J. Phycol. **30,** 999–1011 (1994)

IDENTIFICATION OF GROUP- AND STRAIN-SPECIFIC GENETIC MARKERS FOR GLOBALLY DISTRIBUTED *ALEXANDRIUM* (DINOPHYCEAE). II. SEQUENCE ANALYSIS OF A FRAGMENT OF THE LSU rRNA GENE[1]

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ABSTRACT

A fragment of the large-subunit (LSU) ribosomal RNA gene (rDNA) from the marine dinoflagellates Alexandrium tamarense *(Lebour) Balech,* A. catenella *(Whedon et Kofoid) Balech,* A. fundyense *Balech,* A. affine *(Fukuyo et Inoue) Balech,* A. minutum *Halim,* A. lusitanicum *Balech, and* A. andersoni *Balech was cloned and sequenced to assess inter- and intraspecific relationships. Cultures examined were from North America, western Europe, Thailand, Japan, Australia, and the ballast water of several cargo vessels and included both toxic and nontoxic isolates. Parsimony analyses revealed eight major classes of sequences, or "ribotypes," indicative of both species- and strain-specific genetic markers. Five ribotypes subdivided members of the* A. tamarense/catenella/fundyense *species cluster (the "tamarensis complex") but did not correlate with morphospecies designations. The three remaining ribotypes were associated with cultures that clearly differ morphologically from the tamarensis complex. These distinct sequences were typified by 1)* A. affine, *2)* A. minutum *and* A. lusitanicum, *and 3)* A. andersoni. *LSU rDNA from* A. minutum *and* A. lusitanicum *was indistinguishable. An isolate's ability to produce toxin, or lack thereof, was consistent within phylogenetic terminal taxa. Results of this study are in complete agreement with conclusions from previous work using restriction fragment-length polymorphism analysis of small-subunit rRNA genes, but the LSU rDNA sequences provided finer-scale species and population resolution.*

The five divergent lineages of the tamarensis complex appeared indicative of regional populations; representa-

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tives collected from the same geographic region were the most similar, regardless of morphotype, whereas those from geographically separated populations were more divergent even when the same morphospecies were compared. Contrary to this general pattern, A. tamarense *and* A. catenella *from Japan were exceptionally heterogeneous, displaying sequences associated with Australian, North American, and western European isolates. This diversity may stem from introductions of* A. tamarense *to Japan from genetically divergent sources in North America and western Europe.* Alexandrium catenella *from Japan and Australia appeared identical, suggesting that these two regional populations share a recent, common ancestry. One explanation for this genetic continuity was suggested by* A. catenella *cysts transported from Japan to Australia via ships' ballast water: the cysts contained LSU rDNA sequences that were indistinguishable from those of known populations of* A. catenella *in both Japan and Australia. Ships ballasted in South Korea and Japan have also fostered a dispersal of viable* A. tamarense *cysts to Australia, but their LSU rDNA sequences indicated they are genetically distinct from* A. tamarense/catenella *previously found in Australia and genetically distinct from each other, as well. Human-assisted dispersal is a plausible mechanism for inoculating a region with diverse representatives of the tamarensis complex from geographically and genetically distinct source populations. The D1-D2 region of* Alexandrium *LSU rDNA is a valuable taxonomic and biogeographic marker and a useful genetic reference for addressing dispersal hypotheses.*

Key index words: Alexandrium; *biogeography; large-subunit rRNA; PCR; Pyrrophyta; red tide*

Marine dinoflagellates within the genus *Alexandrium* (= *Protogonyaulax* Taylor; Steidenger and Moestrup 1990) include a number of species responsible for paralytic shellfish poisoning ("red tides"; Prakash et al. 1971). A central concern in many ongoing studies of the taxonomy, biogeography, population dynamics, and toxigenesis of these organisms is their underlying genetic variability (Destombe et al. 1992, Scholin et al. 1993, Scholin and Anderson 1994). This is especially true for the *A. tamarense, A. catenella,* and *A. fundyense* species complex (the "tamarensis complex"), a group of closely related organisms found in many coastal regions of the world (Taylor 1984, Balech 1985, Balech and Tangen 1985). Researchers have long agreed that the conserved morphological features of these species belie a largely unexplored genetic diversity. However, disagreement remains as to how this diversity correlates with morphospecies designations and whether morphotypes actually represent "true species" or a continuum of closely related strains (Taylor 1985, Cembella et al. 1987, Hayhome et al. 1989, Sako et al. 1990, 1993).

A number of laboratories have attempted to resolve this debate using biochemical markers to assess independently the genetic relatedness of isolates representing the different morphotypes, but results of such comparisons have not been consistent. For example, *A. tamarense* and *A. catenella* from Japan have been heralded as a paradigm of the validity of morphospecies designations; overall, isolates defined by morphological features were positively correlated with groups defined by subcelluar criteria such as isozyme electrophoretic patterns, toxin composition, and cell surface antigens (Fukuyo 1985, Sako et al. 1990, 1993). In contrast, biochemical characterization of *A. tamarense/catenella/fundyense* from North America and other regions of the world revealed no consistent correlations with morphotype (Cembella and Taylor 1986, Cembella et al. 1987, 1988, Hayhome et al. 1989). Elsewhere, we have argued that these conflicting conclusions have arisen because different regional populations of the same (or closely related) morphospecies are genetically divergent (Scholin and Anderson 1993, 1994).

In an effort to identify genetic markers that could provide additional resolution, sequences of genomic small-subunit (SSU) ribosomal RNA (rRNA) genes (rDNA) from a variety of *Alexandrium* species and populations, with particular emphasis on the tamarensis complex, were compared by restriction fragment-length polymorphism (RFLP) analysis (Scholin and Anderson 1994). That study revealed distinctive genetic characteristics that delineate some morphospecies as well as populations (strains) of individual species. Here, those observations are extended using sequence analysis of a fragment of a different rRNA gene, the 5′ portion of the large-subunit (LSU) rDNA. The particular region of LSU rDNA chosen encompasses the so-called "D1" and "D2" hypervariable domains, some of the most rapidly evolving portions of rRNA-encoding eukaryotic DNA (Michot et al. 1984, Michot and Bachellerie 1987, Lenaers et al. 1989, 1991). Accelerated rates of nucleotide substitutions within the D1 and D2 domains have rendered these sequences species- and, in some cases, strain-specific, making this fragment an informative genetic marker for addressing both taxonomic and biogeographic questions.

MATERIALS AND METHODS

Cultures used in this study (Table 1) represent a variety of *Alexandrium* species and some of their globally distributed populations. All were maintained in f/2 medium as modified and described by Anderson et al. (1984). Total nucleic acids from each culture were isolated, quantified, and stored as described (Scholin and Anderson 1994). Note that throughout this text the term "clone" refers to a recombinant plasmid whereas "isolate" refers to a specific *Alexandrium* culture.

Polymerase chain reaction amplifications. Approximately 700 base-pairs of the LSU rDNA were amplified by polymerase chain reaction (PCR) (Saiki et al. 1988) using primers targeted toward conserved positions 24–43 ["D1R" (forward); 5′ACCCGCTGAATTTAAGCATA3′] and 733–714 ["D2C" (reverse); 5′CCTTGGTCCGTGTTTCAAGA3′], relative to the *Prorocentrum micans* Ehrenberg LSU rRNA (Lenaers et al. 1989). Amplifications were carried out in duplicate or triplicate as previously described (Scholin and Anderson 1994) except that the

TABLE 1. *Strain numbers, species designations, isolation locales, toxicity, number of LSU rDNA clones isolated and sequenced, length of PCR-amplified LSU rDNA fragment, and culture sources.*

Geographic block	Strain[a]	Species designation[b]	Isolation locale	Toxic?[b]	No. LSU rDNA clones isolated[c]	Length of LSU rDNA fragment[d]	Culture source[e]
North America							
West Coast	PW06*	*A. tamarense*	Port Benny, AK	Yes	5, 7	668	S. Hall
	PI32*	*A. tamarense*	Porpoise Isl., AK	Yes	7	668	S. Hall
	BGt1*	*A. fundyense*	Russian River, CA	Yes	10	668	D. Anderson
East Coast	AFNFA3*	*A. fundyense*	Newfoundland	Yes	8	665/668	D. Anderson
	AFNFA4*	*A. fundyense*	Newfoundland	Yes	11	665/668	D. Anderson
	Gony. #7	*A. fundyense*	Bay of Fundy	Yes	14	665/668	A. White
	Gt429	*A. fundyense*	Ipswitch Bay, MA	Yes	13	665/668	C. Martin
	GtCA29*	*A. fundyense*	Cape Ann, MA	Yes	7	665/668	D. Anderson
	GtMP*	*A. fundyense*	Orleans, MA	Yes	12	665/668	D. Anderson
	GtPP01	*A. tamarense*	Falmouth, MA	Yes	13	665/668	D. Anderson
	GtPP06	*A. tamarense*	Falmouth, MA	Yes	10	665/668	D. Anderson
	GtCN16	*A. tamarense*	Groton, CN	Yes	9	665/668	D. Anderson
	GtLI21	*A. tamarense*	Babylon, NY	Yes	12	665/668	D. Anderson
	TC02*	*A. andersoni*	Eastham, MA	No	11	661	D. Anderson
Western Europe							
United Kingdom	Pgt183*	*A. tamarense*	Plymouth	No	7	669	M. Taylor
Spain	PE1V*	*A. tamarense*	Galicia	Noξ	12	669	I. Bravo
	PE2V*	*A. tamarense*	Galicia	No	14	669	I. Bravo
	PA5V*	*A. affine*	Galicia	No	12	671	I. Bravo
Portugal	GtPort*	*A. lusitanicum*		Yes	9	676	L. Provasoli
Japan							
North	OF041*	*A. tamarense*	Ofunato Bay	Yes	13	666/668	Y. Sako
	OF051*	*A. tamarense*	Ofunato Bay	Yes	10	666/668	Y. Sako
	OF101*	*A. catenella*	Ofunato Bay	Yes	10	669	Y. Sako
South	TN-9*	*A. catenella*	Tanabe Bay	Yes	10	669	Y. Sako
	WKS-1*	*A. tamarense*	Tanabe Bay	No	12	669	M. Kodama
	WKS-8*	*A. catenella*	Tanabe Bay	Yes	10	669	M. Kodama
Thailand	CU-1*	*A. affine*	Gulf of Thailand	No	10	671	M. Kodama
	CU-13*	*A. tamarense*	Gulf of Thailand	Yes	14	666	M. Kodama
Australia							
Mainland	ACPP01*	*A. catenella*	Port Phillip Bay, Vic.	Yes	12	669	G. Hallegraeff
	ACPP02*	*A. catenella*	Port Phillip Bay, Vic.	Yes	9	669	G. Hallegraeff
	ACPP03	*A. catenella*	Port Phillip Bay, Vic.	Yes	11	669	G. Hallegraeff
	ACPP09	*A. catenella*	Port Phillip Bay, Vic.	Yes	11	669	G. Hallegraeff
	AMAD01*	*A. minutum*	Port River, S.A.	Yes	12	676	G. Hallegraeff
	AMAD06*	*A. minutum*	Port River, S.A.	Yes	14	676	G. Hallegraeff
Tasmania	ATBB01*	*A. tamarense*	Bell Bay	Noξ	12	669	G. Hallegraeff
	AABB01/*2	*A. affine*	Bell Bay	No	10	671	G. Hallegraeff
Ballast water	I72/21#2	*A. tamarense*	Muroran, Japan[f]	Yes	10	665/668	G. Hallegraeff
	I72/24#1*	*A. tamarense*	Muroran, Japan[f]	Yes	7	665/668	G. Hallegraeff
	ACJP03*	*A. catenella*	Kashima, Japan[f]	Yes	10	669	G. Hallegraeff
	G. Crux*	*A. catenella*	Singapore[g]	Yes	11	669	G. Hallegraeff
	G. Hope1*	*A. catenella*	Samchonpo, S. Korea[f]	Yes	5	669	G. Hallegraeff
	G. Hope2*	*A. catenella*	Samchonpo, S. Korea[f]	Yes	4	669	G. Hallegraeff

[a] Strain listings from the D. M. Anderson culture collection; * = isolates whose sequences were used to construct the phylogenetic tree (Fig. 5); all cultures are clonal except for BGt1, GtMP, PE1V, PE2V, and G. Hope 1 and 2.

[b] Determined by mouse bioassay and/or HPLC analysis; ξ = may contain trace amounts of toxin (D. Kulis, pers. commun.).

[c] Number of LSU rDNA clones isolated and pooled prior to sequencing; LSU rDNA from PW06 was cloned on two separate occasions.

[d] Sequence lengths (basepairs) of PCR-amplified LSU rDNA fragment excluding amplification primers; 665/668 = cultures harboring the 148 and 590–591 length heterogeneities; 666/668 = cultures harboring the 590–591 length heterogeneity only.

[e] Individuals who supplied the culture.

[f] Origin of ballast water (Hallegraeff and Bolch 1992).

[g] Hailing port of vessel; origin of ballast water uncertain (Hallegraeff and Bolch 1992).

final concentration of each primer was 0.1 μM and primer annealing was at 42°–45° C. Following amplification, replicate reactions from a given culture were pooled, purified, concentrated, and stored as noted (Scholin and Anderson 1994).

Cloning. PCR-amplified LSU rDNA was cloned using Invitrogen's T/A cloning kit (cf. Holton and Graham 1991, Marchuck et al. 1991) according to the recommendations of the manufacturer. Generally, 16 bacterial clones potentially containing plasmids with an insert were screened for each *Alexandrium* culture examined. Plasmids from those clones were isolated from 1.5 mL of an overnight culture using the modified Birnboim procedure (Ausubel et al. 1987), rinsed with 80% ethanol (EtOH), and then

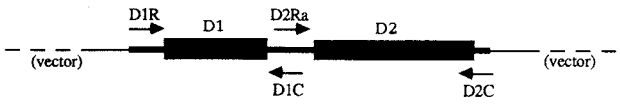


FIG. 1. LSU rDNA clone sequencing strategy. Thin and dashed line represents plasmid sequences; thick line represents the inserted LSU rDNA fragment. Darkened boxes indicate the relative position and size of the D1 and D2 hypervariable domains. Relative location of sequencing primers are also shown; arrows indicate direction of sequence polymerization.

resuspended in 50 μL TE + RNase. One microliter of each plasmid was digested with *Hind*III (New England Biolabs) in a final volume of 10 μL to verify cloning. Clones containing a single LSU rDNA insert were identified by comparing their mobility to size standards. Plasmid clones were stored separately at −20° C.

Sequencing. Several precautions were taken in order to minimize sequencing errors. Two to three replicate PCR amplifications were pooled prior to cloning, multiple clones from each *Alexandrium* isolate were pooled prior to sequencing to gauge the homogeneity of the products and identify the locations of ambiguities or length heterogeneities, and both strands of the cloned molecules were sequenced (Sogin 1990). In some cases, clones from a given isolate were individually sequenced to resolve heterogeneities and ambiguities.

Aliquots of rDNA clones for a given dinoflagellate strain were pooled to yield a final volume of 120 μL. Plasmids were denatured by adding 120 μL of 0.6 N NaOH and incubating at room temperature for 5 min. Denatured templates were neutralized and precipitated by adding 9 μL of 2 M ammonium acetate (pH 4.5) and 900 μL of 100% EtOH. This solution was vortexed, immediately divided among four separate tubes, and chilled at −20° C for at least 2 h [each tube contained approximately 30 μL (~2–3 μg) of denatured plasmid]. When analyzing single clones, 10–30 μL of an individual plasmid preparation was used per sequencing reaction, and volumes of NaOH, NH_4OAc, and EtOH were adjusted accordingly. Denatured plasmid precipitates were pelleted by centrifugation and rinsed in 70% EtOH. Immediately prior to sequencing, supernatant was removed, and pellets were allowed to air-dry.

All sequencing reactions were carried out using United States Biochemical (USB) Corp. Sequenase version 2.0 sequencing kit reagents and Amersham dATP [α^{35}S] label (10 μCi·μL^{-1}). The sequencing strategy is shown in Figure 1. Both strands of the LSU rDNA inserts were sequenced using the amplification primers [D1R (forward) and D2C (reverse)] and two internal primers, "D1C" (reverse; 5'ACTCTCTTTTCAAAGTCCTT 3'; corresponds to *P. micans* LSU rRNA positions 388–369) and "D2Ra" (forward; 5'TGAAAAGGACTTTGAAAAGA3'; corresponds to *P. micans* LSU rRNA positions 365–384; Lenaers et al. 1989).

Denatured, precipitated plasmid clones were resuspended with 8 μL primer (0.5 pmol·μL^{-1} in 10 mM Tris-HCl, pH 7.5) and 2 μL 5× reaction buffer (USB), mixed, and incubated for 10 min at 37° C. During primer annealing, ice-cold labeling mix for three sequencing reactions was prepared by combining 2.1 μL ddH_2O, 3.0 μL 100 mM DTT (USB), 6.0 μL labeling mix (USB; diluted 1:4 with ddH_2O), 3.0 μL dATP [α^{35}S] (10 μCi μL^{-1}), 1.0 μL Sequenase version 2.0 (USB), and 0.5 μL pyrophosphatase (USB). Sequenase and pyrophosphatase were added immediately prior to the completion of the hybridization reactions.

Five microliters of labeling mix was added to the 10-μL hybridization reaction, mixed by gentle pipetting, and incubated for 1 min at room temperature. The remainder of the procedure followed the standard USB sequencing protocol except that termination reactions were allowed to proceed for 10 min. Products of the reactions were resolved on standard 6% polyacrylamide (19:1 acrylamide:*bis*-acrylamide), 8.3 M urea, 1× TBE gels. Gels were fixed in 10% methanol/10% glacial acetic acid for 30 min, dried onto Whatman 3MM paper at 80° C with applied vacuum, and exposed to either XAR-5 or XRP-5 X-ray film (Kodak) from periods of overnight to 2 days.

Sequence analysis. Consensus sequences from each culture were compiled using the editor function of PAUP (Swofford 1993) and then aligned with the help of conserved elements interspersed throughout the molecules. The alignment was subjected to a variety of phylogenetic analyses using heuristic methods (PAUP 3.1.1; Swofford 1993). The phylogenetic tree shown in Figure 5 was constructed using the following parameters: all characters weighted equally; sequence gap=missing data; stepwise addition; closest addition sequence; 1 tree held at each step during stepwise addition; tree-bisection-reconnection (TBR) branch-swapping performed; MULPARS option in effect; steepest descent option not in effect; maxtrees=200; branches having maximum length zero collapsed to yield polytomies; topological constraints not enforced; multi-state taxa interpreted as uncertainty; trees rooted using outgroup taxa AMAD01, AMAD06, GtPort, and TC02; and ACCTRAN character state optimization. Bootstrap analysis (Felsenstein 1985; 250 rounds) of the alignment matrix was also carried out with the same parameters as above, except that maxtrees=10 per replicate bootstrap (Fig. 5).

RESULTS

An average of 10 LSU rDNA clones (range 4–14) were obtained for each *Alexandrium* isolate. LSU rDNA cloned from different *Alexandrium* isolates varied slightly in length. In some cases, clones from a single isolate contained length heterogeneities and sequence ambiguities (Figs. 2, 3; Table 1).

The most dramatic example of length heterogeneities was found in all cultures of *A. tamarense* and *A. fundyense* from eastern North America, two Japanese *A. tamarense* from Ofunato Bay (OF041 and OF051), and two ballast water *A. tamarense* originating from Muroran, Japan (I72/21#2, I72/24#1). These organisms displayed an identical 2-basepair (TG) deletion at aligned positions 590–591 (Figs. 2A, 4). All isolates that harbored this heterogeneity contained at least two distinct classes of LSU rDNA: one that carries the 590–591 TG deletion and one that does not (Fig. 2B). With the exception of the Ofunato Bay *A. tamarense* (OF041 and OF051), cultures exhibiting the 590–591 deletion contained additional heterogeneity over aligned positions 106–110 and a single-basepair (G) deletion at position 148 (Figs. 3A, 4). These heterogeneities also reflect two classes of LSU rDNA (Fig. 3B), presumably the same two identified by the 590–591 deletion. To illustrate these sequences and their phylogenetic relationships, LSU rDNAs cloned from AFNFA3 (*A. fundyense*) have been denoted "AFNFA3.1" [identical to the reference sequence (PW06) at positions 106–110, 148, and 590–591] and "AFNFA3.2" [different from the reference sequence at positions 106–110, 148, and 590–591 (Fig. 4)].

Sequences from 33 *Alexandrium* cultures were subjected to phylogenetic analyses (Table 1). The proposed alignment is shown in Figure 4. Since both variants of AFNFA3 were included (AFNFA3.1 and AFNFA3.2), a total of 34 sequences were compared. Six eastern North American *A. tamarense/fundyense* and one ballast water *A. tamarense* (I72/21#2) were

excluded because the 148 and 590–591 deletions obscured portions of the sequencing ladders. Partial sequences from these cultures nevertheless made it clear that they are very similar, if not identical, to other eastern North American *A. tamarense/fundyense* and the ballast water *A. tamarense* (I72/24#1). Those organisms containing the 106–110, 148, and/or 590–591 heterogeneities that were incorporated into the final alignment are shown with the alternative nucleotides (relative to PW06 and AFNFA3.1) in order to identify them as a group of cultures that share unique LSU rDNA characteristics. Two Australian *A. catenella* (ACPP03 and ACPP09) were excluded from the final analysis because their sequences exhibited only one or two base differences from other Australian *A. catenella* (ACPP01 and ACPP02; Scholin 1993).

Eight distinct classes of sequences, or "ribotypes," were found among the 33 *Alexandrium* cultures compared (Fig. 5). The search resulted in over 200 equally parsimonious trees. The multitude of trees arose from small differences in terminal groups and the polychotomy of North American and temperate Asian sequences. Topologies of trees and significance of branching patterns were examined in several ways. First, PAUP outputs of "ensemble statistical indices" (Swofford 1993) were considered to gauge the "fit" of the sequence data and the tree topology. Trees had relatively high values of consistency and retention indices, suggesting a high degree of congruence between the resolution of major *Alexandrium* groups and their sequence characteristics (Wiley et al. 1991, Swofford 1993). Second, consensus trees were constructed to evaluate *Alexandrium* groupings common to all equally parsimonious trees (cf. Wiley et al. 1991, Swofford 1993). The consensus trees (strict, Adams, and majority-rule) revealed the same major groupings as depicted in Figure 5, indicating that rival trees resolve the same major classes of *Alexandrium* sequences (not shown). Third, the tree-building program was also initiated using "simple," "random," and "as is" addition sequences (Swofford 1993), all of which resulted in trees equivalent to that shown in Figure 5. Finally, bootstrap analysis was performed as a statistical test of branching patterns (Felsenstein 1985). Results of this test also support the existence of the same eight major *Alexandrium* ribotypes proposed in Figure 5. The particular tree chosen for publication reflects these major classes of sequences as well as several minor classes of sequences (Fig. 5, Table 2). Delineation of minor classes of sequences ("subribotypes") was based on fine-scale LSU rDNA variations such as those shown in Figures 2 and 3 (cf. Scholin 1993).

Five ribotypes subdivided members of the *A. tamarense/catenella/fundyense* species complex. The three remaining ribotypes were associated with cultures that clearly differ morphologically from this group; these distinct sequences were typified by 1) *A. affine*, 2) *A. minutum* and *A. lusitanicum*, and 3) *A. andersoni*. LSU rDNA from *A. minutum* and *A. lusitanicum* were identical.

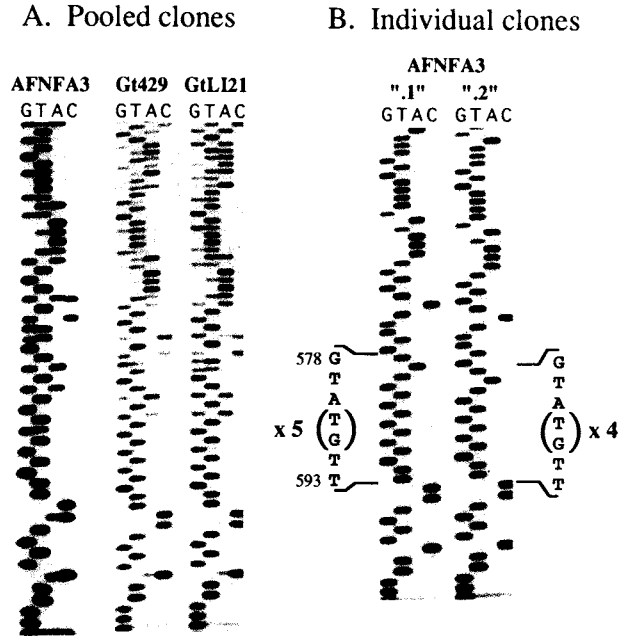


FIG. 2. Examples of the 590–591 TG length heterogeneity found in LSU rDNA cloned from all cultures of *A. tamarense* and *A. fundyense* from eastern North America, two Japanese *A. tamarense* from Ofunato Bay (OF041 and OF051), and two ballast water *A. tamarense* (I72/21#2, I72/24#1). Shown are D2C-primed sequencing ladders obtained using A) pooled or B) individual LSU rDNA clones from indicated isolates; sequences extend from aligned positions 606 (bottom) to 550 (top). Sequences from Gt429 and GtLI21 provide an example of clonal biasing: heterogeneity is evident in both ladders, but each emphasizes a different class of LSU rDNA. In contrast, pooled clones from AFNFA3 show the same two variants in an approximate 1:1 ratio. Individual clones from AFNFA3 reveal the heterogeneity to arise from a 2-basepair deletion at aligned position 590–591: .2 and .1 are designations referring to those molecules that do and do not carry this deletion, respectively.

The five distinct ribotypes within the tamarensis complex were named with reference to the geographic origin of the isolates: "North American," "Western European," and "Temperate Asian" designations reflect the origins of the majority of cultures within each cluster; "Tasmanian" and "Tropical Asian" designations reflect the origins of the only cultures defining those ribotypes. *Alexandrium* species designations were used to identify the three remaining ribotypes: "affine," "minutum," and "andersoni" (Table 2).

DISCUSSION

Sequences of LSU rDNA from geographically diverse representatives of the *Alexandrium* tamarensis species complex revealed the existence of at least five distinct lineages of sequences (Fig. 5, Table 2). These groups ("ribotypes") do not strictly correspond to morphospecies designations, suggesting that morphological features of *A. tamarense, A. catenella,*

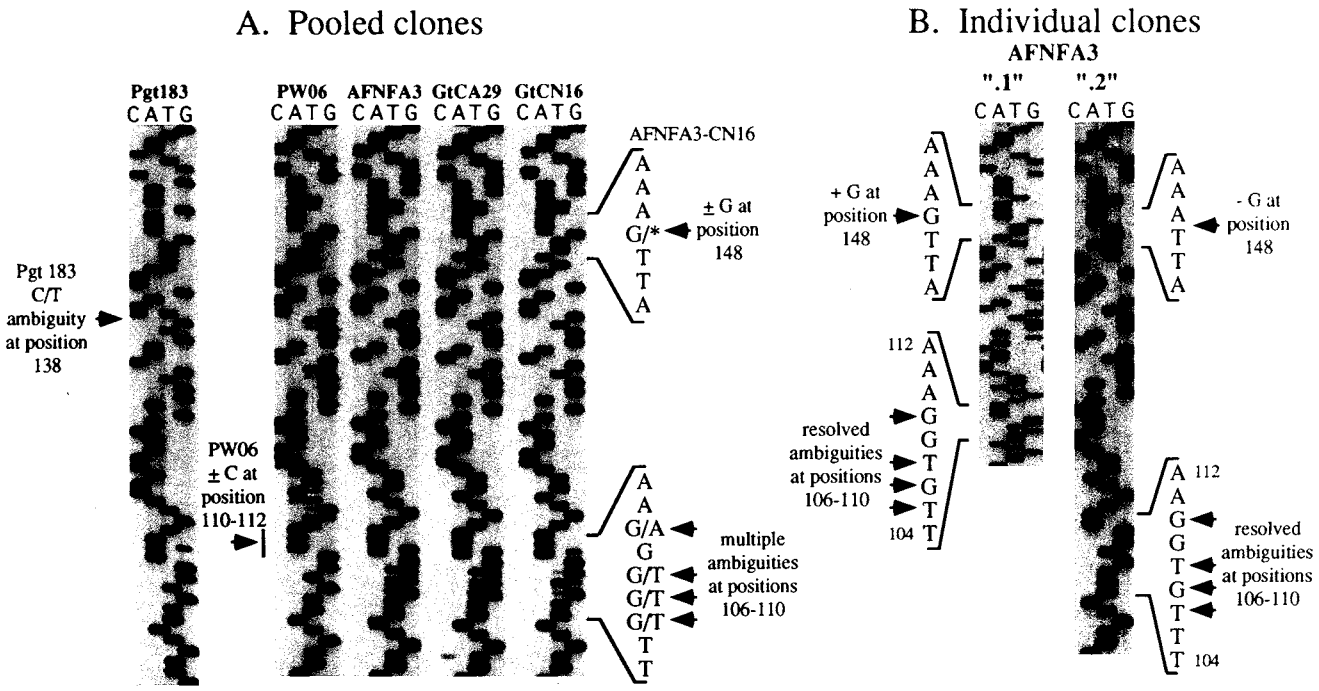


FIG. 3. Fine-scale LSU rDNA sequence heterogeneities seen in the D1 domain of *Alexandrium* LSU rDNA. A) Examples of length heterogeneities, sequence ambiguities, and clonal biasing observed in D1R-primed sequences of pooled LSU rDNA clones from indicated isolates. B) Resolution of some of these heterogeneities by sequencing individual LSU rDNA clones. The region shown extends from aligned positions 98 (bottom) to 164 (top). Several ambiguities are visible in the Pgt183 ladder, but only the C/T ambiguity is confirmed by complementary strand (D1C-primed) sequence (not shown). Single-base, C deletion within aligned positions 110–112 is evident in LSU rDNA from PW06; this heterogeneity is unique to PW06 and has not been recorded in Table 1 or Figure 4. With the exception of the two Ofunato Bay *A. tamarense* (OF041 and OF051), all cultures exhibiting the 590–591 deletion (Fig. 2) contain multiple ambiguities over aligned positions 106–110 and a single-basepair deletion at aligned position 148. Note example of clonal biasing seen in pooled clones from GtCA29 and GtCN16 (A). Analysis of individual clones from AFNFA3 reveal the same two classes of molecules (.1 and .2) emphasized in ladders obtained from GtCN16 and GtCA29, respectively (B).

and *A. fundyense* are less specific indicators of the organisms' relationships than are their LSU rDNA characteristics. The same discrepancy between rDNA sequence and morphological criteria is also evident for other dinoflagellate taxa (Lenaers et al. 1991, Rowan and Powers 1991). Particular regional populations of *A. tamarense, A. catenella,* and *A. fundyense* appear to have distinct sequence characteristics, although some of these regions (e.g. western Europe) are undersampled and currently represented by only a few or single isolates. Based on the isolates examined thus far, however, members of the tamarensis complex collected from the same geographic region are the most similar regardless of morphospecies designations, whereas those from geographically isolated populations are more divergent even when the same morphospecies are compared. *Alexandrium tamarense* and *A. catenella* from Japan are notable exceptions to this general trend, possibly because these organisms are the descendants of introduced species (see later). Likewise, the genetic identity of Japanese and Australian *A. catenella* suggests these two regional populations share a recent, common ancestry. LSU rDNA sequences from *A. affine, A. minutum, A. lusitanicum,* and *A. andersoni* show that these organisms are distinct from the tamarensis group. It is possible that *A. minutum, A. lusitanicum,* and *A. andersoni* are members of another species complex, with *A. affine* being clearly separable from this cluster as well as from the tamarensis complex.

Relationships among the *Alexandrium* isolates used in this investigation were previously assessed by RFLP analysis of SSU rDNA (Scholin and Anderson 1994). The restriction tests were specifically designed to screen cultures for the presence of two distinct classes of SSU rDNA, the "A gene" and "B gene," found in a North American *A. fundyense* (GtCA29; Scholin et al. 1993). The enzymes used in that study detected only a few differences between the A and B sequences but nonetheless were useful in typing a variety of *Alexandrium* species and populations. SSU rDNA RFLP patterns revealed three subdivisions (termed Groups I–III) within the tamarensis complex. Here we resolve at least five LSU rDNA ribotypes in that same group: North American, Western European, Temperate Asian, Tasmanian, and Tropical Asian (Fig. 5, Table 2). Interestingly, a subset of isolates that harbor the A and B genes (all Group I and some Group II) also

TABLE 2. *Comparison of SSU rDNA RFLP groups, LSU rDNA ribotypes, toxicity, species designations, and isolation locales of* Alexandrium *isolates.*

SSU RFLP group[a]	LSU ribotype[b]	Strain	Toxic?[c]	Species designation	Isolation locale
I	North American Eastern[d]	AFNFA3	Yes	*A. fundyense*	Newfoundland
		AFNFA4	Yes	*A. fundyense*	Newfoundland
		GtCA29	Yes	*A. fundyense*	Cape Ann, MA
		GtMP	Yes	*A. fundyense*	Orleans, MA
		I72/24#1	Yes	*A. tamarense*	Ballast water (Muroran, Japan)[e]
II	Western[d]	PW06	Yes	*A. tamarense*	Port Benny, AK
		PI32	Yes	*A. fundyense*	Porpoise Isl., AK
		BGt1	Yes	*A. catenella*	Russian River, CA
	Alternate[d]	OF041	Yes	*A. tamarense*	Ofunato Bay, Japan
		OF051	Yes	*A. tamarense*	Ofunato Bay, Japan
III	Western European	Pgt183	No	*A. tamarense*	Plymouth, U.K.
		PE1V	Noξ	*A. tamarense*	Galicia, Spain
		PE2V	No	*A. tamarense*	Galicia, Spain
		WKS-1	No	*A. tamarense*	Tanabe Bay, Japan
	Temperate Asian Japanese[d]	OF101	Yes	*A. catenella*	Ofunato Bay, Japan
		TN9	Yes	*A. catenella*	Tanabe Bay, Japan
		WKS-8	Yes	*A. catenella*	Tanabe Bay, Japan
		ACPP01	Yes	*A. catenella*	Port Phillip Bay, Victoria
		ACPP02	Yes	*A. catenella*	Port Philip Bay, Victoria
		ACJP03	Yes	*A. catenella*	Ballast water (Kashima, Japan)[e]
		G. Crux	Yes	*A. catenella*	Ballast water (Singapore?)[e]
	Korean[d]	G. Hope 1	Yes	*A. tamarense*	Ballast water (Samchonpo, S. Korea)[e]
		G. Hope 2	Yes	*A. tamarense*	Ballast water (Samchonpo, S. Korea)[e]
	Tasmanian	ATBB01	Noξ	*A. tamarense*	Bell Bay, Tasmania
	Tropical Asian	CU13	Yes	*A. tamarense*	Gulf of Thailand
IV	"affine"	AABB01/2	No	*A. affine*	Bell Bay, Tasmania
		CU1	No	*A. affine*	Gulf of Thailand
		PA5V	No	*A. affine*	Galicia, Spain
V	"minutum"	AMAD01	Yes	*A. minutum*	Port River, South Australia
		AMAD06	Yes	*A. minutum*	Port River, South Australia
		GtPort	Yes	*A. lusitanicum*	Portugal
	"andersoni"	TC02	No	*A. andersoni*	Eastham, MA

[a] Based on results of the A/B gene restriction tests (Scholin and Anderson 1994).
[b] Subdivisions based on LSU rDNA phylogeny (Fig. 5).
[c] Determined by mouse bioassay and/or HPLC analysis; ξ = may contain trace amounts of toxin (D. Kulis, pers. commun.)
[d] Preliminary "subribotype" designations based on fine-scale LSU rDNA sequence variation (Figs. 2, 3, 5; Scholin 1993).
[e] Origin of ballast water (Hallegraeff and Bolch 1992).
[f] Hailing port of vessel; origin of ballast water uncertain (Hallegraeff and Bolch 1992).

contain at least two distinct classes of LSU rDNA (Figs. 2, 3; Table 2). In addition, the SSU rDNA RFLP assay differentiated between *A. affine* (Group IV) and *A. minutum/lusitanicum/andersoni* (Group V) but the latter group of species shared identical restriction patterns. LSU rDNA sequences, on the other hand, clearly indicate that *A. andersoni* is distinct from the *A. minutum/lusitanicum* cluster. Thus, ribotypes ascribed by LSU rDNA sequences are in complete agreement with and offer a finer-scale resolution of groups defined by the SSU rDNA RFLP analyses.

Toxic *Alexandrium* cluster at several different termini on the phylogenetic tree (Fig. 5). The North American, Temperate Asian, and minutum groups thus far consist exclusively of toxic isolates. In contrast, the Western European group encompasses only nontoxic organisms. Terminal taxa classified as Australian, Tropical Asian, and andersoni are also nontoxic. Preliminarily, this suggests that an organism's ability to produce toxin is correlated with its evolutionary history or LSU rDNA phylogenetic lineage. As the data base of sequences from toxic and nontoxic *Alexandrium* species grows, it will be possible to address this potential correlation. Because toxic representatives of the tamarensis complex are known to inhabit western Europe (Blanco et al. 1985), we expect this correlation will either break down, or those isolates will appear unique, or show close relationships to other regional populations.

LSU rDNA ribotypes, protein electrophoretic patterns, and cell surface antigens appear to delineate the same groups of related strains. For example, isozyme banding patterns of eastern North American *A. tamarense/fundyense*, a western European *A. tamarense* (Pgt 183), a Spanish *A. affine* (PA5V), and an eastern North American *A. andersoni* (TC02) showed the eastern North American *A. tamarense/fundyense* group to be a single, closely related cluster; relative to that group, Pgt183, PA5V, and TC02 are progressively more divergent (Hayhome et al. 1989). Here we show that LSU rDNA sequences for

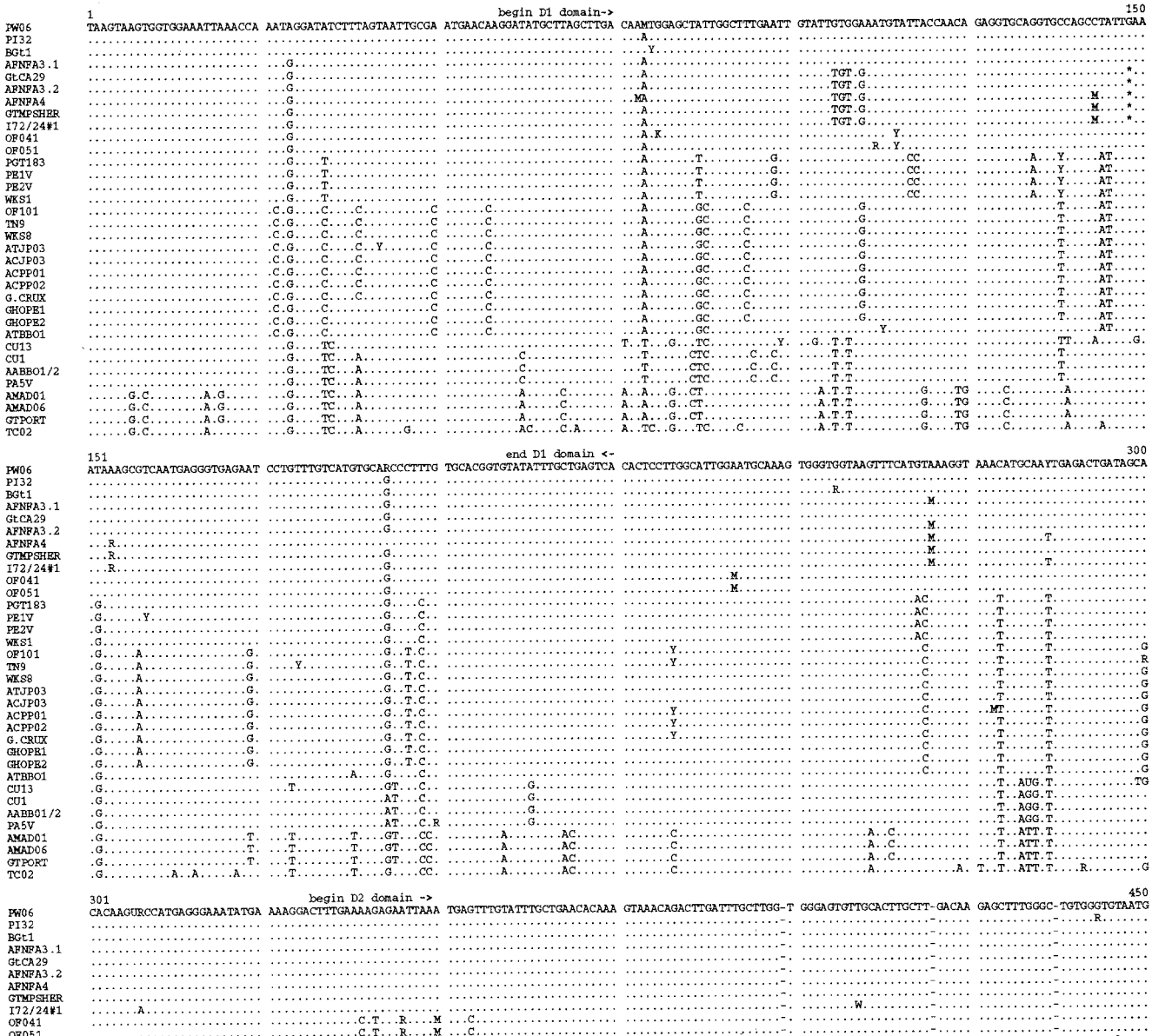
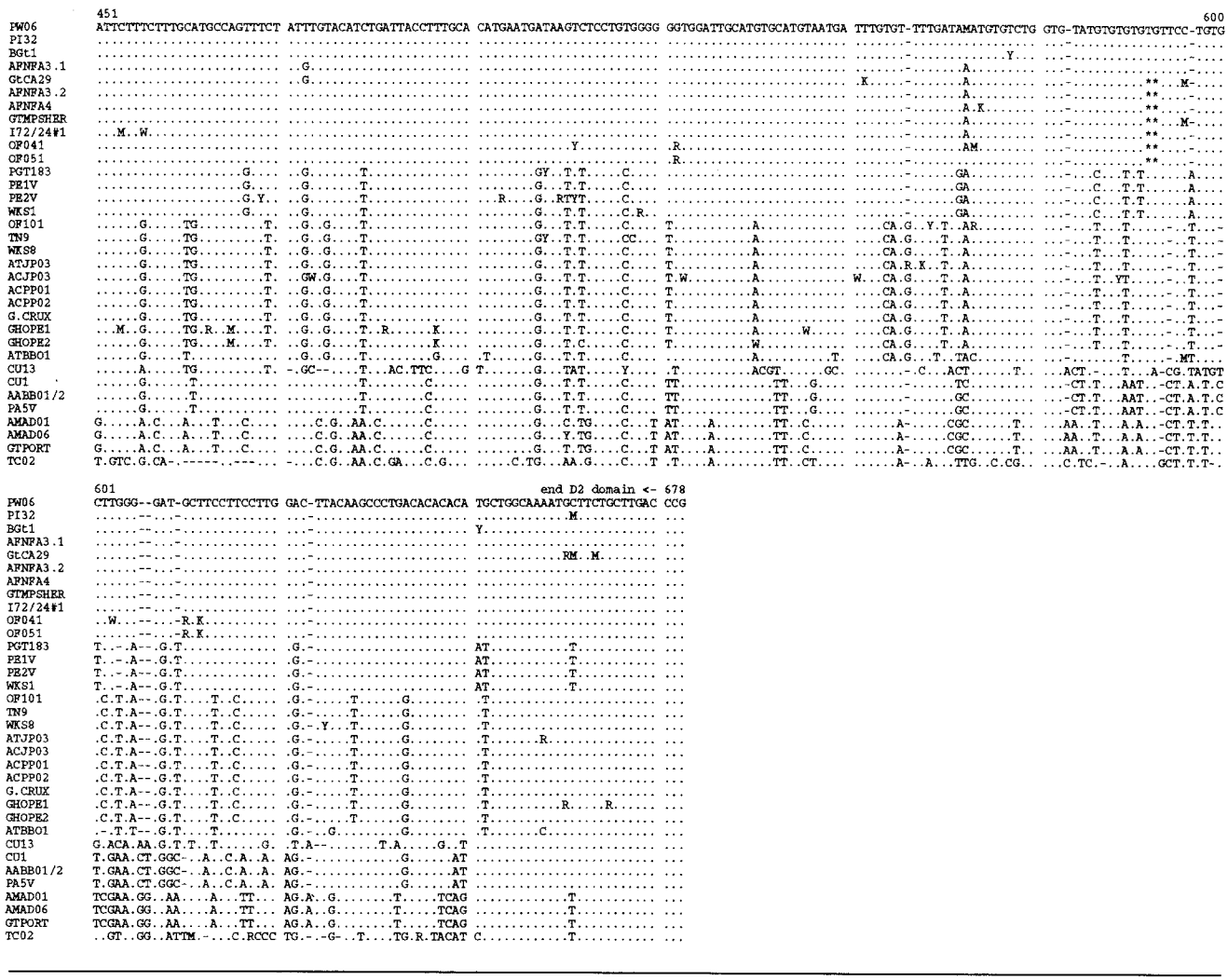


FIG. 4. Proposed LSU rDNA sequence alignment exclusive of amplification primers for *Alexandrium* cultures (see Table 1 for species designations and isolation locales). Alignment position 1 corresponds to *P. micans* LSU rRNA position 44 (Lenaers et al. 1989). PW06 (*A. tamarense*; Alaska) is used as the reference sequence; all equivalent positions are indicated by a period. Dashes represent inserted alignment gaps. Two sequences for AFNFA3 (*A. fundyense*; Newfoundland) are shown: AFNFA3.1 is similar to PW06 at positions 106–

these same cultures reveal an identical tree topology [Fig. 5, Table 2 (North American > Western European > affine > andersoni)]. In a separate study, Sako et al. (1990, 1993) reported that OF041 (*A. tamarense*) and OF101 (*A. catenella*), both from Japan, are distinguishable on the basis of their isozyme



←

110, 148, and 590–591; AFNFA3.2 differs from PW06 at positions 106–110 and harbors the 148 (*) and 590–591 (**) deletions (see Figs. 2, 3). Those organisms containing the 106–110 ambiguities, 148, and/or 590–591 length heterogeneities are shown here as the AFNFA3.2-like variant. Approximate boundaries of the D1 and D2 hypervariable domains are noted. Sequence ambiguities are reported using standard IUPAC nomenclature (R = A or G; Y = C or T; M = C or A; K = G or T; W = A or T).

electrophoretic patterns and their reactivity toward monoclonal antibodies. In our study, their rDNA sequences place them into two different ribotypes: the North American and Temperate Asian groups, respectively (Table 2). Thus, biochemical and molecular characterizations of *Alexandrium* species have yielded equivalent results.

There is no strict correlation among *A. tamarense*, *A. catenella*, and *A. fundyense* morphospecies designations and the ribotypes of their globally distributed representatives. The various morphotypes can appear genetically similar or genetically distinct, depending on the particular strains (populations) compared (Fig. 5, Table 2; for the purposes of this discussion, "morphotype" refers to the ensemble of genes responsible for morphology, whereas "genotype" refers to specific subcellular characteristics, such as rDNA sequence). For example, GtCA29 (*A. tamarense*; North America) is genetically distinct from ACPP01 (*A. catenella*; Australia). However, the former isolate is also genetically divergent from other *A. tamarense* (G. Hope 1 and 2 from South Korea, and Pgt183 from western Europe; Fig. 5, Table 2). Many examples of agreements and disagreements between ribotype and morphotype are evident (Table 2). This pattern is not attributable to differences between taxonomists (Scholin and Anderson 1994). Instead, it appears to arise from phenotypic overlap between genetically distinct populations (e.g. North American and western European *A. tamarense*) as well as phenotypic plasticity within regional populations (e.g. western North American *A. tamarense/catenella/fundyense*).

Results of this study therefore offer an explanation as to how parallel investigations of the relatedness of *A. tamarense*, *A. catenella*, and *A. fundyense*

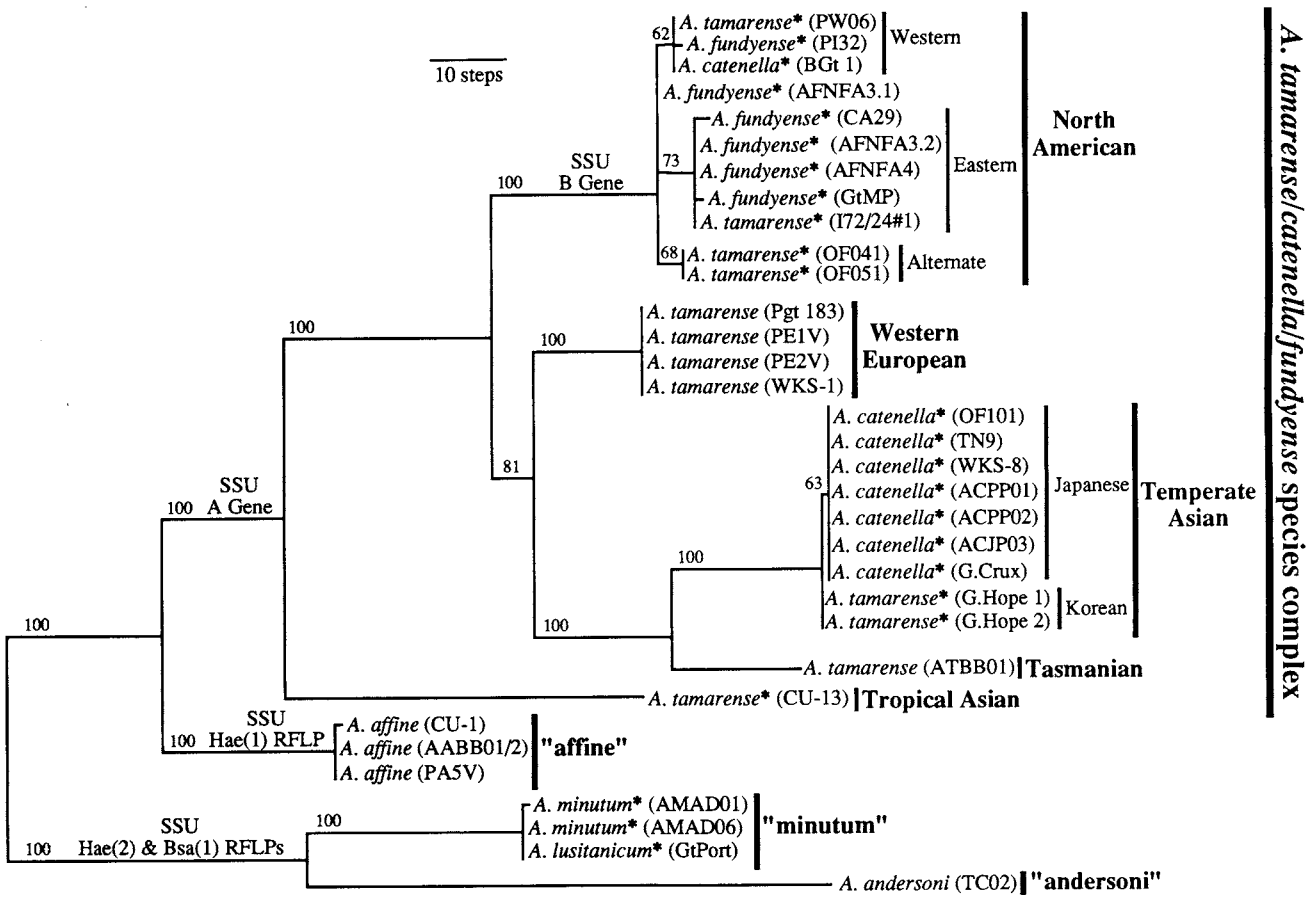


FIG. 5. Parsimony phylogenetic tree inferred from aligned *Alexandrium* LSU rDNA sequences generated by PAUP 3.1.1 (Swofford 1993). Tree statistics are as follows: length = 403; consistency index (CI) excluding uninformative characters = 0.802; rescaled CI (RC) = 0.790; retention index (RI) = 0.948. The tree was rooted using the outgroup method, with *A. minutum*, *A. lusitanicum*, and *A. andersoni* defined as outgroup taxa. These isolates were chosen as the outgroup because their sequences share a common SSU rDNA restriction pattern that is distinct from *A. affine* and members of the tamarensis complex (Table 2) and because they are the most divergent taxa relative to representatives of the tamarensis complex. Horizontal branch lengths reflect the relatedness of the sequences; scale bar represents a divergence of 10 steps. North American, Western European, Temperate Asian, Tasmanian, Tropical Asian, "affine," "minutum," and "andersoni" are proposed ribotype designations of terminal taxa; western, eastern, and alternate and Japanese and Korean are subribotypes of the North American and Temperate Asian groups, respectively. * denotes toxic isolates. SSU rDNA RFLP characteristics for the cultures are also shown on the appropriate branches; note correspondence between those patterns and the LSU rDNA phylogeny (see also Table 2). Numbers indicate the frequency that taxa to the right of the value were found to group together upon bootstrap analysis (250 iterations; Felsenstein 1985, Swofford 1993).

have yielded both positive and negative correlations between morphospecies designations and biochemical/genetic characteristics. Generally speaking, groups of genetically similar isolates are associated with specific geographic regions, and each of these groups can contain one or more morphospecies (Fig. 5, Table 2). The overall resolution afforded by the LSU rDNA phylogeny is thus one of *geography*, not morphology, indicating that different regional populations of the tamarensis complex can have unique combinations of "morphotypes" and "genotypes." Consequently, comparative studies of biochemical and morphological characteristics of isolates (i.e. tests of the morphospecies concept) could yield vastly different results depending on which regional population(s) are sampled and which particular cultures are chosen for comparison. Studies to date have indeed revealed such confusing relationships: isolates collected primarily from North America show no consistent correlation between morphotype and biochemical properties (Cembella et al. 1987, 1988, Hayhome et al. 1989), yet isolates collected from Japan reveal the exact opposite (Sako et al. 1990, 1993). The latter example, however, appears to be a special case where strains examined not only differed morphologically but also (despite their present coexistence in a region) were fortuitously derived from different primordial populations (see later). We conclude that tests of the morphospecies concept must be interpreted in the context of global population biogeography and natural history (Scholin et al. unpubl.). In this light, it is apparent that the tamarensis complex is comprised of multiple, genetically distinct strains whose morphological fea-

tures alone do not adequately describe their relationships.

We believe that the confusing associations between morphotype and genotype for members of the tamarensis complex are rooted in the evolutionary history of the organisms. Cembella et al. (1988) reasoned that morphological variation within this group is not attributable to independently evolved, distinct ancestral lines, a conclusion that agrees with our results. We favor the hypothesis that *A. tamarense/catenella/fundyense* arose monophyletically and over millions of years was dispersed to various regions of the world. Further, we suggest that the resulting populations were geographically isolated and diverged genetically but maintained an overall conserved morphology. This hypothesis predicts that phylogenetic lineages of the tamarensis complex reflect the independent evolution of isolated populations, not genetically distinct morphospecies (Scholin et al. unpubl.).

Among the isolates examined thus far, this prediction is largely met. Geographically cooccurring *A. tamarense, A. catenella,* or *A. fundyense* appear to be closely related, while geographically separated populations of any one of these species are divergent. For example, isolates from Australia, North America, or western Europe are distinguishable from one another, but within each of these regions there is a high degree of similarity, or even identity. However, two exceptions to this general pattern are noteworthy. First, *A. tamarense* and *A. catenella* collected from Japan are found in Temperate Asian, North American, or Western European ribotypes. Second, toxic *A. catenella* from Australia are identical to the Temperate Asian strains found in Japan.

One explanation for genetic continuities between geographically isolated populations is suggested by sequences from *A. tamarense/catenella* cysts collected from the ballast water of ships. In one instance, a cargo vessel ballasted in Japan during an *A. catenella* bloom arrived in Australia containing viable *A. catenella* cysts genetically identical to known populations of this species in both countries. In two other separate cases, ships ballasted during *A. tamarense* blooms in Japan and South Korea also inadvertently transported viable resting cysts to Australia. However, these cysts proved to be genetically distinct from *A. tamarense/catenella* previously found in Australia and genetically distinct from each other, as well (Table 2). Thus, human-assisted dispersal of *A. tamarense/catenella* from genetically and geographically diverse populations could occur and is a plausible mechanism whereby a region may be inoculated with one or more ribotypes. Exchange of shellfish stocks and natural movements of water masses are also important vectors of dispersal, both of which could foster the immigration of one or more tamarensis complex representatives to new locations (Anderson 1989, Hallegraeff and Bolch 1991). Further discussion of the evolution and global dispersal of the tamarensis complex will be presented at a later date.

Representatives of *A. affine, A. minutum, A. lusitanicum,* and *A. andersoni* were included in the analysis in order to assess the fidelity of LSU rDNA sequences to discriminate what are considered to be distinct species from those within the tamarensis group. In accordance with current morphotaxonomic designations, *A. affine, A. minutum, A. lusitanicum,* and *A. andersoni* are clearly divergent from representatives of that large species complex. The distinction between *A. affine* and the *A. minutum/lusitanicum/andersoni* cluster, and further differentiation between *A. andersoni* and the *A. minutum/lusitanicum* group, also agree with established taxonomic criteria. However, the LSU rDNA sequences fail to differentiate between *A. minutum* and *A. lusitanicum.* Hallegraeff (pers. commun.) has suggested that *A. minutum, A. lusitanicum,* and *A. andersoni* are closely related and that their morphological differences may not warrant unique species designations. In part, his view is supported by the LSU rDNA sequence data and toxicity determinations: *A. minutum* and *A. lusitanicum* share the same ribotype and are both toxic. However, the *A. andersoni* sequence is clearly different, and *A. andersoni* is also nontoxic. Recent work by Franco et al. (1994) indicates that *A. minutum* and *A. lusitanicum* may indeed by synonyms. Thus, a growing body of data supports a distinction between the *A. minutum/lusitanicum* group and *A. andersoni,* but not between *A. minutum* and *A. lusitanicum.* It seems possible that *A. minutum, A. lusitanicum,* and *A. andersoni* are members of a species complex that is analogous to the tamarensis group. Morphological and genetic characterization of additional *A. minutum, A. lusitanicum,* and *A. andersoni* from diverse source populations is needed to address this possibility.

In summary, the recognition of genetically distinct *Alexandrium* species and populations offers a new reference point from which debates concerning the relationships between morphological and biochemical characters may be viewed. Results of the present study could foster a resolution to this long-standing controversy, and thus a unified systematic scheme may now be in reach. The definition of genetic markers for certain regional populations also sets the stage for their use in testing dispersal hypotheses. To facilitate the latter, a LSU rDNA-based RFLP assay is currently under development to simplify and speed analyses of additional isolates for biogeographic studies (Judge et al. 1993, Scholin et al. unpubl.).

An encouraging aspect of the growing SSU and LSU rDNA data base is the identification of *Alexandrium* genus-, species- and strain-specific "signature sequences." Oligonucleotide probes designed to recognize each of these markers are now being tested. It is conceivable that this series of probes could be used to rapidly identify, enumerate, and

separate whole cells collected in culture or field samples (e.g. Amann et al. 1990). The reactivity of organisms toward certain probes may also be useful for making predictions about their isozyme characteristics, toxin production capability, antibody cross-reactivity, or mating type affinity if strong associations between particular ribosomal signatures and biochemical characteristics actually exist.

The authors thank I. Bravo, S. Hall, G. Hallegraeff, M. Kodama, L. Provasoli, Y. Sako, and A. White for providing cultures used in this investigation. S. Blackburn, C. Bolch, and D. Kulis are thanked for their many contributions to the culturing efforts and toxicity determinations as well as light and electron microscopy. G. Lenaers and other members of Laboratoire Arago inspired the LSU rDNA sequencing and generously provided unpublished sequences of PCR and sequencing primers. E. DeLong kindly contributed equipment and technical advice. This work was supported by grants from the National Science Foundation No. OCE 8911226 (D.M.A.), French Centre National de la Recherche Scientifique No. URA 117 (M.H.), Woods Hole Oceanographic Institution Ocean Ventures Fund (C.A.S.), and the Vettelsen Foundation. Contribution No. 8259 from the Woods Hole Oceanographic Institution.

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