome *b*) and COX1 (6 His; ligands for heme *a*, Cu_B, and heme a_3) of other organisms were identified in the *D. acuminata* proteins.

mRNA editing characteristics. Comparison of the colinear genomic and corresponding cDNA sequences of *D. acuminata* revealed edited sites for *cob* and *cox1*. Five TOEs were detected for *cob*; A→G, U→C, and C→U were predominant, followed by G→C and G→A (Table 2). For *cox1*, six TOEs were observed, with additional A→C edits in comparison to *cob*. Similar to the case for *cob*, *cox1* editing was dominated by A→G and U→C, followed by G→C and C→U. There were 31

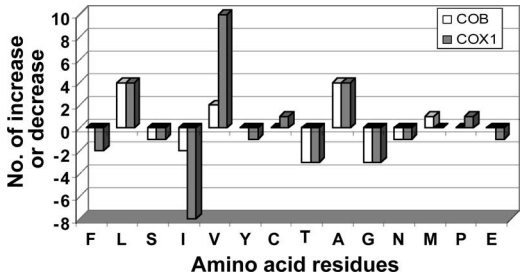

FIG. 3. Editing-mediated increase (+) or decrease (−) in the content of the 20 amino acid substituents of COB and COX1. Amino acids are denoted by their standard one-letter abbreviations.

editing events in *cob* and 30 in *cox1*, accounting for 3.35% and 2.24%, respectively, of the sequence length covered in the study. These edited sites were distributed in four (Fig. 1) and three (Fig. 2) discrete clusters for *cob* and *cox1*, respectively, as found in other dinoflagellates. Unique to *D. acuminata* was a silent U→C change that occurred in the gap between clusters II and III (Fig. 1). In *cox1*, there were two unique editing sites (site 3 and 17, both A→G) in *D. acuminata*; however, they resided in clusters I and II, respectively. Similar to the case for other dinoflagellates documented previously (21, 43), editing in both *cob* and *cox1* of *D. acuminata* occurred predominantly in the first and second positions of the affected codons, resulting in changes in the encoded amino acid residues. These changes did not result in an increase of similarity in amino acid sequences between *Dinophysis* and other lineages (not shown). Rather, editing caused some changes in the proportions of several amino acid residues (Fig. 3). For COB in *D. acuminata*, leucine and alanine increased and threonine and glycine decreased most markedly. For COX1, the greatest increase occurred in valine, leucine, and alanine whereas, isoleucine, threonine, and glycine decreased. These changes led to only a small increase in the average hydrophobicity for both COB and COX1 (from 8.4 to 9.1 and from 9.0 to 9.3, respectively, as estimated using PepTool [BioTools, Inc., Alberta, Canada]).

18S rDNA-*cob-cox1* three-gene phylogeny. Maximum-likelihood analysis of the three-gene concatenated DNA alignment yielded the tree topology shown in Fig. 4A. *Dinophysis* appeared to be an independent lineage that is sister (<60% bootstrap support) to the cluster consisting of Gonyaulacales lineages. Assessment of alternate positions for *D. acuminata* in the three-gene tree using the AU test did not turn up strong evidence in favor of the "best" position shown in Fig. 4A ($P = 0.783$) over several other divergence points. For example, the position of *D. acuminata* on the branch uniting *Adenoides eludens* and the Prorocentrales was also supported ($P = 0.325$). Many other alternate positions were also not rejected by the

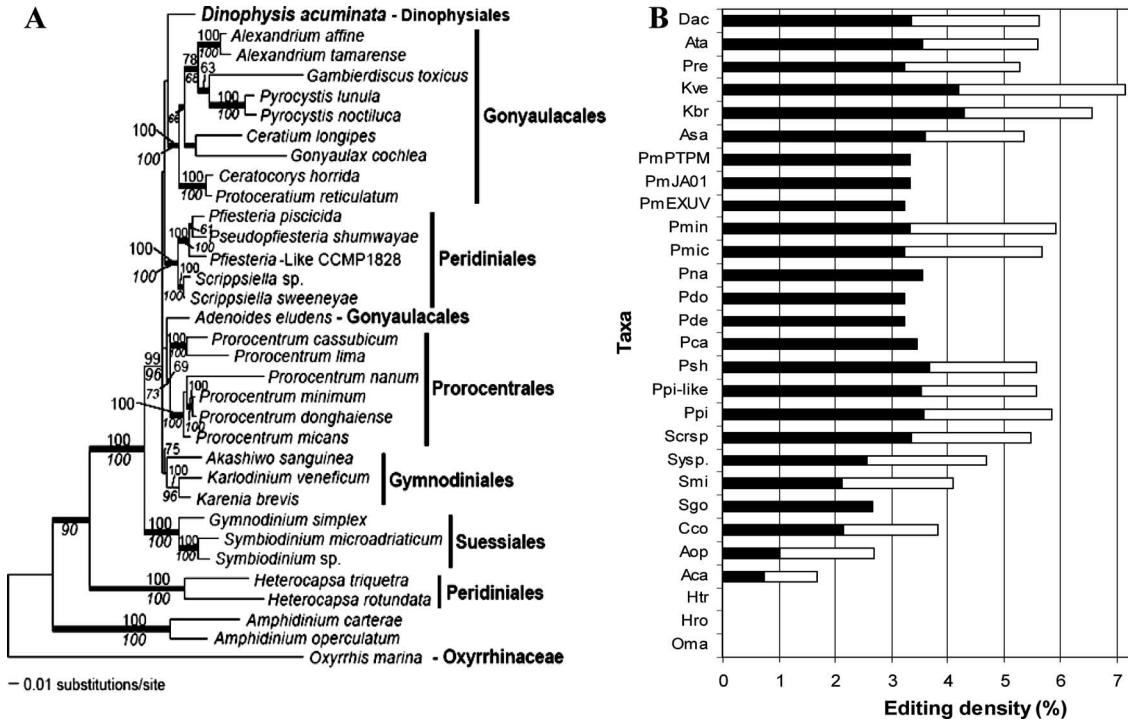

FIG. 4. Maximum-likelihood tree inferred from 18S rDNA-*cob-cox1* three-gene concatenated data set (A) and mapping of editing density (B). (A) The three-gene maximum-likelihood tree was inferred using PAUP with an ssGTR model (four categories of nucleotide substitution rates). Bootstrap support was assessed using PHYML with a GTR + I + Γ model (numbers above branches) and MP analysis (numbers below branches). Posterior probabilities from Bayesian analysis when ≥0.95 are indicated as thick branches. The tree is rooted with *Oxyrrhis*, the lineage that is consistently placed at the base of all dinoflagellates when trees are rooted with the apicomplexan *Plasmodium*. (B) Editing density is plotted against taxa (abbreviations of species names are as in Table 1), with filled bars of *cob* stacked by open bars of *cox1*. Note that in the phylogenetic tree, there is a well-supported early-to-later branching order from *Oxyrrhis*, to *Amphidinium*, to *Heterocapsa*, to *Symbiodinium*, to the clade of *Akashiwo*/*Karlodinium*/*Karenia*. Accordingly, editing density increases in this order. For the remaining lineages, phylogenetic resolution is less clear, and editing densities among those lineages are similar.

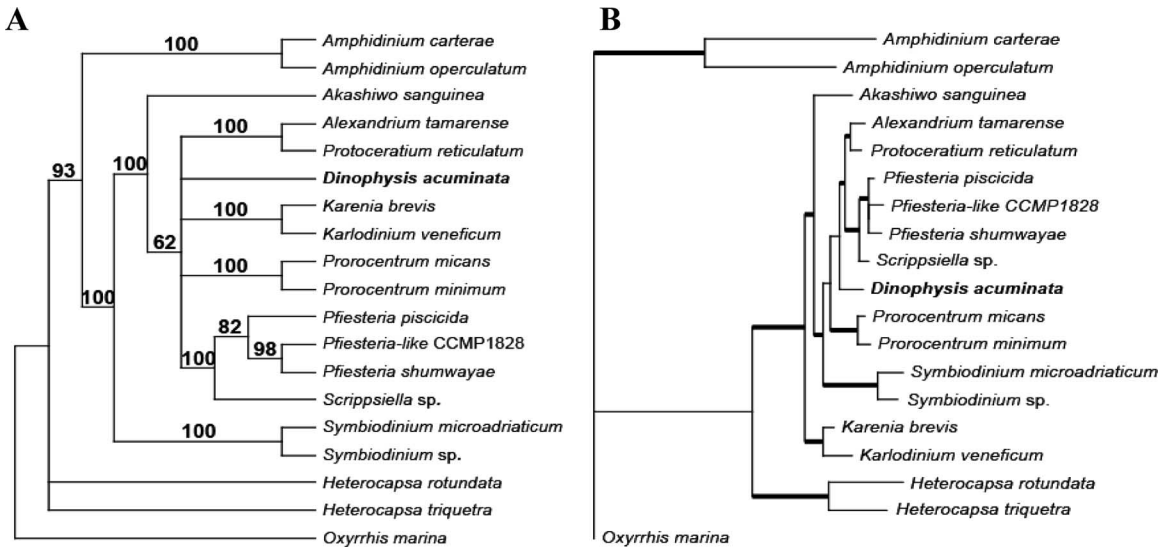

FIG. 5. (A) Bayesian consensus tree that was inferred from the concatenated editing data. The posterior probabilities that were calculated after the standard deviation between the two runs was <0.05 (i.e., 11,000 generations) are shown at the branches. (B) Bayesian consensus tree (burn-in = 9,000 generations) inferred using the unlinked models for editing (+ Γ) and for each protein partition (cpREV + I + Γ). The average standard deviation between two runs was <0.01. Posterior probabilities of ≥0.95 are indicated by thick lines.

AU test. These results left in question the phylogenetic placement of *D. acuminata*. Similar results were found with maximum-likelihood and MP analysis of the COB-COX1 protein data (data not shown).

Phylogenetic trend of editing density. The mRNA editing of *cob* and *cox1* was analyzed for 13 non-*Dinophysis* dinoflagellates (Table 1). Editing density, calculated for each species as the percentage of nucleotides in the gene that are edited, was mapped to the three-gene phylogenetic tree (Fig. 4B). This procedure revealed that editing did not occur in basal lineages (e.g., *Oxyrrhis*), and its density increased progressively in later-diverging lineages, reaching an equilibrium density of ca. 3% in the derived dinoflagellates in our study. Therefore, based on the editing density of *Dinophysis* and the general phylogenetic trend of increasing editing density over evolutionary time, *Dinophysis* most likely does not occupy a basal position among dinoflagellates and is more closely related to a derived lineage (i.e., as in Fig. 4A). In addition, if these editing data reflect true phylogenetic signals, then they clearly support the early divergence of *Amphidinium* spp. and *Heterocapsa* spp. (46), two long-branched taxa that have little or no editing of mitochondrial *cob* and *cox1*.

Editing-based phylogeny. The Bayesian consensus tree based on the concatenated data (i.e., six categories) of editing information is generally congruent with the three-gene DNA tree although providing far less resolution of branching order among derived dinoflagellate clades due to the paucity of informative editing characteristics for these taxa (Fig. 5A versus Fig. 4A), However, again we found no significant Bayesian support for a specific affiliation of *D. acuminata* (Fig. 5A). These data showed that *D. acuminata* diverged somewhere in the region occupied by gonyaulacoid, gymnodinioid, prorocentroid, and peridinioid lineages that formed a multifurcation (Fig. 5A). Similarly, the Bayesian consensus tree (burn-in = 9,000 generations) inferred using the unlinked models for editing (+ Γ) and for each protein partition (cpREV + I + Γ) revealed the affiliation of *D. acuminata* in the neighborhood of the prorocentroid, gonyaulacoid, and peridinioid clusters (Fig. 5B).

DISCUSSION

This is the first report of *cob*/*cox1* genomic and cDNA sequences from *Dinophysis* or any other dinoflagellate natural population. The effort was made possible with the use of flow cytometric analysis and dinoflagellate-specific PCR approaches. This approach can be used for other microbial organisms that are currently not amenable to single-species culture, such as heterotrophic and mixotrophic species and many unclassified picoplanktonic dinoflagellates that have recently been discovered (19, 22, 26, 32).

Amplifying multiple genes (nuclear and mitochondrial) and deciphering characteristics of editing density proved useful in characterizing dinoflagellates, albeit still not allowing us to elucidate the phylogenetic relationships of *D. acuminata*. Low taxon sampling within this genus may explain this result. It has already been demonstrated that a combination of *cob* and nuclear genes provides robust phylogenetic trees for alveolates and other organisms (30, 34) and that *cob* and *cox1* can be used for inferring dinoflagellate phylogeny (44, 45). Our study indicates that the characteristics of editing density bolster the results of the multigene phylogeny approach even for a natural population.

In our analysis of the editing of *cob* and *cox1*, the putative basal lineages exhibited low (*Amphidinium*) or no (*Oxyrrhis* and *Heterocapsa*) editing, whereas more-derived lineages (including *Dinophysis*) consistently had higher levels of editing. This result extends the previous observation of *cob* mRNA editing density increasing from basal to derived lineages of dinoflagellates (43, 46). The editing alone and editing-plus-

protein phylogenetic trees are generally similar to the gene sequence tree, in that *Oxyrrhis*, *Heterocapsa*, *Noctiluca*, and *Amphidinium* occupied basal positions in the tree. The clustering of *Pfiesteria* and related taxa with *Scrippsiella* is in full agreement with our current understanding that *Pfiesteria* and related lineages are peridinioid, and the affiliation of *Karenia* with *Karlodinium* in all of the trees is also consistent with our current understanding of their phylogenetic positions (see, e.g., references 9, 41, and 45). These data allowed us to draw two important conclusions. First, *D. acuminata* is not related to any of the basal lineages identified in this study based on the phylogenetic analyses and the frequency and distribution of edited sites in the *cob* and *cox1* genes. In support of this conclusion are the intermediate level of editing density and the fact that *D. acuminata* was never placed near the root in any of the phylogenetic trees inferred using various combinations of data.

Second, *D. acuminata* is no more closely related to Prorocentrales than to Gonyaulacales or Gymnodiniales. This is despite the fact that Dinophysiales and Prorocentrales share major synapomorphies. Both lineages have a theca divided into lateral halves and have two apical pores, one large and the other small, with both flagella arising from the larger pore (39), and many *Dinophysis* and some *Prorocentrum* species (e.g., *P. lima*) produce polyether-type toxins (27). The apparently close relationship between the two dinoflagellate orders based on these features is not strongly supported by phylogenies based on multiple genes and editing characteristics. This weak correspondence stresses the need to include as many analytic approaches as possible and to use caution when inferring relationships between groups of organisms. Indeed, increasing evidence shows that algal lineages that produce the same type of metabolites can be phylogenetically distantly related. For instance, some cyanobacteria (e.g., *Anabaena circinalis*) and *Alexandrium* species produce saxitoxin (3, 29). *Amphidinium* and *Karlodinium* produce linear polyketide toxins (2, 8, 17). Our study provides another piece of evidence that similar toxin-producing attributes do not necessarily indicate a close phylogenetic affiliation, in our case between *Prorocentrum* and *Dinophysis*. The AU test rejects the positioning of *D. acuminata* at the base of the Prorocentrales ($P = 0.007$) or as sister to any of its members ($P < 0.01$).

In summary, our study presents a novel approach that could prove successful in characterizing environmental samples, especially when harmful algal bloom species are considered. Currently, our approach is time-consuming and requires highly skilled workers, and it thus can be used only for research purposes. Nevertheless, it can contribute to the development of molecular markers specific to certain target organisms, especially when genes involved in toxin production are incorporated in the approach.

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