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LSU rDNA-BASED RFLP ASSAYS FOR DISCRIMINATING SPECIES AND STRAINS OF *ALEXANDRIUM* (DINOPHYCEAE)[1]

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ABSTRACT

In a previous study large-subunit ribosomal RNA gene (LSU rDNA) sequences 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 were compared to assess inter- and intraspecific relationships. Many cultures compared in that study contained more than one class of LSU rDNA. Sequencing pooled clones of rDNA from single cultures revealed length heterogeneities and sequence ambiguities. This complicated sequence comparisons because multiple rDNA clones from a single culture had to be sequenced individually to document the different classes of molecules present in that culture. A further complication remained as to whether or not the observed intraculture sequence variations were reliable genetic markers or were instead artifacts of the polymerase chain reaction (PCR) amplification, cloning, and / or sequencing methods employed. The goals of the present study were to test the accuracy of* Alexandrium *LSU rDNA sequences using restriction fragment-length polymorphism (RFLP) analysis and to devise RFLP-based assays for discriminating among representatives of that group. Computer-assisted examination of the sequences allowed us to identify a set of restriction enzymes that were predicted to reveal species, strain, and intraculture LSU rDNA heterogeneities. All groups identified by sequencing were revealed independently and repeatedly by RFLP analysis of PCR-amplified material. Five ambiguities and one length heterogeneity, each of which ascribes a unique group of* Alexandrium *species or strains, were confirmed by restriction digests. Observed intraculture LSU rDNA heterogeneities were not artifacts of cloning and sequencing but were instead a good representation of the spectrum of molecules amplified during PCR reactions. Intraculture LSU rDNA heterogeneities thus serve as unique genetic markers for particular strains of* Alexandrium, *particularly those of* A. tamarense, A. catenella, *and* A. fundyense. *However, some of these "signature heterogeneities" represented a smaller portion of PCR product than was expected given acquired sequences. Other deviations from predicted RFLP patterns included incomplete digestions and appearance of spurious products. These observations indicate that the diversity of sequences in PCR product pools were greater than that observed by cloning and sequencing. The RFLP tests described here are useful tools for characterizing* Alexandrium *LSU rDNA to define the evolutionary lineage of cultures and are applicable at a fraction of the time, cost, and labor required for sequencing.*

Key index words: Alexandrium; *biogeography; Dinophyceae; LSU rDNA; paralytic shellfish poisoning; PCR; red tide; RFLP*

Dinoflagellates of the genus *Alexandrium* (Halim) Balech include a number of species that produce saxitoxin and its derivatives, potent neurotoxins responsible for a syndrome commonly referred to as paralytic shellfish poisoning, or PSP (Prakash et al. 1971). In an effort to better define species boundaries and to assess intraspecific genetic divergence among representatives of this genus, researchers have increasingly turned to comparisons using both morphological and subcellular criteria (e.g. Cembella et al. 1988, Hayhome et al. 1989, Sako et al. 1990, 1993, Destombe et al. 1992, Adachi et al. 1994, Anderson et al. 1994). In earlier studies, we and others have shown that a number of *Alexandrium* species as defined morphologically, including *A. affine* (Fukuyo et Inoue) Balech, *A. andersoni* Balech, and *A. minutum* Halim, are also recognizable as distinct groups on the basis of nuclear-encoded small- and large-subunit ribosomal RNA (SSU and LSU rRNA) gene (rDNA) sequences. In contrast, representatives of *A. tamarense* (Lebour) Balech, *A. catenella* (Whedon et Kofoid) Balech, and *A. fundyense* Balech (the "*tamarensis* complex") are not distinguished as three distinct groups using the same subcellular criteria (Scholin et al. 1994a). Instead, globally distributed populations of the latter species are comprised of at least five major evolutionary lineages. These lineages do not reflect the development of different morphotypes but instead reveal a monophyletic radiation and independent evolution of geographically isolated regional populations (Scholin et al. 1995).

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Collection and analysis of rDNA sequences from a variety of *Alexandrium* species has proven labor-intensive and difficult. In many cases, clonal, unialgal isolates contained multiple classes of rDNA not attributable to culture contaminants. Thus far we have documented a SSU pseudogene (Scholin et al. 1993), a SSU pseudogene harboring an insertion (direct repeat), chimeras of SSU rDNA containing segments representative of both expressed and pseudogene sequences (Scholin and Anderson 1994), and multiple classes of LSU rDNA as defined by sequence ambiguities and length heterogeneities. Adachi et al. (1996) have also found two classes of molecules in a fragment of the rDNA cistron that includes the internal transcribed spacer regions (ITS1 and 2) and 5.8S coding sequence. The objective of this work was to devise restriction fragment-length polymorphism (RFLP) analyses to identify terminal taxa (termed "ribotypes" and "subribotypes") given acquired LSU rDNA sequences. Preliminary results of this work were presented by Judge et al. (1993). The principles governing this type of assay are described in detail elsewhere (Scholin and Anderson 1994, Scholin et al. 1994b, and references therein).

Ribotypes and subribotypes of *Alexandrium* are defined on the basis of the most prevalent class of LSU molecule cloned from the organisms and also on unique combinations of heterogeneous classes of rDNA (Table 1; Scholin et al. 1994a). For example, the ribotype North American is comprised of three distinct subgroups, or subribotypes, all of which were defined by unique sequence ambiguities and length heterogeneities (Fig. 1a–c). Subribotypes of the temperate Asian group were defined similarly (Fig. 1d, e). Multiple classes of rDNA in some *Alexandrium* representatives hinders sequence comparisons because multiple rDNA clones from each culture must be sequenced individually to document different classes of molecules in that culture (Scholin et al. 1993). Furthermore, clonal biasing (i.e. the random selection of one sequence variant over another from a common pool of polymerase chain reaction [PCR] products) affects the appearance of sequences generated for a given culture when relatively few clones are pooled prior to sequencing (Scholin et al. 1994a; e.g. Fig. 1b). The result is that one may obtain sequencing ladders in which a particular class of molecule is over- or underrepresented relative to its actual abundance in the pool of PCR products. Errors may thus be propagated in the sequence data base, in turn affecting all downstream applications of that information.

Despite these obstacles, comparison of rDNA sequences from different populations and species of *Alexandrium* remains a valuable tool for taxonomic studies and biogeographic surveys. Ironically, the sequence heterogeneities and length variants that complicate and slow analyses also offer fine-scale resolution of distinct groups contained within larger regional populations. This fine-scale resolution appears useful for studying the evolutionary history of the organisms and routes of natural and human-assisted dispersal (Scholin et al. 1995). However, a complication remains as to whether or not these fine-scale sequence differences are in fact reliable genetic markers or are instead artifacts of the amplification, cloning, and/or sequencing methods used thus far.

In this article, we report on efforts to test and improve the accuracy of our *Alexandrium* LSU rDNA sequence data base. To this end we pose two questions: 1) Were sequences acquired by a PCR/cloning strategy a fair representation of the total pool of molecules found in PCR products? 2) Can we speed characterization of LSU rDNA from additional *Alexandrium* isolates to facilitate studies of their molecular diversity, biogeography, and dispersal? Results of this work are discussed with respect to the accuracy of the existing *Alexandrium* phylogeny, clonal biasing of acquired sequence data, and future applications of rDNA-based RFLP assays.

MATERIALS AND METHODS

A list of isolates used in this investigation is presented in Table 1. Each isolate represents a unique phylogenetic group as described by Scholin and Anderson (1994) and Scholin et al. (1994a).

GenBank accession numbers for reference sequences (Table 1) are as follows: western North American (*A. tamarense*, clone PW06) U44927; eastern North American ".1" (*A. fundyense*, clone AFNFA3.1) U44926; eastern North American ".2" (*A. fundyense*, clone AFNFA3.2) U44928; alternate North American [*A. tamarense* (AFNFA3.2-like variant), clone OF041] U44929; western European (*A. tamarense*, clone Pgt183) U44930; Japanese temperate Asian (*A. catenella*, clone OF101) U44931; Korean temperate Asian (*A. catenella*, clone AtGHope 1) U44932; Tasmanian (*A. tamarense*, clone ATBB01) U44933; Tropical Asian (*A. tamarense*, clone CU13) U44934; *affine* (*A. affine*, clone CU1) U44935; *minutum* (*A. minutum*, clone AMAD06) U44936; and *andersoni* (*A. andersoni*, clone TC02) U44937. The sequences AFNFA3.1 and AFNFA3.2 are thought to reside in rDNA operons that harbor the SSU A and B genes (Scholin and Anderson 1994), respectively. The AFNFA3.1 class is expressed as a stable LSU rRNA molecule, whereas the AFNFA3.2 class is not (unpubl. data).

Reference sequences were scanned for restriction sites using MacDNASIS Pro software (v. 1.0) and then compared to determine which enzymes would delineate one or more ribotypes; *Afl* III (New England Biolabs; NEB), *Apa* LI (NEB), *Hinc* II (NEB), *Mse* I (NEB), and *Nsp* I (United States Biochemical Corp.) were chosen for further testing.

Two fragments of LSU rDNA, "D1R/D2C" and "D1R/D3Ca," were digested with the preceding enzymes. The former fragment is the same as that analyzed by cloning and sequencing as described by Scholin et al. (1994a). The latter is a longer fragment that includes the D1R-D2C region as well as ~200 basepairs (bp) distal to the D2C primer target (Scholin et al. 1994b). Both fragments encompass variable as well as conserved sequence motifs (Lenaers et al. 1989). Sequences of the D1R, D2C, and D3Ca primers, their relative target positions within the LSU rDNA, and their relationship to other taxa are given by Scholin et al. (1994a, b and references therein).

PCR amplifications were accomplished as described previously (Scholin et al. 1994a) with the following exceptions: template concentration was varied from 1–5 ng; amplifications for each isolate were done in duplicate; and a Perkin Elmer 4800 ther-

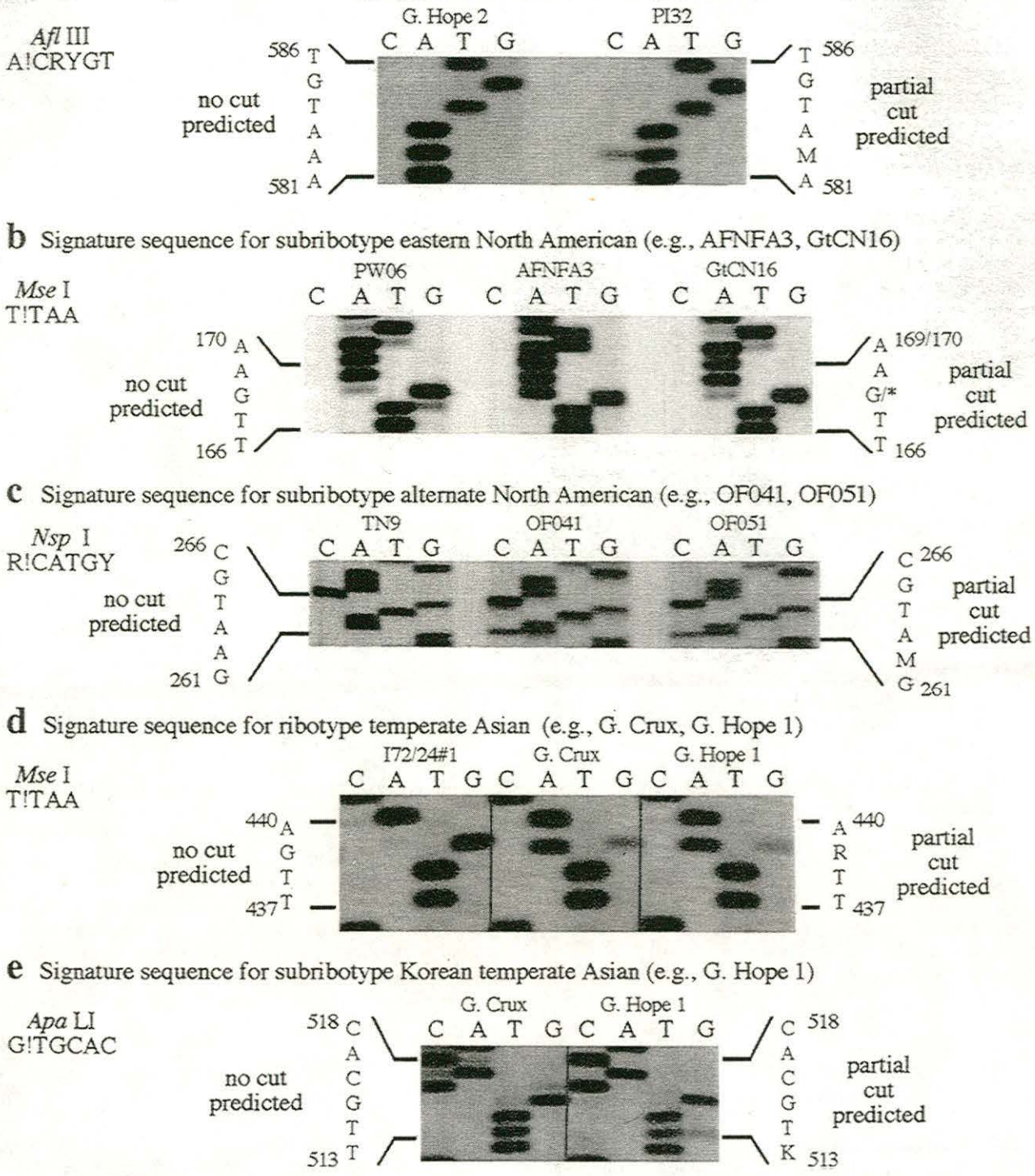


FIG. 1. Autoradiographs of sequencing gels showing examples of LSU rDNA "signature heterogeneities" present in *tamarensis* complex representatives of the North American and temperate Asian ribotypes (Table 1), and the restriction enzymes used to detect those signature nucleotides (Tables 2, 3). The sequence ambiguities and length heterogeneity shown were observed using a PCR amplification/cloning/sequencing methodology (Scholin et al. 1994a). Each panel presents two or more sequencing ladders to illustrate the type of results that were obtained. Sequences representative of the a) western North American, b) eastern North American, c) alternate North American, d) temperate Asian, and e) Korean temperate Asian groups (right side) are compared to other members of the *tamarensis* complex that lack their signature heterogeneity (left side). Cultures from which the sequences were derived are coded at the top of the sequencing ladders (see Scholin et al. 1994a for culture code information). Numbers refer to nucleotide position within the D1R/D2C-primed fragment (bp), inclusive of the D1R primer sequence. Sequence ambiguities are reported using standard IUPAC nomenclature (R = A or G; Y = C or T; M = C or A; K = G or T); * A single base deletion. An example of clonal biasing is seen in

TABLE 1. Alexandrium *cultures representative of phylogenetic groups as defined by Scholin et al. (1994a) that were used as standards for RFLP analyses. Shown are their associated ribotype, subribotype, culture code, and species designations. For additional information regarding these isolates and others, see Scholin et al. (1994a).*

Phylogenetic group	Ribotype	Subribotype[a]	Representative culture	Species designation
1	North American	Western	PW06	*A. tamarense*
2	North American	Eastern	AFNFA3	*A. fundyense*
3	North American	Alternate	OF041	*A. tamarense*
4	Western European		Pgt183	*A. tamarense*
5	Temperate Asian	Japanese	OF101	*A. catenella*
6	Temperate Asian	Korean	G. Hopel	*A. tamarense*
7	Tasmanian		ATBB01	*A. tamarense*
8	Tropical Asian		CU13	*A. tamarense*
9	*affine*		CU1	*A. affine*
10	*minutum*		AMAD06	*A. minutum*
11	*andersoni*		TC02	*A. andersoni*

[a] Cultures representing eastern and alternate subribotypes of the North American cluster contain at least two classes of LSU rDNA, termed ".1" and ".2." Both variants cloned from AFNFA3 were submitted to GenBank; the sequence from OF041 was submitted as the AFNFA3.2-like variant (see Scholin et al. 1994a).

mocycler was programmed for 30 cycles of 94° C, 1 min; 50°–55° C, 1.5 min; 72° C, 1 min, with an autoextension (5 per cycle) of the 72° C step. Thermocycling ramp times between target temperatures were as fast as the machine allowed. Duplicate 100-μL amplification reactions were pooled and loaded into a Centricon ultrafilter (100K molecular weight cutoff; Amicon), spun at 500 × *g* for 10–15 min at 12° C, and then rinsed with 200 μL of dH_2O. Recovery of the PCR products followed manufacturer specifications, and the volume of retentate was adjusted to 20–30 μL with dH_2O. Purified products were stored at −20° C.

All restriction digests followed recommendations of the manufacturers and utilized buffers supplied with the enzyme. Water, buffer, and enzyme were prepared in a master mix; 10 μL of this was added to 2 μL aliquots of purified PCR product, each of which represented 1 of the 11 *Alexandrium* ribotypes (Table 1). Digests were thus prepared using 2–5 units of restriction enzyme and approximately 0.5–1.0 μg PCR product per 12 μL reaction; cuts proceeded for 4–18 h in a 37° C water bath. Tops of the tubes were covered with a styrofoam block to inhibit condensation (prevented excessive loss of water from small-volume digests). Restriction products were stored at 4° C.

Products of digestions were resolved using a 3:1 mixture of Nusieve:SeaPlaque agarose (FMC Corp.), 1× TAE buffer, and ethidium bromide incorporated in both the gel and running buffer (Ausubel et al. 1987). Samples were mixed with 2 μL of gel loading dye. The entire sample was applied to the gel and then run at 4 volts per centimeter for 1–2 h using a Fisher mini gel system. Gels were photographed using an MP-4 camera system and 667 print film. Sizes of PCR and digestion products were estimated by comparing their mobility to standards (BioMarker Low; Bio Ventures, Inc., Murfreesboro, Tennessee).

RESULTS

Over 40 enzymes showed promise as tools to distinguish signature nucleotides indicative of one or more terminal groups, and five of these were chosen for further analysis: *Afl* III, *Apa* LI, *Hinc* II, *Mse* I, and *Nsp* I. Restriction sites within the D1R/D2C-primed fragment for each of these enzymes and all *Alexandrium* ribotypes and subribotypes are given in Table 2. The choice of enzyme was based on predicted capacity to delineate as many unique groups as possible, to confirm/deny length variants or sequence heterogeneities considered diagnostic for certain ribotypes and subribotypes, and to yield digestion products in the range visible on a standard agarose gel (Fig. 1, Table 2).

D1R/D2C PCR products. Representatives of the temperate Asian and *andersoni* groups exhibited "signature amplifications." The former always appeared with higher and lower molecular weight bands in addition to the major product of ~700 bp. The *andersoni* representative always gave rise to a minor product slightly greater than 700 bp, in addition to the smaller but major product predicted on the basis of sequence data (Fig. 2a). The preceding patterns were observed consistently when template concentrations were varied from 1 to 5 ng per 100 μL amplification reaction and when primer annealing was set between 42° and 55° C while holding all other parameters constant as described by Scholin et al. (1994a). In contrast, PCR products from all other ribotypes appeared homogeneous using the same set of PCR reagents and same range of amplification conditions (Fig. 2a).

D1R/D2C digestion products. There was excellent agreement between predicted and observed RFLP patterns. All ribotypes and subribotypes as defined

←

b: sequences from both AFNFA3 and GtCN16 show heterogeneity at the same position (168), but each ladder emphasizes a different ratio. Clones from AFNFA3 reveal an approximate 1:1 ratio of molecules that do and do not carry the G deletion. In contrast, clones from GtCN16 are skewed more toward that class of molecule that does not carry the deletion. Also shown in b, the ladder for PW06 is out of register by 1 base on account of a single base deletion at positions 130–132 ("upstream" from the sequence shown here). For additional examples of clonal biasing and the effect of single and double base deletions on the resultant sequencing ladders, see Scholin et al. (1994a).

TABLE 2. *Restriction sites predicted for the D1R/D2C fragment of LSU rDNA from indicated* Alexandrium *ribotypes and subribotypes.*

Enzyme	Ribotype[a]	Subribotype	Restriction sites[b]
Afl III	North American	Western	±582
Apa LI	North American	Western	221
	North American	Eastern	220/221
	North American	Alternate	221
	Western European		221
	Temperate Asian	Japanese	221
	Temperate Asian	Korean	221, ±514
	Tasmanian		221, 514
	Tropical Asian		221, 319
	affine		221
	minutum		221
	andersoni		221
Hinc II	North American	Alternate	±368
	Tropical Asian		639
Mse I	North American	Western	13, 39, 367
	North American	Eastern	13, 39, ±167, 367
	North American	Alternate	13, 39, 367
	Western European		13, 39, 367
	Temperate Asian	Japanese	13, 39, 367, ±438, 581
	Temperate Asian	Korean	13, 39, 367, ±438, 581
	Tasmanian		13, 39, 367
	Tropical Asian		13, 39, 367, 648
	affine		13, 39, 58, 367
	minutum		13, 58, 367
	andersoni		13, 58, 296, 367
Nsp I	North American	Western	299, 480, 552, 558, ±582, 659
	North American	Eastern	298/299, 479/480, 551/552, 557/558, 656/659
	North American	Alternate	±262, 299, 480, 552, 558, 657/659
	Western European		480, 552, 558
	Temperate Asian	Japanese	558, 572, 644
	Temperate Asian	Korean	558, 572, 644
	Tasmanian		558, 572, 671
	affine		82, 553
	minutum		555, 585
	andersoni		545

[a] See Table 1; the full-length sizes of the templates (inclusive of the D1R and D2C amplification primers) are western North American, 708; eastern North American, 705/708; alternate North American, 706/708; Western European, 709; Japanese temperate Asian, 709; Korean temperate Asian, 709; Tasmanian, 709; tropical Asian, 706; *affine*, 711; *minutum*, 716; *andersoni*, 701.

[b] Nucleotide position within the D1R/D2C-primed LSU rDNA fragment distal to the site of cleavage; ± indicates sites where partial digestion is expected (see Table 3).

by sequencing were also revealed independently and repeatedly by restriction digests of PCR-amplified material (Figs. 1, 2, Tables 2–4). In particular, five ambiguities and one length heterogeneity (Table 3), as recorded by sequencing limited numbers of LSU rDNA clones (Fig. 1), were confirmed by RFLP analyses. These sites represent signature sequences indicative of multiple classes of LSU rDNA that define the ribotypes North American and temperate Asian, as well as their associated subribotypes (Table 1).

TABLE 3. *Examples of "signature heterogeneities" indicative of multiple classes of LSU rDNA found in cultures representing North American and temperate Asian ribotypes and subribotypes of* Alexandrium. *The ambiguities and single-base deletion listed are considered diagnostic for indicated groups and were all identified by sequencing PCR-amplified / cloned material (Scholin et al. 1994a). Digestion of PCR products with indicated restriction enzymes should reveal these signature nucleotides as shown in Figure 1 and Tables 2 and 4 if acquired sequences do in fact represent the diversity of molecules present in PCR product pools.*

Ribotype	Subribotype	Enzyme	Signature heterogeneity sampled[a]	Example sequencing ladder
North American	Western	*Afl* III	C/A ambiguity (582)	Figure 1a
	Eastern	*Mse* I	G deletion (168)	Figure 1b
	Alternate	*Hinc* II	G/A, C/A ambiguities (365, 370)	Not shown
	Alternate	*Nsp* I	C/A ambiguity (262)	Figure 1c
Temperate Asian		*Mse* I	G/A ambiguity (439)	Figure 1d
	Korean	*Apa* LI	G/T ambiguity (513)	Figure 1e

[a] Parentheses indicate nucleotide position within the D1R/D2C-primed LSU rDNA fragment, inclusive of the D1R amplification primer.

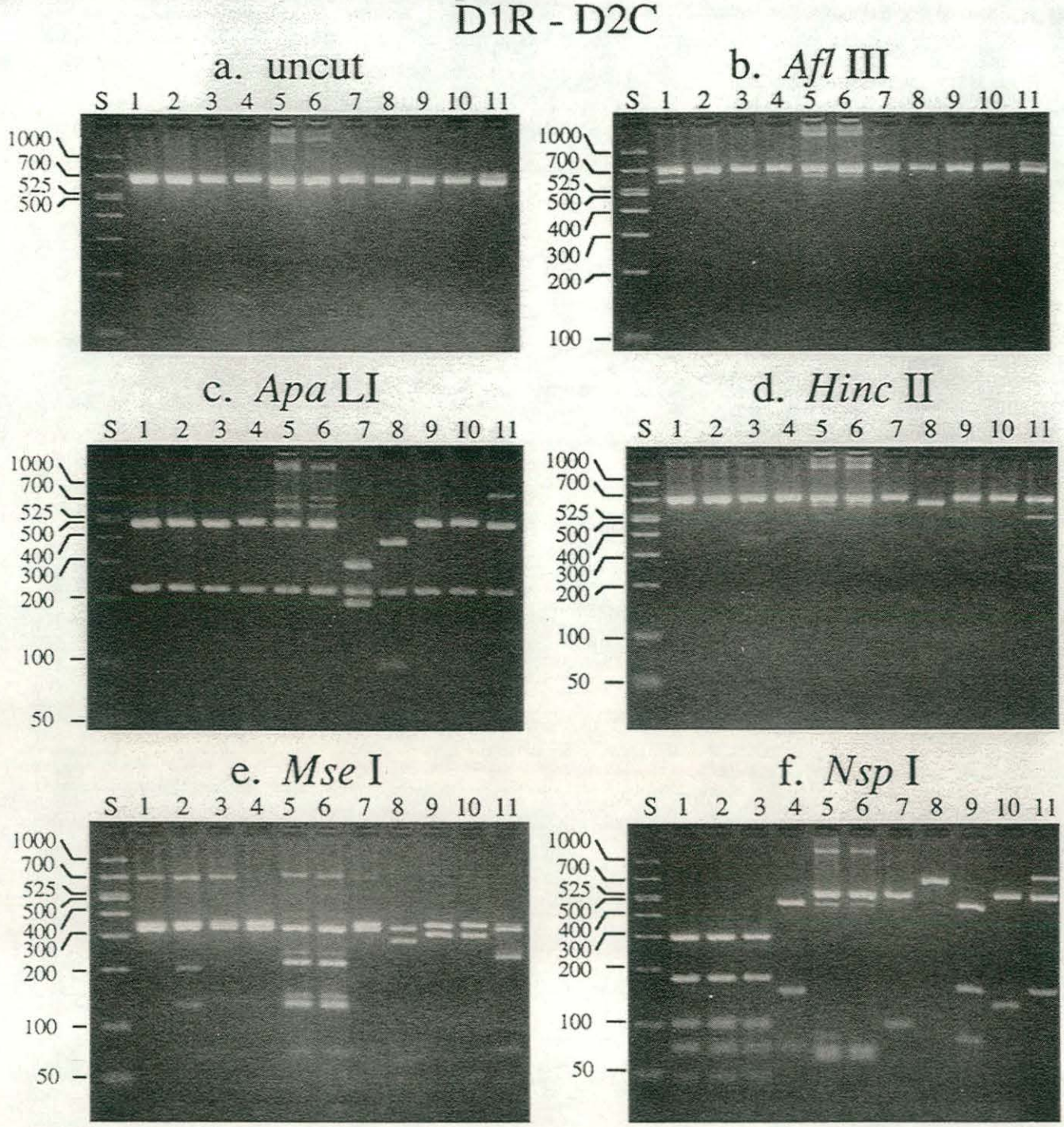


FIG. 2. Agarose gels showing D1R/D2C a) PCR amplification products and restriction patterns produced when that material is digested with b) *Afl* III, c) *Apa* LI, d) *Hinc* II, e) *Mse* I, and f) *Nsp* I. Lane designations are as follows: S = size standards, 1 = western North American, 2 = eastern North American, 3 = alternate North American, 4 = Western European, 5 = Japanese temperate Asian, 6 = Korean temperate Asian, 7 = Tasmanian, 8 = tropical Asian, 9 = *affine*, 10 = *minutum*, and 11 = *andersoni* (see Tables 1, 4).

However, some ambiguities and length heterogeneities confirmed by RFLP analyses represent a smaller portion of the total PCR product pool than was expected given acquired sequences (see later).

Exceptions to the agreement between predicted and observed RFLP patterns fell in two categories: 1) incomplete digestion of full-length PCR product, and 2) appearance of spurious digestion products. Regarding the former, *Mse* I digests of North American and temperate Asian samples appeared incomplete (Fig. 2e, lanes 1–3, 5, 6), contrary to the prediction deduced from sequence analyses (Table 4).

TABLE 4. *Sizes of predicted and observed restriction fragments for the D1R/D2C-primed LSU rDNA fragment from indicated* Alexandrium *ribotypes and subribotypes (see Tables 1–3, Figs. 1, 2).*

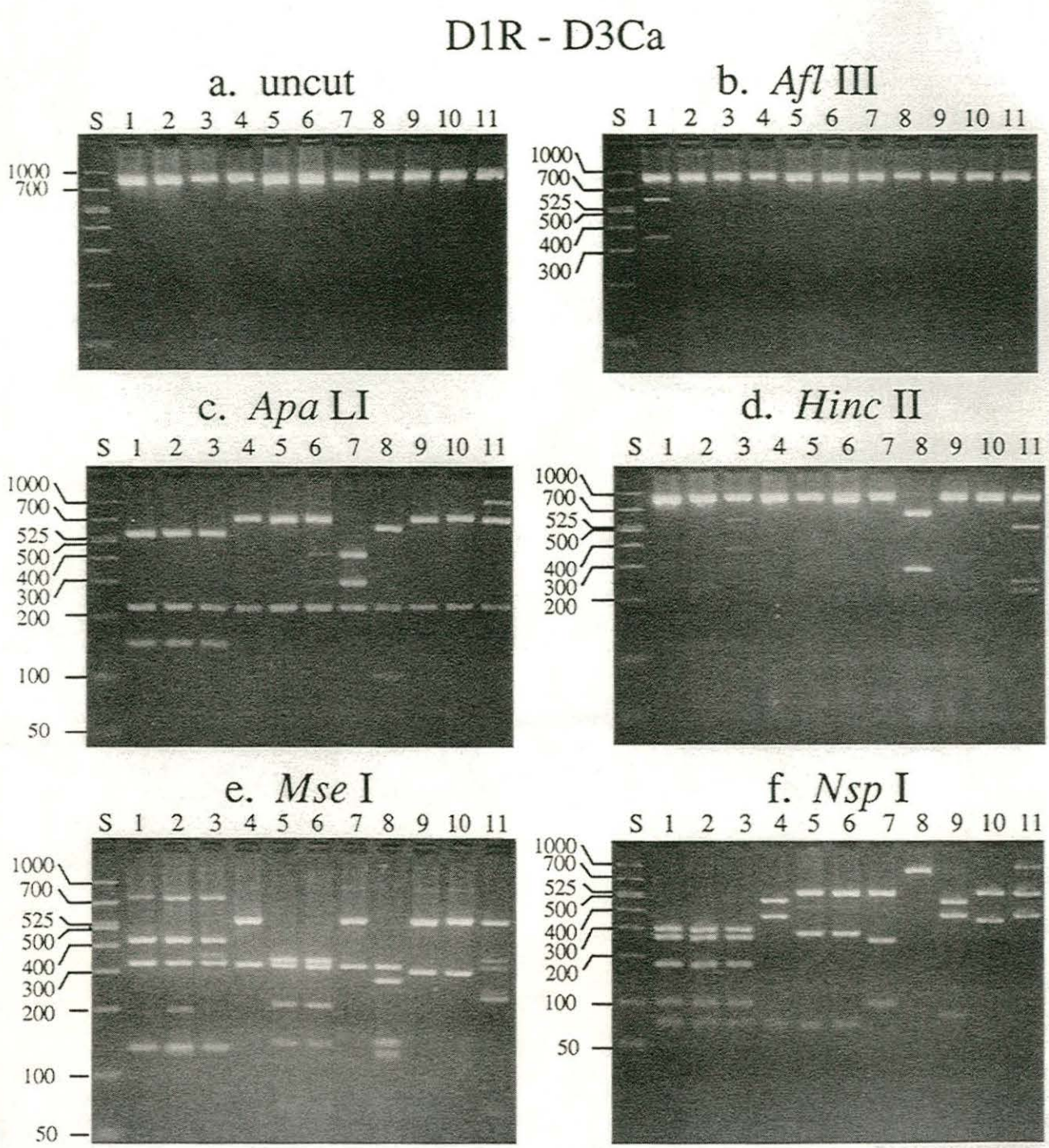
Enzyme	Figure 3 gel lane	Ribotype	Subribotype	Predicted fragments (bp)[a]	Observed fragments (bp)[b]
Afl III	1b	North American	Western	708	700
				(581)	590
				(127)	130*
	2–11b	All others		No cut	No cut
Apa LI	1–4c	North American		486–489	480
		Western European		219–220	225
	5c	Temperate Asian	Japanese	489	[750, 700]
				220	480
					225
	6c	Temperate Asian	Korean	489	[750, 700]
				(293)	480
				220	290*
				(196)	225
					200*
	7c	Tasmanian		293	290
				220	225
				196	185
	8c	Tropical Asian		388	380
				220	225
				98	95
	9, 10c	*affine, minutum*		491, 496	500
				220	225
	11c	*andersoni*		481	[750]
				220	480
					225
Hinc II	1, 2d	North American	Western, eastern	No cut	No cut
	3d	North American	Alternate	706–708	700
				(367)	360*
				(339, 341)	345*
	4–7d	Western European Temperate Asian Tasmanian		No cut	No cut
	8d	Tropical Asian		638	680
				68	70*
	9–11d	*affine, minutum*		No cut	No cut
	11d	*andersoni*		No cut	No cut
					[520]
					[260]
Mse I	1, 3e	North American	Western, alternate	340–342	[700]
				328	350
				26	330
				12	
	2e	North American	Eastern	340–342	[700]
				328	350
				(200)	330
				(127)	210
				26	130
				12	
	4e	Western European		343	350
				328	330
				26	
				12	
	5, 6e	Temperate Asian		328	[700]
				214	330
				(143)	[260]
				128	210
				(71)	150
				26	130
				12	78
	7e	Tasmanian		343	350
				328	330
				26	
				12	
	8e	Tropical Asian		328	330
				281	290
				62	68*
				59	
				38	
	9e	*affine*		345	350

TABLE 4. *Continued.*

Enzyme	Figure 3 gel lane	Ribotype	Subribotype	Predicted fragments (bp)[a]	Observed fragments (bp)[b]
				309	300
				26	
				19	
				12	
	10e	*minutum*		350	350
				309	300
				45	52*
				12	
	11e	*andersoni*		335	[350]
				238	340
				71	[260]
				45	240
				12	75
					52*
Nsp I	1f	North American	Western	298	300
				181	180
				101	100
				(77)	75
				72	45
				50	
				(24)	
				6	
	2f	North American	Eastern	297–298	300
				181	180
				99–101	100
				72	75
				50	45
				6	
	3f	North American	Alternate	298	300
				(261)	270*
				181	180
				99–101	100
				72	75
				50	45
				(41)	
				6	
	4f	Western European		479	500
				152	160
				72	75
				6	
	5f	Temperate Asian	Japanese	557	[1000+]
				72	560
				66	[480]
				14	[82]
					70
	6f	Temperate Asian	Korean	557	[1000+]
				72	560
				66	[480]
				14	70
	7f	Tasmanian		557	560
				99	100
				39	40*
				14	
	8f	Tropical Asian		No cut	No cut
	9f	*affine*		471	460
				159	160
				81	82
	10f	*minutum*		554	560
				132	130
				30	
	11f	*andersoni*		544	[750]
				157	545
					150

[a] Deduced from sequences; see Table 2. Parentheses denote fragments predicted to arise as a result of ambiguities and the length heterogeneity shown in Figure 1. All fragment sites are given in basepairs.

[b] Estimated from agarose gel photos by comparing mobility of size standards to those of digestion products. All fragment sizes are given in bp. Brackets indicate digestion products whose appearance was not predicted on the basis of acquired sequences. * Fragments that appear as weak bands on ethidium bromide–stained gels. Results reported represent those of several independent RFLP trials.



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This was observed repeatedly when enzyme concentration was varied from 2- to 5-fold in excess of that theoretically required and when digestion time was varied from 4 to 18 h. Concerning (2), *Apa* LI, *Mse* I, and *Nsp* I digests of temperate Asian ribotype material, and *Hinc* II and *Mse* I cuts of *andersoni* template yielded unexpected digestion products (Table 4). Fortuitously, the unpredicted product observed from *Nsp* I digests of the temperate Asian ribotype sample revealed a difference between the Japanese and Korean subribotypes of this group: the unpredicted ~82-bp product was diagnostic for the Japanese subribotype (Fig. 2f lanes 5, 6, Table 4).

D1R/D3Ca PCR and digestion products. Results ob-

tained using the D1R/D3Ca-primed fragment were similar to those already discussed but did reveal several notable differences. First, the "signature amplification" indicative of the temperate Asian ribotype was lost (compare Figs. 2a and 3a, lanes 5, 6). Second, *Mse* I digests of North American ribotype LSU rDNA remained incomplete as before, whereas those of the temperate Asian sample were fully digested (compare Figs. 2e and 3e, lanes 1–3, 5, 6;). Third, the overall clarity of RFLP groups was improved, particularly the resolution of North American subribotypes using *Mse* I (compare Figs. 2e and 3e, lanes 1–3). Fourth, the fortuitous distinction between temperate Asian Japanese and Korean subribotypes using *Nsp* I was lost (compare Figs. 2f and 3f, lanes 5, 6).

DISCUSSION

The distinctions between *Alexandrium* ribotypes and subribotypes as defined by a PCR amplification/cloning/sequencing methodology were confirmed by RFLP analyses. There is excellent agreement between predicted and observed RFLP patterns of the LSU D1R/D2C-primed fragment (Table 4, Fig. 2), but some discrepancies did arise (see later). Application of the same enzymes to a larger portion of LSU rDNA (the D1R/D3Ca-primed fragment) gave results similar to those earlier (Fig. 3; Table 5), revealing unique RFLP patterns for each terminal group shown in Table 1. Results of this study provide further evidence that intraculture fine-scale sequence differences such as those shown in Figure 1 are useful genetic markers that should not be ignored. Although multiple classes of rDNA from a single *Alexandrium* isolate complicate sequence analyses, such "complications" also provide a basis for improving resolution of genetically divergent species and populations. In the discussion that follows, we address each of these conclusions in greater detail and outline their implications with respect to future rDNA analyses to characterize cultured as well as naturally occurring *Alexandrium*.

Validation of the Alexandrium *LSU rDNA phylogeny.* The phylogeny presented by Scholin et al. (1994a) is based on sequences from an average of 10 PCR-amplified/cloned LSU rDNA representatives from each culture examined. The extent of intraculture sequence heterogeneity observed among those clones was surprising. This led us to question whether or not the sequences acquired were in fact a good representation of those in corresponding pools of PCR products. To test the accuracy of acquired sequences, we identified a set of restriction enzymes that were predicted to reveal signature nucleotides indicative of major ribotype divisions, as well as subribotype divisions (Fig. 1, Tables 2, 3). By digesting PCR products with this set of enzymes, it was possible to test these predictions empirically.

Results presented here show that sequences deduced from a limited number of LSU rDNA clones from each culture were indeed a good representation of the spectrum of sequences present in corresponding pools of PCR products, lending further support to the distinction of terminal groups as described earlier. Elsewhere (Scholin et al. 1994a), we note that these divisions agree with and further resolve clades defined by isozyme electrophoresis (Hayhome et al. 1989, Sako et al. 1990), immunogenicity (Sako et al. 1993), and RFLP analyses of SSU rDNA (Scholin and Anderson 1994) as well as internal transcribed spacer (ITS) regions (Adachi et al. 1994, 1996). These studies and others (Cembella et al. 1988, Anderson et al. 1994) leave no doubt that representatives of *A. tamarense*, *A. catenella*, and *A. fundyense* are genetically diverse and that morphological-based species definitions for these organisms may agree or disagree with groups as defined by subcellular criteria. Nevertheless, the *tamarensis* complex as a whole is clearly distinct from *A. affine*, *A. minutum*, and *A. andersoni* (Scholin et al. 1994a) as well as *A. pseudogonyaulax* and *A. insuetum* (Adachi et al. 1994, 1996).

Results of this study also show that characterization of LSU rDNA from some *Alexandrium* lineages is incomplete. Comparison between predicted and observed RFLP patterns indicates that at least some of the *Alexandrium* sequences collected to date are clonally biased (see later). This in turn has introduced error into the phylogenetic tree, but the extent of this error is not clear. Although ribotype and subribotype divisions of *Alexandrium* are valid groupings, we believe that branch lengths separating these terminal taxa may not provide an accurate representation of their actual divergence.

Evidence for clonal biasing. Multiple classes of LSU rDNA were identified in isolates representing the North American and temperate Asian ribotypes (Scholin et al. 1994a). In some cases, the ambiguities and length heterogeneities fall fortuitously on restriction enzyme sites, making it possible to check the efficacy of the sequences by RFLP analyses (Scholin and Anderson 1994). Sites of this nature examined here are shown in Figure 1 and Table 3. In all cases, restriction digests revealed the same heterogeneities as were defined by sequencing.

However, in some cases what appear to be abundant classes of molecules by sequence analyses do not yield highly visible, diagnostic RFLP markers. Instead, the characteristic bands were much fainter than expected given the intensity of signature nucleotides seen on sequence ladders. For example, sequences of the alternate North American subribotype show a clear C/A ambiguity (Fig. 1c), suggesting two classes of molecules that occur in an approximately 1:1 ratio. Given this, we predict that an enzyme that recognizes that site should yield an RFLP pattern in which roughly half of the molecules harboring that site are cut and half are not. Instead, only a tiny fraction of the molecules were cleaved (Fig. 2f, lane 3, Table 4), indicating that the se-

TABLE 5. *Sizes of observed restriction fragments for the D1R/D3Ca-primed LSU rDNA fragment from indicated* Alexandrium *ribotypes and subribotypes (Table 1, Fig. 3).*

Enzyme	Figure 4 gel lane	Ribotype	Subribotype	Observed fragments (bp)*
Afl III	1b	North American	Western	950
				580
				360
	2–11b	All others		950
Apa LI	1–3c	North American		560
				220
				145
	4c	Western European		700
				220
	5c	Temperate Asian	Japanese	700
				220
	6c	Temperate Asian	Korean	700
				400*
				300*
				220
	7c	Tasmanian		400
				300
				220
	8c	Tropical Asian		610
				220
				95
	9, 10c	*affine, minutum*		700
				220
	11c	*andersoni*		950
				700
				220
Hinc II	1, 2d	North American	Western, eastern	950
	3d	North American	Alternate	950
				600*
				360*
	4–7d	Western European Temperate Asian Tasmanian		950
	8d	Tropical Asian		650
				280
	9, 10d	*affine, minutum*		950
	11d	*andersoni*		950
				500
				240
				210
Mse I	1e	North American	Western	760
				440
				340
				130
	2e	North American	Eastern	760
				440
				340
				205
				130 × 2
	3e	North American	Alternate	760
				440
				360
				340
				130
	4e	Western European		550
				340
	5, 6e	Temperate Asian		360
				340
				220
				140
				70*
	7e	Tasmanian		550
				340
	8e	Tropical Asian		340
				275
				145
				120

TABLE 5. *Continued.*

Enzyme	Figure 4 gel lane	Ribotype	Subribotype	Observed fragments (bp)[a]
	9e	*affine*		550
				310
	10e	*minutum*		550
				310
				45*
	11e	*andersoni*		550
				340
				320
				245
				225
				65*
				45*
Nsp I	1–3f	North American		300
				260
				180
				100
				70
	4f	Western European		500
				350
				70
	5, 6f	Temperate Asian		550
				280
				70
	7f	Tasmanian		550
				250
				95
	8f	Tropical Asian		950
	9f	*affine*		460
				350
				80
	10f	*minutum*		540
				330
	11f	*andersoni*		950
				540
				350

[a] Estimated from agarose gel photos by comparing mobility of size standards to those of digestion products. All fragment sizes are given in basepairs. *Fragments that appear as weak bands on ethidium bromide–stained gels. Results reported represent those of several independent RFLP trials.

quence ladder overrepresented the class of molecule containing the "C variant." In sharp contrast, RFLP predictions for other heterogeneities shown in Figure 1 were confirmed. For example, we predicted that restriction fragments diagnostic for the Korean temperate Asian ribotype would appear weak, whereas those of the western North American subribotype would appear stronger. This prediction was based on the fact that the signature ambiguity diagnostic for the former (Fig. 1e) is weaker than that of the latter (Fig. 1a). This prediction was met (compare Figs. 2 and 3, panels b, lane 1, and c, lane 6; the faint bands in each lane are those that arose from the signature heterogeneities shown in Fig. 1). A similar pattern is seen when comparing intensities of diagnostic restriction fragments that delineate eastern North American and Korean temperate Asian subribotypes; those of the former are much brighter than those of the latter (Fig. 2e lane 2, and 2c, lane 6), as expected (Fig. 1b, and e).

Acquired sequences for most groups are thus a good representation of the diversity of molecules present in the PCR product pool. In other cases, however, minor classes of sequence in the PCR pool as judged by RFLP analysis are overrepresented in sequencing ladders. This discrepancy might arise from sequencing error, in which case a minor class of molecule in the reaction would have to serve as a more efficient dideoxy terminator than other, more abundant templates present in the same mixture. Alternatively, overrepresentation of rare classes of molecules could arise from clonal biasing. Regarding the latter, cloning of the LSU rDNA fragments imposes a selection on which classes of sequence are ultimately represented in sequencing ladders. We would expect that the most abundant class of molecule in the PCR product pool would also be the most frequently cloned molecule if selections are random. However, if one class of molecule is cloned more efficiently than others, or if rare classes of molecules were inadvertently yet preferentially selected, then the resultant clonal pool will be biased. In other words, clones selected will not reflect the actual frequencies of molecules present in PCR

products. By selecting only a few clones for a given culture out of a large and heterogeneous PCR product pool, the extent of clonal biasing may be exaggerated. An example of this is seen in Figure 1b for cultures AFNFA3 and GtCN16 (see also Scholin et al. 1994a). We believe that random errors associated with sampling are one explanation for this observation. However, some classes of molecule were apparently selected for or excluded from the population of stable bacterial recombinants, and the selections imposed occurred repeatedly. At present, we do not understand the molecular basis of this phenomenon.

Other inconsistencies between predicted and observed RFLP patterns. Clonal biasing is also evidenced by incomplete digestions of full-length PCR product and appearance of spurious products, neither of which were expected on the basis of acquired sequences. In this case, selection of a limited number of LSU rDNA clones resulted in a loss of some classes of molecule prior to sequencing. For example, digestions of the North American and temperate Asian ribotype D1R/D2C fragments with *Mse* I always appear incomplete (Fig. 2e, lanes 1–3, 5, 6). This was observed repeatedly despite using different batches of PCR product, a severalfold excess of restriction enzyme, and adequate time for digestions. Moreover, it is only observed in North American and temperate Asian samples even though all PCR amplifications, PCR product purification, and restriction digests were processed in batch mode and employed a common set of reagents. In addition, the same pattern is seen in multiple representatives of these ribotypes acquired from independent laboratories in different regions of the world (unpubl. data). Therefore, for members of the North American and temperate Asian ribotypes, PCR products contain more heterogeneity than was observed by cloning and sequencing.

Choice of amplification primers also affected the diversity of molecules in PCR products. Use of the D1R/D2C primer set with temperate Asian template repeatedly gave higher and lower molecular weight products in addition to the predicted product (Fig. 2a, lanes 5, 6). This amplification pattern is unique to members of the temperate Asian ribotype; use of the D1R/D3Ca primer set eliminates this (Fig. 3a, lanes 5, 6). Once the higher molecular weight products are eliminated, the apparent incomplete digestion with *Mse* I is no longer seen. This suggests that the "undigested, full-length PCR product" seen with the D1R/D2C-primed fragment stems from higher molecular weight material. We believe that higher and/or lower molecular weight amplification products are also the source of unpredicted fragments when D1R/D2C-primed temperate Asian samples are digested with *Apa* LI, *Mse* I, and *Nsp* I. However, these "spurious products" are not necessarily a liability. For example, digestion of temperate Asian D1R/D2C fragments with *Nsp* I allows for a distinction between the Japanese and Korean subribotypes of this group. In contrast, the same enzyme applied to the "cleaner" D1R/D3Ca fragment does not (compare Figs. 2f and 3f, lanes 5, 6).

LSU rDNA amplified from *A. andersoni* also yielded heterogeneous products. Two distinct size classes of molecules are evident using both the D1R/D2C and D1R/D3Ca primer sets as seen in electrophoretic patterns of uncut material, as well as *Apa* LI, *Hinc* II, *Mse* I, and *Nsp* I digests (Figs. 2, 3, lane 11). At this time, only a single representative of this species is held in culture; thus, it is not possible to confirm that these two classes of molecules are unique to that culture or are instead a character shared among all representatives of that species. Presently, we can only conclude that the heterogeneity does not stem from an obvious culture contaminant.

The RFLP assays described here and elsewhere (Adachi et al. 1994, Scholin and Anderson 1994) are applicable to a host of research topics. For example, if distinct ribotypes or subribotypes can be mated, one could track defined genetic elements through meiotic divisions using an RFLP assay to measure rDNA partitioning among the progeny. Another application concerns mapping the biogeographic distributions of known ribotypes. This could speed tests of evolutionary concepts and dispersal hypotheses as discussed elsewhere (Scholin et al. 1995). Screening isolates representative of global populations not yet examined (e.g. South America, South Africa) are in progress in hopes that novel ribotypes can be identified.

CONCLUSIONS

We have described a set of RFLP standards for 11 *Alexandrium* LSU rDNA ribotypes, each of which defines unique species and strain-specific markers. The RFLP groups agree with and further resolve cultures compared on the basis of other regions of rDNA, including the SSU (Scholin and Anderson 1994), 5.8S, and ITS regions (Adachi et al. 1994, 1996). The RFLP assays available for characterizing *Alexandrium* representatives make it possible to screen many cultures simultaneously with relative ease to define their rDNA evolutionary lineage. This information can be cross-referenced with a variety of other data, such as morphology, toxicity, immunogenicity, isozyme electrophoretic patterns, and breeding group affinities.

Clonal biasing has introduced error into the *Alexandrium* LSU rDNA sequence data base, but the extent of this error is not known. Future sequencing efforts should take this observation into account, placing greater emphasis on analysis of a larger clonal pool, or direct sequencing of PCR products or purified rRNA. Because multiple classes of rDNA are present in a number of *Alexandrium* species, one must take care that rRNA-targeted probe design focus on the expressed, stable gene product(s), not transcriptionally silent classes of molecules.

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