The Application of a Molecular Clock Based on Molecular Sequences and the Fossil Record to Explain Biogeographic Distributions Within the *Alexandrium tamarense* "Species Complex" (Dinophyceae)

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The cosmopolitan dinoflagellate genus *Alexandrium*, and especially the *A. tamarense* species complex, contain both toxic and nontoxic strains. An understanding of their evolution and paleogeography is a necessary precursor to unraveling the development and spread of toxic forms. The inclusion of more strains into the existing phylogenetic trees of the *Alexandrium tamarense* species complex from large subunit rDNA sequences has confirmed that geographic distribution is consistent with the molecular clades but not with the three morphologically defined species that constitute the complex. In addition, a new clade has been discovered, representing Mediterranean nontoxic strains. The dinoflagellates fossil record was used to calibrate a molecular clock: key dates used in this calibration are the origins of the Peridiniales (estimated at 190 MYA), Gonyaulacaceae (180 MYA), and Ceratiaceae (145 MYA). Based on the data set analyzed, the origin of the genus *Alexandrium* was estimated to be around late Cretaceous (77 MYA), with its earliest possible origination in the mid Cretaceous (119 MYA). The *A. tamarense* species complex potentially diverged around the early Neogene (23 MYA), with a possible first appearance in the late Paleogene (45 MYA). A paleobiogeographic scenario for *Alexandrium* is based on (1) the calculated possible ages of origination for the genus and its constituent groups; (2) paleogeographic events determined by plate movements, changing ocean configurations and currents, as well as climatic fluctuations; and (3) the present geographic distribution of the various clades of the *Alexandrium tamarense* species complex.

Introduction

Alexandrium is a much-studied goniodomacean dinoflagellate genus that currently contains 29 species, nine of which are known to produce paralytic shellfish poisoning (PSP) toxins (Balech 1995). Harmful algal blooms (HABs) involving these organisms are responsible for a wide variety of environmental and public health problems (Smayda et al. 1990; Hallegraeff 1993) and have a world-wide occurrence. Moreover, for reasons yet to be explained fully, such blooms appear to be increasing in frequency, intensity, and distribution (Hallegraeff 1993, 1995).

The genus *Alexandrium* is subdivided primarily on the basis of differences of shape of particular plates; the presence or absence of a ventral pore; ornamentation in a few species, plus cell size, shape, and chain formation (Balech 1995). Within the genus *Alexandrium*, *A. tamarense*, *A. fundyense*, and *A. catenella* comprise a closely related cosmopolitan toxigenic grouping of morphology-based species (''morphospecies'')—the ''*Alexandrium tamarense*'' species complex—that play a prominent role in HABs. Individual morphospecies are identified by differences in cell shape and in the geometry of the apical pore complex (APC), by the presence (in *A. tamarense*) or absence (in *A. catenella*/*A. fundyense*) of a ventral pore on the apical plate (1′), and by the tendency to form chains (in *A. catenella*) or not (in *A. tamarense*/*A. fundyense*). Although the tabulational differences are sometimes very slight, they remain consistent in cultures, aberrant individuals being very rare (Taylor 1975, 1987*a*).

Key words: *Alexandrium tamarense*/*Alexandrium catenella*/*Alexandrium fundyense* species complex, biogeography, dinocysts, dinoflagellates, evolution, harmful algal blooms, molecular clock, phylogeny, toxic algae.

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Phylogenetic studies of the *Alexandrium tamarense* species complex, based on 18S rDNA (Scholin 1993), the D1/D2 region of 28S rDNA (Scholin et al. 1994; Medlin et al. 1998; Higman, Stone, and Lewis 2001) and ITS sequences (Adachi, Sako, and Ishida 1996*a*), have yielded results that contrast with the conventional morphological approach. These studies have identified strains within the *A. tamarense* species complex that are distributed geographically rather than by morphospecies. Indeed, several of the ribotypes contain specimens that would be assignable to each of the three morphospecies of the *A. tamarense* species complex (Scholin, Hallegraeff, and Anderson 1995). Thus, at least for molecular phylogenetic purposes, the three morphospecies are generally referred to collectively as the *A. tamarense* ''species complex.''

Within the *A. tamarense* species complex, five different ribotypes/geographic clades have been previously identified: western European (WE), North American (NA), temperate Asian (TA), Tasmanian (TASM), and tropical Asian (TROP) clades. The NA, TA, and TROP clades consist only of toxic strains, whereas the WE and TASM clades are exclusively nontoxic. A new Mediterranean nontoxic clade (ME) is reported here for the first time.

Many dinoflagellate species produce zygotic cysts as part of their sexual cycle, some of which (about 13%–16%) are fossilizable (Head 1996). This fossil record, even though incomplete, yields important information that can be used to calibrate the timing of divergences in the lineage leading to *Alexandrium*. Although biological and biogeochemical evidence suggests an origin for the dinoflagellate lineage dating back to the late Proterozoic, which ended 545 MYA, the earliest fossils confidently determined to be dinoflagellates date from about 240 MYA (Fensome et al. 1996; Fensome, Saldarriaga, and Taylor 1999). Around the same time, dinoflagellates appear to have diverged in a true radiation event (Fensome et al.

1996). *Alexandrium* belongs to the family Goniodomaceae, within the order Gonyaulacales. The order Gonyaulacales appeared in the late Triassic (about 200–210 MYA), but no confirmed members of the Goniodomaceae predate the Cretaceous—about 140 MYA (Fensome et al. 1993, 1996), and no fossils attributable to the genus *Alexandrium* have ever been recognized. However, fossil cyst–based genera, such as *Dinopterygium* and *Xiphophoridium*, reflect a tabulation very similar to that of *Alexandrium*, and first appear in the Albian age of the Cretaceous period, about 105 MYA. This date can therefore be used to provide some constraint on possible estimated dates for the divergence of *Alexandrium*-like morphotypes.

Unfortunately, only a few species that produce fossilizable cysts have been sequenced, most sequences deriving from species with no fossil record. Molecular data can be used to reconstruct the phylogenetic relationships of recent organisms, however, and those organisms with a fossil record can be used to calibrate a molecular clock that can be used to extrapolate to potential divergence times of taxa lacking a fossil record. Certain biases exist in calculating a molecular clock: they are (1) the potential inaccuracy of fossil dates, (2) the possible misalignment of sequence data, (3) the algorithm chosen for tree construction, (4) unequal rates of evolution between lineages, and (5) unequal rates of evolution within a lineage through time. Software packages are available to correct for biases 2 and 3. Lintree (Takezaki, Rzhetsky, and Nei 1995) checks the molecular clock constancy for the given data set to eliminate quickly or slowly evolving sequences. Using Lintree, the rate of evolution is linearized to average the rate of evolution through time and between lineages. Thus, although a universal molecular clock may not exist, and although base substitution rates probably vary within lineages and genes (Ayala 2000), by correcting some biases, it is possible to use molecular data to estimate organism divergence times. Nevertheless, the fossil dates will always be underestimates because they record the first appearance of a taxon and not its molecular divergence. Hence, all molecular clocks underestimate divergence times.

The main objective of this study is to use information from both molecular sequences and the fossil record to construct a molecular clock and thus model the historical biogeography of *Alexandrium* and the *A. tamarense* species complex. An integral part of this objective is the development of an evolutionary scenario for the distribution of the *Alexandrium tamarense* species complex that is consistent with paleoceanographic regimes, paleoclimates, and the present geographic distribution of the molecularly identified clades. In the course of this study, we have also analyzed several new strains of *Alexandrium* with respect to their phylogenetic relationships within the genus *Alexandrium*.

Materials and Methods

Strains and Culturing Conditions

For DNA extraction, unialgal strains of various taxa were cultured (table 1). Cultures were grown in 500-ml Erlenmeyer flasks in IMR/2 growth medium (Eppley, Holmes, and Strickland 1967), supplemented with 10 nM selenite (for *Alexandrium tamarense*, *A. catenella*, *A. fundyense*, *A. pseudogoniaulax*, *A. taylorii*, and *A. minutum*), or in K medium (Keller et al. 1987) for *A. ostenfeldii*. All cultures were maintained at 15°C in a controlled growth chamber with a 14:10 h light:dark photocycle, at a photon flux density of 150 μM m^{-2} s^{-1}, except for *A. ostenfeldii* (90 μM m^{-2} s^{-1}).

DNA Extraction, Amplification of rRNA Genes, and Sequencing

DNA extractions were made from 500 ml of culture in logarithmic growth phase with a 3% CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle and Doyle 1990). Thereafter, the DNA was treated with 10 μl RNase A (10 mg ml^{-1}) (QIAGEN, Hilden, Germany) for 30 min incubated at room temperature, followed by a 90-min incubation in a thermoshaker at 37°C with 20 μl of proteinase K (10 mg ml^{-1}), and purified using phenol: chloroform extraction with alcohol precipitation. DNA concentration was measured spectrophotometrically at 260 nm, and integrity was verified by agarose-gel electrophoresis. Polymerase chain reaction (PCR) conditions for amplifying the small subunit (SSU) rDNA gene and the D1/D2 region of the large subunit (LSU) rDNA gene follow the methodologies of Medlin et al. (1988) and Scholin et al. (1994), respectively. Three PCR products of amplified SSU genes and LSU D1/D2 regions, respectively, were pooled, purified, and then sequenced using the Long Read kit (Biozym, Hessisch Oldendorf, Germany) on a LiCor 4000L automatic sequencer (MWG, Ebersberg, Germany). Sequence alignment was done with ClustalX software, and improved by eye for the SSU and LSU sequences. Full alignments for both genes can be obtained from the authors by request.

Sequence Analyses

The data set for the D1/D2 region of the LSU rDNA contained 70 taxa and 635 unambiguously aligned bp out of 720 bases and was rooted, with *Prorocentrum minimum* as an outgroup. Hierarchical likelihood ratio tests (hLRTs) were performed using Modeltest Version 3 (Posada and Crandall 1998, 2001) to determine the best model among 56 different models of evolution that best fit the data for the maximum likelihood (ML) analysis.

Maximum likelihood phylogenies were reconstructed with PAUP* 4.08b (Swofford 1998) constrained with the following Modeltest parameters. The model selected for the LSU rDNA data set was the General Time Reversible model with a gamma distribution (GTR + G) with base frequencies of A = 0.2486, C = 0.1706, G = 0.2586, T = 0.3222; base substitution rates of G T = 1.0000, A C = 0.8472, A G = 1.8546, A T = 0.8128, C G = 0.5084, CT = 2.8610, GT = 1.0000; proportion of invariable sites I = 0; and gamma distribution shape parameter = 0.5980.

For the SSU rDNA sequence data set containing 34 taxa and 1751 bp, we used *Tetrahymena thermophila* as an outgroup. Hierarchical likelihood ratio tests gave a GTR model allowing for invariant sites and a gamma distribution (GTR + I + G) as the model that fit best the data set.

Table 1
List of Species Used in This Study

Species	Strain or Abbreviation as Used in This Study	Gene: SSU[a]	Gene: LSU[a]	Geographic Clade	Geographic Origin	Collector
Alexandrium affine	A.affine		L38630			
(Inoue and Fukuyo) Balech	Alexaffi		AAU44935			
Alexandrium catenella	BAHME215		AJ535361*	TA	Tarragona (Spain)	M. Delgado
(Whedon and Kofoid) Balech						
	BAHME217	AJ535392*	AJ535362*	TA	Tarragona (Spain)	M. Delgado
	BAHME222		AJ535359*	TA	Tarragona (Spain)	M. Delgado
	ALexcat1		AF019408	TA		
	ALexcat3		AF042818	TA		
	ALcatHK1		AF118547	TA		
	ALcatHK2		AF118546	TA		
Alexandrium concavum	ALexconc		AF032348			
(Gaarder) Balech						
Alexandrium fundyense Balech	Alexfund	U09048		NA		
Alexandrium lusitanicum Balech	A.lusita					
Alexandrium margalefii Balech	ALexmarg	U27498	AF033531			
Alexandrium minutum Halim	AL1T		AJ535352*		Gulf of Trieste (Italy)	A. Beran
	AL3T	AJ535388*	AJ535353*		Gulf of Trieste (Italy)	A. Beran
	AL8T		AJ535350*		Gulf of Trieste (Italy)	A. Beran
	AL9T		AJ535360*		Gulf of Trieste (Italy)	A. Beran
	L20/2		AJ535351*		Gulf of Trieste (Italy)	A. Beran
	Alexminu	U27500			Gulf of Trieste (Italy)	A. Beran
	Alexmin1	U27499				
Alexandrium ostenfeldii	AOSH1		AJ535358*		Nova Scotia (Canada)	A. Cembella
(Paulsen) Balech and Tangen	Alexostf	U27500				
	K0324	AJ535381*	AJ535363*		Limfjord (Denmark)	P.J. Hansen
	K0287	AJ535382*	AJ535356*		Limfjord (Denmark)	P.J. Hansen
	BAHME136		AJ535357*		Timaru (New Zealand)	N. Berkett
	Alexoste		AF033533			
Alexandrium pseudogoniaulax	AP2T		AJ535355*		Gulf of Trieste (Italy)	A. Beran
(Biecheler) Horiguchi, Yuki & Fukuyo						
Alexandrium tamarense	Alextama	X54946	AF033534			
(Lebour) Balech	Aletamar	AF022191				
	OF842332.4		AJ535364*	NA	Ofunata Bay (Japan)	Kodama
	AT-9		AJ535364*	NA	Ofunata Bay (Japan)	Kodama
	SZN01	AJ535387*	AJ535368*	ME	Gulf of Naples (Italy)	M. Montresor
	SZN08		AJ535369*	ME	Gulf of Naples (Italy)	M. Montresor
	SZN19	AJ535386*	AJ535370*	ME	Gulf of Naples (Italy)	M. Montresor
	SZN21		AJ535374*	ME	Gulf of Naples (Italy)	M. Montresor
	UW53		Higman et al. 2001	WE	Belfast (Nord Ireland)	W. Higman
	UW42		Higman et al. 2001	WE	Belfast (Nord Ireland)	W. Higman
	31/4	AJ535391*	Higman et al. 2001	WE	Cork Harbor (Ireland)	W. Higman
	31/9		Higman et al. 2001	WE	Cork Harbor (Ireland)	W. Higman
Alexandrium tamiyavanichii	Atamiy	AF113935	AF174614			
Balech						
Alexandrium taylorii Balech	AY1T	AJ535390*	AJ535347*		Lagoon of Marano (Italy)	A. Beran
	AY2T	AJ535385*	AJ535348*		Lagoon of Marano (Italy)	A. Beran
	AY4T	AJ535389*	AJ535349*		Lagoon of Marano (Italy)	A. Beran
Ceratium fusus (Ehrenberg)	Cerafus2	AF022153				
Dujardin						
Ceratium tenue	Certenue	AF022192				
Ostenfeld et Schmidt						
Gonyaulax spinifera	Gonyspin	AF022155				
(Claparède et Lachmann) Diesing						
Noctiluca scintillans	Noct.ilu	AF022200				
(Macartney) Kofoid et Swezy						
Peridinium bipes Stein	Peri.bip	AF231805				
Peridinium sp.	Peridini	AF022199				
Peridinium willei Huitfelt-Kaas	Periwill	AF274272				
Perkinsus marinus	Perkmar	X75762				
Perkinsus sp.	Perksp	L07375				
Prorocentrum micans Ehrenberg	PmicaM04		P385 Sylt			
Prorocentrum minimum	Prormin6		AF042813			
(Pavillard) Schiller						
Protoceratium reticulatum	Protreti	AF274273				
(Claparède et Lachmann)						

Table 1
Continued

Species	Strain or Abbreviation as Used in This Study	Gene: SSU[a]	Gene: LSU[a]	Geographic Clade	Geographic Origin	Collector
Bütschli						
Pyrocystis lunula (Schütt) Schütt	Pyrolunu	AF274274				
Pyrocystis noctiluca Murray ex Haeckel	Pyrocyst	AF022156				
Tetrahymena thermophila	Tetr.the	X56165				

[a] An asterisk indicates a gene produced in this study.

The ML-tree calculation was constrained using base frequencies of A = 0.2781, C = 0.1803, G = 0.2477, T = 0.2939; base substitution rates of: A C = 1.0000, A G = 2.2697, A T = 1.0000, C G = 1.0000, C T = 4.5862, G T = 1.0000; proportion of invariable sites I = 0.2239; and a gamma distribution shape parameter = 0.6120. Bootstrap values (Felsenstein 1985) were generated for the maximum parsimony (MP) and with Neighbor-Joining (NJ) analyses using the ML settings for the distance analysis with 500 replicates for LSU analysis and 1,000 replicates for SSU analysis, respectively. For the SSU data set, 572 sites were informative for the MP analysis, resulting in a tree with a length of 1,970 steps, a 0.6122 CI index and 0.7318 RI index. For the LSU data set, 324 sites were informative for the MP analysis, resulting in a tree with a length of 1,114 steps, a 0.6266 CI index and 0.8930 RI index.

The phylogenetic relationships of the dinoflagellates in general and species of the genus *Alexandrium* in particular were also determined by Bayesian inference (BI) (Huelsenbeck and Ronquist 2001; Huelsenbeck et al. 2001) using the SSU rDNA and the D1/D2 region of the LSU rDNA data sets, respectively. The advantages of BI are that it is relatively fast, even when large data sets are used, and it generates probabilistic measures of tree strength, which give posterior probabilities (PP) for phylogenetic stability (Huelsenbeck et al. 2001 and references therein). These values are more straightforward to interpret than bootstrap values, because they can be taken as the probability that the topology of a tree is correct and represents the best estimated phylogeny. The BI settings for the SSU rDNA sequence data set were GTR + G + I with base frequencies estimated and 1.2×10^6 Markov chain Monte Carlo (MCMC) generations and four simultaneous MCMC chains, for the LSU rDNA GTR + G with base frequencies estimated and 1.5×10^6 MCMC generations and four simultaneous MCMC chains, respectively. The analysis was done using MrBayes (http://morphbank.ebc.uu.se/mrbayes/).

To estimate the approximate divergence times of species and clades with molecular data within the data set, a linearized tree was constructed under the assumption of a molecular clock. Lintree was used to construct an NJ tree with the pairwise distance option of TrN + G, allowing for variable base substitution rates and a gamma distribution. The data set was tested with the two-cluster test, which examines the equality of the average substitution rate for two clusters that are created by each node in the tree. Sequences that evolved significantly (at 1% level) faster or slower compared to the average rate were eliminated from the data set. Because elimination of sequences from the data set affects tree topology, NJ trees and two-cluster tests were repeated iteratively until a data set was obtained with nearly all taxa evolving within a Poisson distribution rate of evolution. Some fast or slow evolving taxa can be retained in the data set if their inclusion is critical for the tree topology and for the analyses (Takezaki, Rzhetsky, and Nei, 1995). Thereafter, a linearized tree for a given topology was constructed for the remaining sequences after using the two-cluster test.

A regression of first appearance dates of the genus *Alexandrium* and the *A. tamarense* species complex from fossil occurrences (Ma) against branch lengths (distance) of taxa and strains in the linearized tree was performed. The average possible age for the undated nodes was estimated by multiplying the length of its average branch by the regression coefficient. The earliest possible age of the undated nodes is taken from the upper 95% confidence limit given the distance of its average branch (Hillis, Moritz, and Mable 1996).

Results

Phylogeny of Alexandrium

Starting with 67 dinoflagellate SSU rDNA sequences, 33 were eliminated because they evolved too fast or too slow at $P > 0.05$ level according to the two-cluster test (Takezaki, Rzhetsky, and Nei, 1995): the resulting SSU tree is shown in figure 1. Dinoflagellate phylogenies constructed with all available sequences can be found in Edvardsen et al. (2003). Our ML tree generated from 34 sequences used *Tetrahymena thermophila* and two *Perkinsus* strains as the closest outgroups to the dinoflagellates. The remaining 31 sequences, belonging to several species of dinoflagellates, were used to analyze the phylogenetic relationship of *Alexandrium* to other dinoflagellates. If *Perkinsus* remains intermediate between apicomplexans and dinoflagellates (Litaker et al. 1999), then *Noctiluca scintillans* is the earliest derived extant dinoflagellate species, diverging before the thecate Peridiniphycidae. In this data set, the Peridiniales, represented by species of *Peridinium*, diverge before the Gonyaulacales. Within the Gonyaulacales, the subfamily Gonyaulacoideae of the family Gonyaulacaceae, represented by species of *Gonyaulax*, diverged first, followed by *Protoceratium*, a gonyaulacacean of the subfamily Cribroperidinioideae;

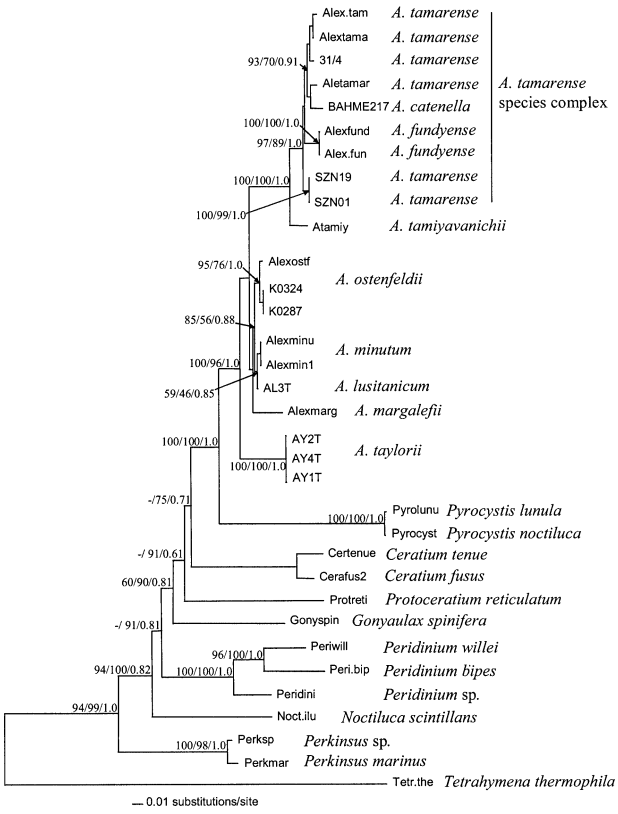

FIG. 1.—Maximum likelihood phylogenetic tree of 18S SSU rDNA sequences from dinoflagellates. *Tetrahymena thermophila* (a ciliate) was used as an outgroup. The tree is generated using PAUP 4.08b with a GTR + I + G model with a number of invariable positions = 0.2239 and a gamma shape = 0.6120. Sequences corresponding to strains that are not listed in table 1 were taken from Destombe et al. (1992) and Scholin, Anderson, and Sogin (1993). Bootstrap values (>50%) from an MP/NJ analysis placed close to each node or arrow show the corresponding node. The BI tree was of similar topology, the third number noted at each of the branches is the posterior probability.

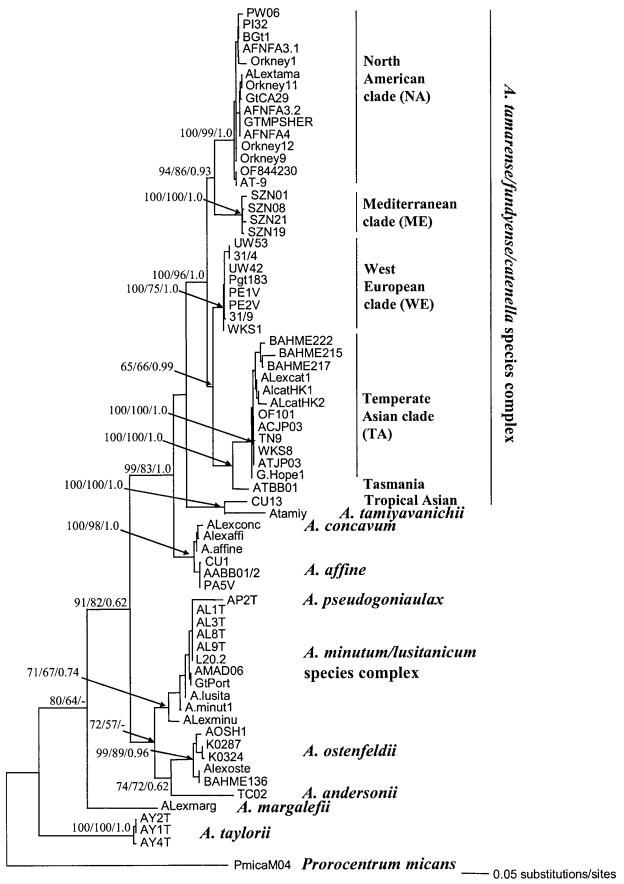

FIG. 2.—Maximum likelihood phylogenetic tree of representatives of the genus *Alexandrium* based on their sequences of the D1/D2 region of the LSU rDNA. *Prorocentrum micans* and *P. minimum* were used as outgroups. The tree was generated using PAUP 4.08b with a GTR + G model with a gamma shape = 0.5980. Sequences corresponding to strains that are not listed in table 1 were taken from Scholin et al. (1994), Medlin et al. (1998), and Higman et al. (2001). Bootstrap values (>50%) from an MP/NJ analysis placed close to each node or arrow show the corresponding node. The BI tree was of similar topology; the third number noted at each of the branches is the posterior probability.

next was the Ceratiaceae represented by *Ceratium*, then the Pyrocystaceae represented by *Pyrocystis*, and lastly the Goniodomaceae represented by *Alexandrium*.

Of species of *Alexandrium* examined to date, *A. taylorii* appears to be the earliest to diverge. Thereafter, species diverge into two clusters. The first cluster consists of *A. margalefii*, *A. ostenfeldii*, and the *A. minutum/lusitanicum* species complex. Within the second cluster, the first species to diverge is *A. tamiyavinichii*, with well-supported bootstrap and PP values, then the *Alexandrium tamarense* species complex. The two SSU sequences of the new Mediterranean clade fall into the *A. tamarense* species complex. Again, the SSU sequences of the *Alexandrium tamarense* species complex do not support the monophyletic nature of the three morphospecies. In contrast to the LSU sequence analysis (see below), no geographic clades were differentiated because the rate of evolution in the SSU gene is much slower than that of the D1/D2 region of the LSU gene.

The analysis of the LSU rDNA data set provides better resolution of the *A. tamarense* species complex (fig. 2). The simplest measure of evolutionary distance in molecular phylogenetics is the number of base differences per species. We have calculated nucleotide differences among strains of the *Alexandrium* species complex. An alignment of 635 bp of 22 *Alexandrium* strains shows that the number of different nucleotides among the sequences of the *A. tamarense* species complex varies from 12 for the WE clade, to over 15 for NA clade sequences, to 19 for the new Mediterranean clade (ME), to 29 for the TA clade. However, the sequence of CU13 strain, formerly designated by Scholin et al. (1994) as the TROP clade, contained 46 nucleotides that distinguish it from the *A. tamarense* species complex. Of these 46 nucleotides, 39 were shared with *A. tamiyavanichii*. *Alexandrium tamiyavanichii* is distinguished from CU13 by 27 nucleotides and 66 nucleotides from the remaining *A. tamarense* species complex. In contrast, there were only two nucleotide differences observed between *A. concave* and *A. affine*. The Tamura and Nei (TrN) distance matrix calculated from sequences shows that, within each geographic clade or ribotype, the distance ranged from 0.006 to 0.024. However, between clades or ribotypes the average distance was 0.103 and ranged from 0.078 between US and NA to

0.165 between TA and ME. The TROP clade showed an average distance to the remaining members of the species complex of 0.182 and a distance to TA of 0.192. Within the TROP clade, the distance between CU13 and *A. tamiyavanichii* was 0.113, whereas the distance between *A. concave* and *A. affine* was only 0.009.

The phylogenetic analysis of the D1/D2 region of the LSU rDNA shows *A. taylorii* as the first divergence in *Alexandrium*. Thereafter, *A. margalefii* diverges, followed by a split into the *A. minutum*/*lusitanicum* species complex with *A. pseudogoniaulax* and a cluster consisting of *A. ostenfeldii* and *A. andersonii* (TCO2). The final divergence in the tree is between the *A. affine*, *Alexandrium tamiyavinichii*, and the *A. tamarense* species complex. In this latter cluster, we see the expected differentiation of the species complex into geographic clades as previously described by Scholin, Hallegraeff, and Anderson (1995); Medlin et al. (1998); and Higman, Stone, and Lewis (2001). All clades and their branching order within the species complex were well supported by bootstrap values (MP/NJ) and posterior probabilities (PP), except for the tropical Asian clade (TROP), which now consists of the strain CU13 of the *A. tamarense* species complex and the species *A. tamiyavanichii*. There is neither bootstrap support nor any posterior probabilities for the position of this clade, and in this analysis it falls unsupported prior to the divergence of the geographic clades of the *A. tamarense* species complex. Analyses using different models resulted in trees in which CU13 and *A. tamiyavanichii* diverge before *A. affine* (data not shown). The major species complex diverges in two clusters, the first cluster containing the nontoxic WE clade and the toxic TA clade and, within it, an early divergence of the Tasmanian strain ATBB01. The second cluster, which diverges slightly after the first one, consists of the toxic NA clade, to which the Orkney Islands (Scotland) isolates belong. The NA clade is sister to the four sequences of our new nontoxic Mediterranean clade (ME).

Linearized Tree

As mentioned, from an original data set of 67 dinoflagellate sequences, we eliminated 33 because their rate of evolution did not fall within a Poisson distribution (Takezaki, Rzhetsky, and Nei 1995). Nevertheless, three taxa were retained in the data set even though their SSU sequences evolved too fast. This is because their inclusion helped to produce a tree topology similar to that of the LSU rDNA tree as well as to the evolutionary tree produced by Fensome et al. (1993) from morphological data. *Perkinsus* was too fast but was used as outgroup in the ML analysis. *Pyrocystis* evolved too fast with respect to *Alexandrium*. Within *Alexandrium*, *A. margalefii* evolved too fast. However, all other clusters evolved at the same average speed and were used for molecular clock calculation. The topology of the linearized TrN + G neighbor-joining tree (fig. 3*A*) compared well with the ML tree (fig. 1), there being only slight differences. *Noctiluca*, *Peridinium*, and *Protoceratium* collapsed to a polytomy. Also, *Ceratium* and *Pyrocystis* could not be separated with this analysis. The topology of the linearized tree is in accord with the classification of Fensome et al. (1993) (fig. 3*B*).

Fossil Dates Plotted on the Geological Time Scale

Times of origin for extant families, genera, and species were obtained from the plots and charts of Fensome et al. (1996); Fensome, Saldarriaga, and Taylor (1999); and Williams et al. (1998, 1999). On the basis of fossil evidence, the divergence between gonyaulacaleans and peridinialeans (the two principal orders of thecate dinoflagellates found as fossils) appears to have occurred early in the Jurassic, about 190 MYA. Hence, we use this date for the origin of the Peridiniales. The order Gonyaulacales, as defined by Fensome et al. (1993), included the atypical rhaetogonyaulacineans, whose range extends back into the late Triassic, to about 210 MYA. However, gonyaulacaleans with a typical gonyaulacacean tabulation first appear around the early/mid Jurassic boundary, about 180 MYA, a date we thus use for the divergence node of *Gonyaulax spinifera* in our linearized tree (fig. 3*A* and *C*). For the family Ceratiaceae, Riding, Poulsen, and Bailey (2000) reported the dinoflagellate *Muderongia simplex* from the late Kimmeridgian *rotunda* Zone (about 145 MYA), which is thus used to date the divergence of the Ceratiaceae. These three dates were plotted onto a geological time scale (fig. 3*C*), with black arrows showing their position in the linearized tree (fig. 3*A*).We used first appearance dates of taxa of higher rank (orders and families) to calibrate our tree, because their first appearance dates are less ambiguous than those of taxa of lower rank.

Calibration of the Molecular Clock

Linearized branch lengths were regressed against the three fossil dates to calculate a molecular clock according to the method described by Hillis, Moritz, and Mable (1996). As already noted, ages derived from the fossil record represent the latest date for an event and are underestimates. We used the dates mentioned above: 190 MYA for the Peridiniales, 180 MYA for the Gonyaulacaceae, and 145 MYA for the Ceratiaceae. The molecular clock thus constructed was then used to extrapolate dates for the nodes of unfossilized taxa, e.g., *Alexandrium* and its species. The average time of origin for the genus *Alexandrium* (77 MYA) and the *Alexandrium tamarense* species complex (23 MYA) was calculated from the average branch lengths of each group. The earliest possible origin of the genus (119 MYA) and the species complex (45 MYA) was calculated from the upper 95% confidence limit, given the lengths of the average branch of each group.

Discussion

We have used the SSU rDNA analysis to investigate relationships within the genus *Alexandrium* because, using this marker, the resolution between major species is appropriate for the comparisons needed. The D1/D2 region of the LSU rDNA is useful only when finer resolution between strains is needed, because it evolves at a much higher rate. Our phylogenetic analysis of the SSU of rDNA sequences was consistent with those of previous studies

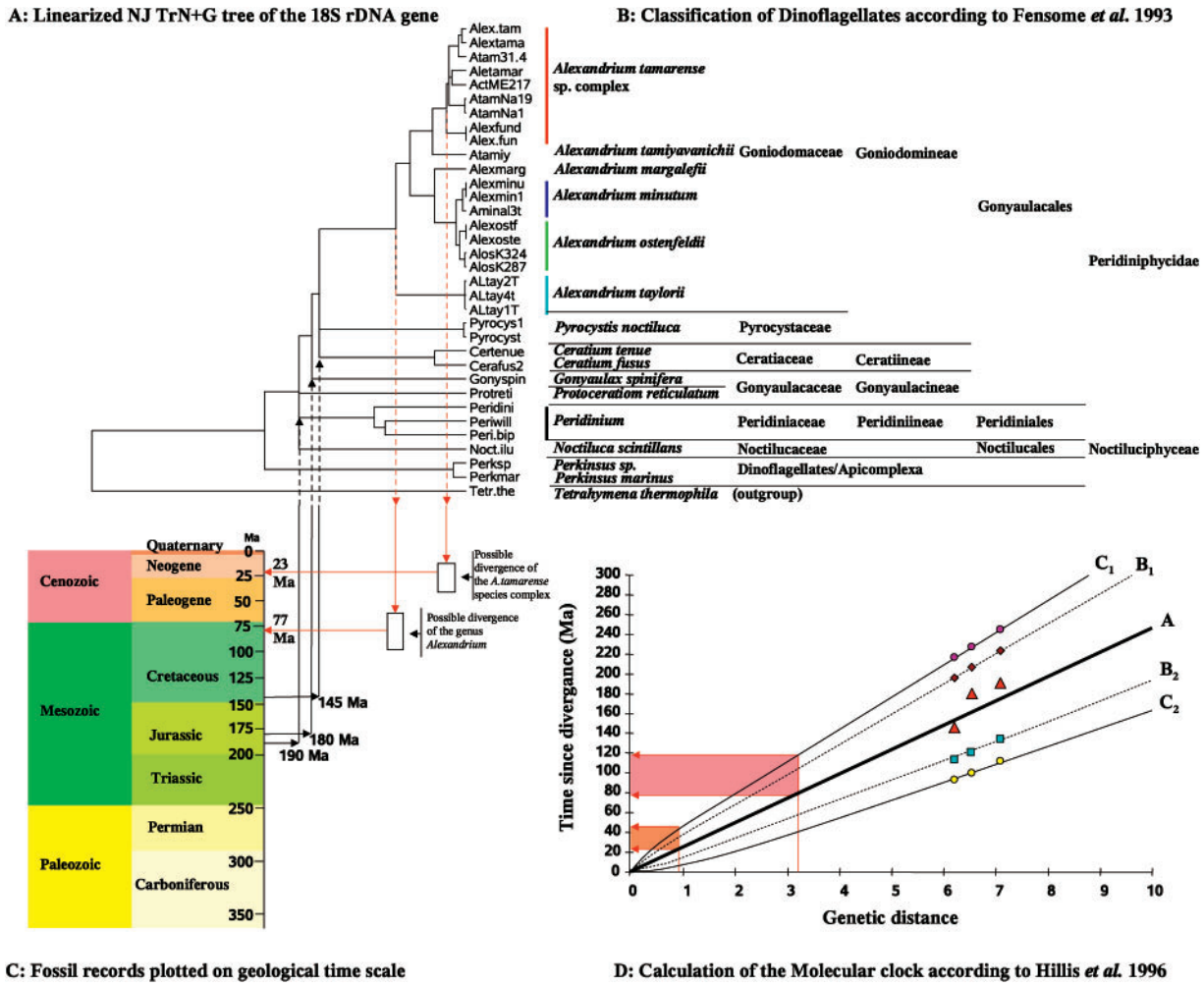

FIG. 3.—*A*, Linearized NJ tree constructed from the Tamura and Nei gamma distribution distances and from an unlinearized NJ tree generated using Lintree (Takezaki et al. 1995) of the SSU rDNA from dinoflagellates. Black arrows mark the fossil events in the linearized tree; the red arrow 1 shows the divergence of the genus *Alexandrium*, and red arrow 2 shows the divergence of the *A. tamarense* species complex. *B*, Systematic classification of dinoflagellates (Fensome et al. 1993). *C*, Fossil events and the calculated divergences of both the genus *Alexandrium* and the *A. tamarense* species complex plotted on a geological time scale. Boxes symbolize the variance in appearance dates: the y-axis shows the possible appearance from the lower 95% confidence (B_2) of regression line (A) to the earliest possible appearance (C_1) calculated using the molecular clock (D); the x-axis has no meaning. Black arrows show the fossil dates and demonstrate their position within the linearized tree (A); the red arrows connect the nodes of divergence of both the genus *Alexandrium* and the *A. tamarense* species complex with the geological time scale according to the calculated dates of the molecular clock. *D*, Molecular clock calibration for the linearized tree in *A*, from the SSU nuclear encoded rDNA gene from dinoflagellates. First appearance of the genus *Alexandrium* and the *A. tamarense* species complex were regressed against measured branch lengths from the linearized tree (*A*). For the molecular clock: A is the regression of estimated time since separation on sequence divergence of SSU rDNA in dinoflagellates, constrained through the origin. B_1 and B_2 are the bounds of the 95% confidence limits of the regression line. C_1 and C_2 are the bounds of the 95% confidence limits for a new predicted value of time given the lengths of an undated node. Arrows show the origin of the groups estimated from the molecular clock. The lower arrow shows the average age of the genus or the species complex, and the upper arrow shows the earliest possible time of origin based on the upper 95% confidence internal (C_1) of an undated node.

(Saunders et al. 1997; Walsh et al. 1998; Litaker et al. 1999; Saldarriaga et al. 2001; Edvardsen et al. 2003). Our analysis also generally agreed with the conventional classification of dinoflagellates by Fensome et al. (1993). The SSU rDNA tree shows that the Goniodomaceae was one of the last families to diverge within the Gonyaulacales; that *Alexandrium* is monophyletic, supported by high bootstrap and posterior probability values; and that there is a clear differentiation of species (or species complexes) within the genus. However, the two subgenera of *Alexandrium*, *Alexandrium* subgenus *Alexandrium* (in which the first apical homologue—*1′—contacts the apical pore complex—apc) and *Alexandrium* subgenus *Gessnerium* (in which *1′ does not contact the apc) form no clear groups in our phylogenetic trees. *Alexandrium taylorii*, *A. margalefii* and, in the case of the LSU analysis, *A. pseudogoniaulax*, all representatives of subgenus *Gessnerium*, formed no distinct group. Instead *Alexandrium pseudogoniaulax* is a sister group of *A. minutum* and *A. lusitanicum*, both members of subgenus *Alexandrium*. We suggest that more species and isolates of subgenus *Gessnerium* should be analyzed in future studies to clarify the phylogenetic status of the two subgenera. The close relationship between *Alexandrium ostenfeldii* and the *A. lusitanicum*/*minutum*

species complex was unexpected, because of their different sizes and morphologies. Also, the *A. tamarense* species complex shares a last common ancestor with *A. tamiyavanichii*, which was also its sister taxon in the LSU tree. Unfortunately, no sequences were obtained from the TROP clade or from *A. affine*. Hence, for the latter species, for which sequences were obtained from our SSU data set, its order of divergence with respect to *A. tamiyavanichii* and the CU13 strain could not be clarified (see the discussion below on the resolution in the LSU tree). The sequences of the new Mediterranean clade fall, as expected, within the *A. tamarense* species complex.

The phylogenetic analysis of the LSU rDNA gene of the *Alexandrium* sequences confirms earlier reports (Scholin et al. 1994; Adachi, Sako, and Ishida 1996*a*; Medlin et al. 1998) that the *A. tamarense* species complex is separated into distinct geographic clades. These are the NA, TA, WE, and ME clades, not the three morphotypes (*A. tamarense*, *A. catenella*, and *A. fundyense*). Hence, of the 29 species that Balech (1995) included in *Alexandrium*, some may not be truly distinct species (Taylor and Fukuyo 1998).

The LSU rDNA sequences of the four isolates from the Mediterranean Sea form a sister group to the North American clade within the *A. tamarense* species complex, with well-supported bootstrap and posterior probability values. Also the nucleotide differences and the distance values of these sequences, compared to the sequences within the other geographic clades, support their recognition as a new clade in the tree. This may not be the last discovery of a new ribotype within the *A. tamarense* species complex: reports of the *A. tamarense* species complex from the southern hemisphere indicate that they are part of the NA clade (reviewed by Taylor 1987*b*; Gayoso 2001; Lilly, Taroncher-Oldenburg, and Anderson 2002). However, to determine whether these new isolates are indigenous or introduced by human activity through ballast water or shellfish stocks (Scholin, Hallegraeff, and Anderson 1995), they will have to be analyzed by the more recently available molecular probes (Adachi, Sako, and Ishida 1996*b*; Scholin et al. 1997; John et al. 2003). As an example, the strains BAHME215, 217, and 222 have been isolated from the Spanish coast, and their sequences group together with ALcatHK1, ALcatHK2, and ALexcat1 isolates from Hong Kong Harbor. This result could indicate that the Spanish isolates have been introduced by human activity.

Earlier studies have shown that the TROP clade represents the ancestral population of the *A. tamarense* species complex (Scholin et al. 1994; Medlin et al. 1998). Adachi, Sako, and Ishida (1996*a*) have suggested that the isolates of the TROP clade might be a different species, because the distance values of the ITS region between isolates of the TROP clade and the NA clade is greater than the distance values between the other clades. Similar relationships of the distance values were obtained in our analysis. Among the NA, ME, WE, and TA clades, the average distance was 0.103, but it was 0.192 between TROP and the other species complex clades. The distance between CU13 and *A. tamiyavanichii* was 0.09. However, *A. tamiyavanichii* is morphologically clearly different from the *A. tamarense* morphotype (Balech 1995), so a misidentification of CU13 is unlikely. Therefore we suggest that at this cladogenesis, the *A. tamarense* morphotype appeared and that CU13 and *A. tamiyavanichii* diverged from a common ancestral taxon, which likely bore the *A. tamarense* morphotype because the CU13 strain bears that morphotype. However, the position of the branch of CU13 and *A. tamiyavanichii* had no bootstrap and posterior probability support. Higman, Stone, and Lewis (2001) and Usup et al. (2002) used the NJ method to construct a phylogenetic tree and showed that the TROP clade diverged before *A. affine*. Higman, Stone, and Lewis (2001) further suggested that these results were obtained because they had only one representative of each in their analysis, and the analytical method used might have affected the outcome as well. Unfortunately, no bootstrap values were presented in their analysis, which makes an interpretation of their results difficult. But the analysis of Usup et al. (2002) shows strong support, from bootstrap values, of the possibility that *A. affine* is the sister group of *A. tamarense* species complex. However, in future studies more sequences of the TROP clade and *A. tamiyavanichii* should be included in the analysis to clarify the position and identity of the true sister group of the *A. tamarense* species complex.

Alexandrium affine and *A. concavum* cluster together, bootstrap values and the posterior probability supporting their position as sister to the *A. tamarense* species complex (see above), with the TROP clade either diverging before or after them, depending on the evolutionary model used. Based on morphological features, *A. affine* and *A. concavum* should diverge before the TROP clade, as shown in figure 2, and by Scholin et al. (1994); Adachi, Sako, and Ishida (1996*a*); and Medlin et al. (1998). Balech (1995) considered the position of *A. concavum* to be uncertain. Despite its exceptionally large size, it is difficult to study because of its delicate theca. Even its biology is poorly understood: it is one of the rarest oceanic *Alexandrium* species. If it has not been misidentified based on the small distance value of 0.006, the divergence between *A. concavum* and *A. affine* must have occurred very recently.

As we generated the linearized tree, 33 taxa were excluded from the data set because the evolution rates of their SSU rDNA gene were too fast. Our final SSU rDNA data set for phylogenetic study of the dinoflagellates, and for the calibration of a molecular clock, included 34 taxa. Similar problems with large variation in the substitution rate of rDNA genes have been shown for foraminifera (Pawlowski et al. 1997). The rDNA of planktonic foraminifera evolves 50 to 100 times faster than that of the benthic foraminifera. There are two hypotheses that might explain these differences in DNA substitution rates: the generation time effect hypothesis (Li et al. 1996); and the metabolic rate hypothesis (Martin 1995). These factors might be responsible for the acceleration of the evolution rate in the planktonic versus the benthic foraminifera. Pawlowski et al. (1997) assumed that a higher reproduction rate, shorter generation time, more exposure to solar UV radiation, and changes in the DNA replication or DNA repair mechanism have resulted in a higher mutation rate for the planktonic foraminifera. Benthic, planktonic,

parasitic, and endosymbiontic species were among the 67 dinoflagellate taxa that were initially used in the two cluster test (Takezaki, Rzhetsky, and Nei 1995). These species exhibited variable generation times and metabolisms, with some being autotrophic, some mixotrophic, and others heterotrophic. Any of these factors might have resulted in a high variance in evolutionary rates among the sequences, and explanations similar to those invoked for foraminifera may also be applicable to dinoflagellates.

Our molecular clock is only a hypothetical model to investigate the biogeographic distribution of the *A. tamarense* ribotypes, because the relationships among the geographic clades exhibit vicariant events rather than dispersal events. We estimate that the average age of the genus *Alexandrium* is 77 Myr (late Cretaceous), and no earlier than 119 Myr (mid-Cretaceous); these dates do not conflict with the 105 Myr date for the closest dinoflagellates with similar tabulation and fossilizable cysts. At 120 MYA, climate and water temperature were much warmer than today. Shallow seas covered much of the continental areas, with sea levels up to 200 m higher than today. These continental areas were arranged such that there was a global circumequatorial current within the Tethys Ocean (Scotese 1991; Marincovich et al. 1990). Between 65 MYA and 55 MYA, two catastrophic events affected global biodiversity: the end-Cretaceous mass extinction event (65 MYA); and the late Paleocene thermal maximum (55 MYA), with a deep-sea temperature increase of 5°–6°C that killed benthic foraminifera and apparently caused planktonic microalgae, including dinoflagellates, to proliferate (Crouch et al. 2001; Zachos et al. 2001). In the early Paleogene (40–65 MYA), the ocean basins were significantly rearranged as Tethys closed, new oceans opened, resulting in lowered sea level and a cooler seasonal global climate. Permanent polar ice sheets formed (Bice et al. 2000; Zachos et al. 2001), and the length of global coastlines and the area of continental shelves both increased. Coastal regions became more heterogeneous in topological, hydrodynamic, and climatic conditions, thus promoting regional differences (Scotese 1997).

Under these mid-Cenozoic conditions, *Alexandrium* likely diverged into several taxa (fig. 1, fig. 3*A*). The *A. tamarense* species complex probably diverged around the early Neogene (23 MYA), but no earlier than the late Paleogene (45 MYA). A global distribution of planktonic species was possible through the eastern Indian Ocean, Tethys, and the Pacific Ocean, with counter currents for anticlockwise distributions. At 36 MYA, the Tasmania-Antarctica and Drake passages opened, forming the Antarctic Circumpolar Current (ACC) and intensifying conditions favorable for the build-up of increasing Antarctic ice sheets and ocean fertility (Zachos et al. 2001 and references therein). When the Tethys Ocean closed, populations became isolated in various ocean basins. This regionalizing effect was enhanced when, from about 3 to 13 MYA, the Isthmus of Panama was uplifted, cutting of the tropical Pacific–Atlantic connection and reorganizing northern hemisphere ocean circulation. As a result, surface waters cooled through north Atlantic deep water formation, which could have increased precipitation in the northern hemisphere and promoted glaciation after 2.5–3 MYA (Haug and Tiedemann 1998). These geological events likely led to allopatric speciation of global planktonic populations.

Given mid-Cenozoic paleoclimatic and geological changes, we propose the following scenario to explain the modern distribution of the strains within the *Alexandrium tamarense* species complex. Our scenario starts with a globally distributed ancestral population (fig. 4*A* and *B*), which diverges first into eastern and western Pacific populations (fig. 4*C* and *D*) as a response to a relatively short but deep glacial maximum around 23 MYA (Paul et al. 2000). The eastern Pacific population was connected to Atlantic populations through the Central American Seaway and its counter currents, whereas the western Pacific population was connected to eastern Atlantic populations through Tethys (fig. 4*C* and *D*). The heterogeneous climatic and oceanic conditions between 40 and 65 MYA likely promoted phenotypic and genetic differentiation within the *A. tamarense* species complex. When the Tethys Ocean closed, the western Pacific population diverged into TA (yellow stars in fig. 4*E*) and WE clades (black stars in fig. 4*E*). As the Isthmus of Panama uplifted, ancestral populations in the subtropical Atlantic (white stars in fig. 4*E*) were separated from those in the eastern Pacific (NA clade: orange stars in fig 4*E*). The closing of Tethys, the formation of the Mediterranean Sea, and the uplift of the Panama Isthmus created significant changes in circulation and paleoclimate (Haug and Tiedemann 1998). Around 5 MYA, the Mediterranean Sea dried up and was subsequently refilled by tropical and subtropical Atlantic water with subtropical Atlantic *A. tamarense* populations. Eventually, indigenous subtropical Atlantic populations became extinct because of unfavorable environmental conditions, leaving relict populations, the ME clade (white stars in fig. 4*F*), in the Mediterranean. Relict populations of the ancient sister group of the *A. tamarense* species complex can be found in tropical waters (red stars in fig. 4*F*) although, as already discussed, the precise species identification of this sister group is still under debate.

Scholin et al. (1998) reported an isolate from the Kamchatka Peninsula that has a TA/NA intermediate genotype, an observation that may support the initial east/west separation in the Pacific. As suggested by Scholin et al. (1995), the North American east coast population may have originated from an ancestral population from the west coast. Veron (1995) stated that as the Panama Isthmus was emplaced, northern Pacific waters were drawn into the north Atlantic. Thus, Pacific populations may have migrated through the Bering Strait into the Arctic Ocean and the Labrador Sea. Alternatively, as Medlin et al. (1998) noted, migration may have been via the Fram Strait and Greenland currents, with later dispersal via the Gulf Stream; this scenario also explains the occurrence of the NA clade along the Scottish coast. The possibility of human introduction of the Scottish occurrence has been discussed (Higman, Stone, and Lewis 2001) but was discounted by Medlin et al. (1998) because of the high number of base substitutions within and between the Scottish isolates. We assume that the relationships uncovered in the LSU rDNA tree show speciation in progress and represent allopatric vicariant populations. Fig 4*F* shows the idealized distribution of the *A. tamarense* species complex popula-

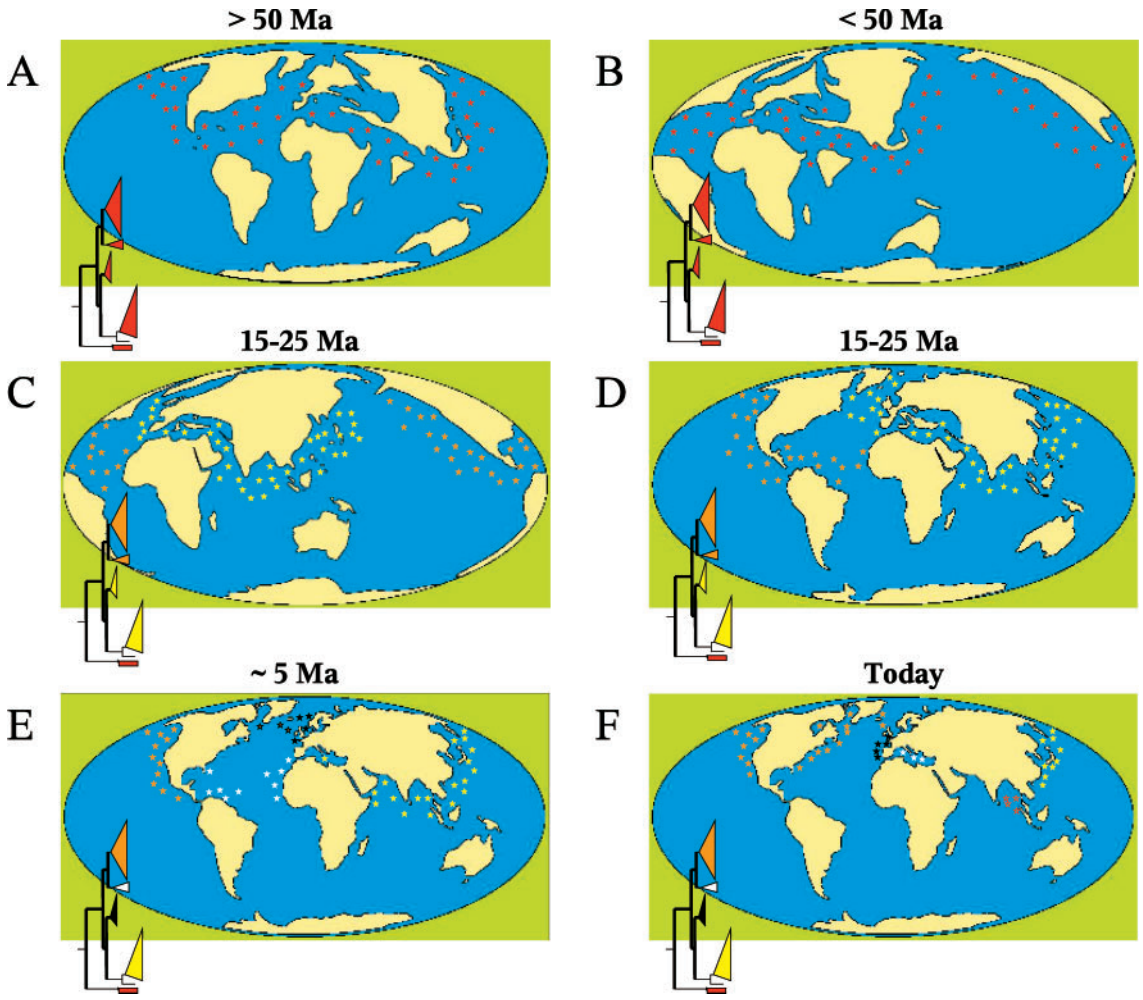

FIG. 4.—Maps showing hypothetical distributions of the populations of the *Alexandrium tamarense* species complex at specified times during the Cenozoic. Stars symbolize *A. tamarense* species complex distribution. Colors of stars correspond to the divergence stage of the *A. tamarense* population according to the modified tree inset of the D1/D2 region of the LSU rDNA phylogenetic tree (fig. 2); also see text. [Paleogeographic reconstructions after Scotese (1997).]

tions. In recent times, populations from different geographic clades have been introduced into new areas via ballast water or shellfish stocks exchange, often into areas where *Alexandrium* populations had never been previously reported (Scholin 1998; Hallegraeff 1998). More intensive examinations of sediment material has uncovered the presence of cysts (Taylor, personal communication)

The *Alexandrium tamarense* morphotype can be found in all ribotypes, and the ribotypes are not fully reproductively isolated: they can still interbreed, even if with lower zygote survival rates (Sako et al. 1990). Based on current data, it is difficult to offer an explanation as to why the three different morphotypes are found in the two toxic ribotypes, whereas the nontoxic ribotypes contain only the *A. tamarense* morphotype. We suggest that the *A. tamarense* morphotype, which is characterized by, for example, the presence of a ventral pore on the first apical plate, is plesiomorphic. The tendency in the *A. catenella* morphotype, for example, to form chains may be an apomorphic feature; this tendency is represented in the TA and NA clades. The *A. fundyense* morphotype, in which a ventral pore is lacking, is present only in the NA clade; thus, this morphotype is probably apomorphic. Both the *A. catenella* and *A. fundyense* morphotypes may indicate an ongoing speciation process. The results at least show that morphological features used to discriminate *A. fundyense* delineate a biologically meaningful clade within the species complex. However, not even these features make an unambiguous identification of the NA clade possible, because this clade also includes *A. tamarense* and *A. catenella* morphotypes. In further studies, taxonomists might examine isolates from the different clades of the *A. tamarense* species complex to seek new morphological features that might reflect the different ribotypes. However, such features may not be obvious, because cryptic speciation appears to be common in unicellular organisms (Medlin et al. 1995; De Vargas et al. 1999).

The observation that ribotypes of *Alexandrium*, rather than morphotypes, reflect geographic areas is not new. Cembella, Taylor, and Therriault (1988) were the first to discuss the distinction between *A. tamarense* and *A. catanella* and, since then, much effort has been made to

understand the geographic and genetic distribution of the *A. tamarense* species complex. Our knowledge of the species complex today results primarily from the work of Scholin (1998). Our discovery of a new ribotype emphasizes that ideas concerning the evolution and distribution of forms within the genus have to be reconsidered continuously. The development of a molecular clock using data from the fossil record helps predict when groups may have diverged, and offers a new hypothesis to explain the extant distribution of clades within the *Alexandrium tamarense* species complex. It has also helped elucidate evolutionary relationships among *Alexandrium* species recovered in our phylogenetic analyses.

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