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IDENTIFICATION OF GROUP- AND STRAIN-SPECIFIC GENETIC MARKERS FOR GLOBALLY DISTRIBUTED *ALEXANDRIUM* (DINOPHYCEAE). I. RFLP ANALYSIS OF SSU rRNA GENES[1]

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ABSTRACT

Two distinct small-subunit ribosomal RNA genes (SSU rDNAs), termed the "A gene" and "B gene," were recently found in the toxic dinoflagellate Alexandrium fundyense *Balech. A restriction fragment length polymorphism (RFLP) assay was developed to rapidly detect the A and B genetic markers. SSU rDNA from 58 cultures with species designations of* A. tamarense *(Lebour) Balech,* A. catenella *(Whedon et Kofoid) Balech,* A. fundyense, A. affine *(Fukuyo et Inoue) Balech,* A. minutum *Halim,* A. lusitanicum *Balech, and* A. andersoni *Balech were screened. These cultures represent toxic and non-toxic isolates from North America, western Europe, Thailand, Japan, Australia, and the ballast water of several cargo ships. The RFLP assay revealed five distinct groups. Three subdivided the* A. tamarense/catenella/fundyense *"species complex" into clusters defined by geographic origin, not by morphospecies designations. The fourth group consisted of* A. affine, *whereas the fifth group was represented by* A. minutum, A. lusitanicum, *and* A. andersoni.

The B gene was only found in A. tamarense, A. catenella, *and* A. fundyense, *but not in all isolates. However, all North American isolates of this closely related group harbored this gene, and it also was found in some* A. tamarense *from scattered locations in Japan and in the ballast water of one ship that operated exclusively between Japan and Australia. Isolates without the B gene appeared to have only a single class of SSU rDNA. The B sequence was not essential for toxin production, but thus far those organisms harboring it were toxic. The* A. tamarense/catenella/fundyense *complex is composed of genetically distinct populations, within which may exist two or all three of the mophotypically defined species. The B gene is a promising taxonomic and biogeographic marker and may be useful for tracking the regional and/or global dispersal of particular populations.*

Key index words: Alexandrium; *biogeography; PCR; pseudogene; Pyrrophyta; red tide; RFLP; small-subunit rRNA*

Marine dinoflagellates within the genus *Alexandrium* (= *Protogonyaulax* Taylor; Steidinger and Moestrup 1990) include a number of species capable of producing potent neurotoxins. These toxins, referred to as paralytic shellfish poisons, can accumulate in filter-feeding shellfish and thereby pose a serious health threat if consumed by humans (Prakash et al. 1971). Toxic *Alexandrium* are found in many regions of the world (Taylor 1984). Compelling evidence from a number of investigators suggests that these organisms have expanded their geographic range by both natural and human-assisted means (Anderson 1989, Hayhome et al. 1989, Hallegraeff et al. 1991, Hallegraeff and Bolch 1991, 1992). Because of this dispersal and the well-known hazards of paralytic shellfish poisons, *Alexandrium* species are receiving increased international attention. Rapid and unequivocal identification of these organisms has become one focal point of toxic dinoflagellate research. Here we report on the application of molecular biological methods for identifying strain-specific genetic markers in toxic and non-toxic *Alexandrium* species and the use of these markers for classifying globally distributed populations.

At present, morphological characters are the primary means of describing *Alexandrium* species (Balech 1985, Steidinger 1990), although their validity

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with respect to species- and strain-level classifications continues to be a subject of debate (Taylor 1985, 1990). An example is the toxigenic *A. tamarense, A. catenella,* and *A. fundyense* species complex (or "tamarensis complex"), which are morphotypically similar organisms differentiated by detailed cellular characteristics such as presence or absence of a ventral pore on the first apical plate, the position of the posterior attachment pore, chain formation, and overall cell shape (Balech 1985, Fukuyo 1985, Taylor 1985). Some consider members of the tamarensis complex distinct species (e.g. Balech 1985, Balech and Tangen 1985), whereas others view them as varieties or strains of a single species (e.g. Taylor 1985, Cembella et al. 1988). Biochemical characters such as isozyme electrophoretic patterns (Cembella and Taylor 1986, Cembella et al. 1988, Hayhome et al. 1989, Sako et al. 1990), toxin composition profiles (Cembella et al. 1987), and cell surface antigens (Sako et al. 1993) have been used to discriminate isolates and to clarify the relationship between morphotype and species-level divisions. In some cases, biochemical markers corroborate morphotaxonomic classifications (Sako et al. 1990, 1993), but in others they do not (Cembella and Taylor 1986, Cembella et al. 1987, 1988, Hayhome et al. 1989). As a consequence, the relationship between fine-scale morphology and biochemical or genetic characters, including toxicity, remains obscure. In turn, the debate over whether tamarensoid, catenelloid, and fundyensoid morphotypes represent species or strains of one species also continues. Complicating matters further is the fact that taxonomic and biogeographic case studies to date have focused primarily on regional rather than globally distributed populations. It thus seems possible that some confusion has arisen because different populations of the same morphospecies are genetically divergent.

Sequence analysis of genomic small-subunit (SSU) and large-subunit (LSU) ribosomal RNA (rRNA) genes (rDNA) is one method that can be used to classify populations of *Alexandrium* (Destombe et al. 1992, Scholin and Anderson 1993, Scholin et al. 1993). Sequences of rRNA and rDNA have been used extensively to evaluate the evolutionary histories of many organisms (Olsen et al. 1986, Sogin et al. 1986, Field et al. 1988, Lenaers et al. 1991) and have gained recognition as species- and strain-specific genetic markers (Gobel et al. 1987, McCutchan et al. 1988, Stahl et al. 1988, Amann et al. 1990, Distel et al. 1991, Schlegel et al. 1991). We reasoned that this well-established method might be useful in settling the dispute over fine-scale *Alexandrium* taxonomic criteria and could help elucidate this group's global population structure. A unified systematic scheme and resolution of intraspecific genetic variation are also necessary references for testing dispersal hypotheses.

A prerequisite for all of these applications is acquiring, compiling, and analyzing sequences from representative *Alexandrium* species collected from many locations throughout the world. As a first step in creating such a data base, we sequenced SSU rDNA from a clonal, toxic *A. fundyense* isolated from eastern North America. This analysis surprisingly revealed the existence of two distinct classes of genes, which we termed the "A gene" and the "B gene." Closer examination of these molecules revealed the B sequence to be a pseudogene (i.e. a non-functional sequence; Scholin et al. 1993).

Because the A and B gene sequences vary little (40 positions out of 1802), it is possible that their divergence records a recent event in the evolutionary history of *A. fundyense.* If so, then the B gene should be a useful taxonomic marker and may also be applicable to questions concerning the organism's biogeography and dispersal. However, the labor involved in identifying and documenting the A and B genes using cloning and sequencing protocols made the prospects of examining a large number of cultures for these markers difficult to justify. A restriction fragment length of polymorphism (RFLP) assay, termed the "A/B restriction test," was therefore developed to facilitate a rapid and cost-effective screening procedure.

The power and simplicity of RFLP assays stem from the recognition and cleavage of specific DNA sequences by restriction enzymes. Sequence heterogeneity among defined DNA molecules can thus be elucidated with relative ease. This method is widely used in diverse areas of research, including taxonomy, ecology, biomedicine, and forensic science (e.g. Curran et al. 1985, Wetton et al. 1987, Goff and Coleman 1988, Moody 1989, Levy et al. 1991, Maeda et al. 1991, Rowan and Powers 1991, Schlegel et al. 1991, De Buyser et al. 1992). Here, the RFLP concept is applied in a search for the A and B genetic markers using endonucleases that discriminate nucleotide differences between the two sequences. We describe the development of this assay, its application to a variety of *Alexandrium* species collected from diverse regions of the world, and results of the tests as they relate to the taxonomy and biogeography of these organisms.

MATERIALS AND METHODS

Cultures used in this study are listed in Table 1; strain and species designations, isolation locale, and available toxicity information are also presented. All were maintained in f/2 medium as modified and described by Anderson et al. (1984). Cultures that were obtained from sources other than Anderson laboratory are as follows: PW05, PW06, PI32, IP02, ACQH01, and ACQH02 (S. Hall); Gony.#7 (A. White); Gt 429 (Provasoli-Guillard Culture Collection); Pgt 183 (North East Pacific Coast Culture collection [NEPCC 183]); PE1V, PE2V, PA5V, and AL2V (I. Bravo); Gt Port (L. Provasoli); AM2 and AM3 (E. Erard-Le Denn); N 239 and N 520 (National Institute for Environmental Studies [NIES Collection, Japan]); ND-1, OK875-1, OF875-8, OF84423D3, WKS-1, WKS-3, WKS-8, CU-1, and CU-13 (M. Kodama); OF041, OF051, OF101, and TN9 (Y. Sako); and ATJP01, ACPP01, ACPP02, ACPP03, ACPP09, AMAD01, AMAD06, ATBBO1, AABB01/2, I72/21#2, I72/22#2, I72/21#4, ACJP03, G. Crux,

G. Hope 1, and G. Hope 2 (G. Hallegraeff). All strains listed in Table 1 are currently maintained at the Woods Hole Oceanographic Institution.

Nucleic acid extraction. Approximately 10–15 mL of a mid-log culture was harvested by gentle centrifugation, and the cell pellet was resuspended in approximately 200 μL of autoclaved Milli-Q water (Millipore Corp.) at room temperature. The cell slurry was transferred to a 1.5-mL sterile microcentrifuge tube and adjusted to contain 1% sodium dodecyl sulfate, 10 mM ethylenediaminetetraacetate (EDTA; pH 8.0), 10 mM Tris-HCl (pH 7.5), and 10 mM NaCl in a final volume of 250 μL. Nucleic acids in this solution were purified by extracting once with Tris-buffered phenol, 2–3 times with phenol : chloroform : isoamyl alcohol (PCI; 24:24:1) and once with chloroform : isoamyl alcohol (CI; 24;1; Ausubel et al. 1987). Total nuclei acids were ethanol (EtOH)-precipitated (Ausubel et al. 1987), rinsed with 80% EtOH, and then resuspended in 10–50 μL of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0). An aliquot of this material was diluted, and the concentration of DNA was determined by reading its absorbance at 260 nm (Ausubel et al. 1987). DNA samples were stored at −20° C.

Polymerase chain reaction amplification of SSU rDNA. Complete SSU rDNAs were amplified using the polymerase chain reaction (PCR; Saiki et al. 1988) with universal eukaryotic primers (Sogin 1990) using a Perkin Elmer Cetus DNA Thermal Cycler and Perkin Elmer GeneAmp PCR Core Reagents as recommended by the manufacturers. Amplifications were typically carried out as follows: denaturing at 92° C, 1.5 min; cooling to 45°–55° C, 30 s; annealing at 45°–55° C, 1.5 min; warming to 72° C, 1.5 min; and extension at 72° C, 2.0 min. This cycle was repeated 30 times with an autoextension of the polymerization cycle (5 s per cycle). Primers were used at a final concentration of 0.01–0.05 μM, with 3 mM $MgCl_2$ and 1 ng of total DNA. Amplification reactions for a given DNA preparation were done in duplicate or triplicate, pooled, and then purified by extracting once with PCI and once with CI. Products were EtOH-precipitated and resuspended in 10–50 μL of TE (pH 7.5); 1 μL of this was run on an agarose gel in order to gauge the success of the reaction and approximate concentration of the products. Amplified SSU rDNAs were stored at −20° C.

A/B gene restriction test. Theoretical restriction maps of the A and B sequences (Scholin et al. 1993) were generated using MacDNASIS Pro (v. 1.0; Hitachi) DNA analysis software. The resultant cleavage sites of each enzyme that recognized one or both of the genes were then compared to determine which enzymes would discriminate the two genes; *Bsa*AI, *Bsr*I, *Hae*III, and *Xba*I (New England Biolabs) were chosen for further analysis. Approximately 50–100 ng of PCR product were digested with each of the enzymes in 10–25-μL reactions for 1–3 h as directed by the manufacturer. Products of the digestions were resolved on 1.0–1.5% agarose gels using 1 × TBE buffer (Ausubel et al. 1987). Digesting the SSU rDNA of a particular isolate with each of the four enzymes, separating the products on an agarose gel, and scoring the resultant pattern constitute the "A/B gene restriction test."

RESULTS

Computer-assisted restriction site analysis of the A and B sequences resulted in the identification of over 100 enzymes that would theoretically cleave at one or more locations in one or both of the genes (data not shown). After initial comparisons, 18 candidate enzymes were identified that should differentially recognize the two sequences, along with two enzymes that were expected to cleave the genes at identical locations (Scholin 1993). From this list, *Bsa*AI and *Bsr*I were chosen to discriminate between the A and B genes, while both *Hae*III and *Xba*I should give identical patterns (Fig. 1a). This suite of enzymes was tested on PCR-amplified SSU rDNA from 58 *Alexandrium* cultures with species designations of *A. tamarense, A. catenella, A. fundyense, A. affine, A. minutum, A. lusitanicum,* and *A. andersoni.* These cultures included both toxic and non-toxic isolates from North America, western Europe, Thailand, Japan, Australia, and the ballast water of several cargo ships (Table 1). The A/B gene restriction test revealed five distinct groups among these cultures (Fig. 1b–f, Table 2).

Three of the RFLP groups subdivided the tamarensis species complex (Groups I–III), an assemblage that included toxic *A. tamarense/catenella/fundyense* as well as non-toxic *A. tamarense* (Table 1). All shared the predicted A gene restriction pattern for each enzyme. The distinguishing characteristics of the groups were based on whether or not their PCR-amplified SSU rDNA included the B gene as well as a class of molecules ~300 bp greater than the expected PCR product of ~1800 bp (Fig. 1a–f, lanes I–III; Table 2). Group I was typified by isolates that harbored both the A and B genes and gave rise to the larger amplification products (Fig. 1b–f, lane I). This included all eastern North American *A. tamarense* and *A. fundyense,* Japanese *A. tamarense* isolated from Okkirai and Noda bays, and *A. tamarense* from the ballast water of one cargo vessel. Group II isolates also harbored the A and B genes but did not display the larger PCR products (Fig. 1b–f, lane II). Group II included all *A. tamarense, A. catenella,* and *A. fundyense* from western North America and several *A. tamarense* from Ofunato Bay, Japan. Both Groups I and II displayed minor *Bsa*AI digestion products not predicted by the computer-generated restriction maps (Fig. 1a, c, lanes I–II). Group III exhibited a restriction pattern for the A gene alone, having neither the B gene patterns, the larger amplification products, nor any unpredicted patterns (Fig. 1b–f, lane III). Group III encompassed *A. tamarense* and *A. catenella* isolated from western Europe, Japan, Australia, and the ballast water of three cargo vessels (Table 2).

A fourth group identified using the RFLP assay consisted of non-toxic *A. affine* from Spain, Tasmania, and Thailand (Group IV). PCR products from these isolates did not cut with *Hae*III, as would be predicted from the known A and B sequences (Fig. 1a, e, lane IV). This restriction pattern was designated "Hae(1)" (Table 2).

The fifth and final group consisted of toxic *A. minutum* and *A. lusitanicum* and non-toxic *A. andersoni* (Group V). Each of these isolates shared sites for *Bsa*AI and *Hae*III that varied from those predicted from the known A and B gene sequences (Fig. 1a, c, e, lane V; Table 2). These restriction patterns were designated "Bsa(1)" and "Hae(2)," respectively.

The B gene was not essential for toxin production.

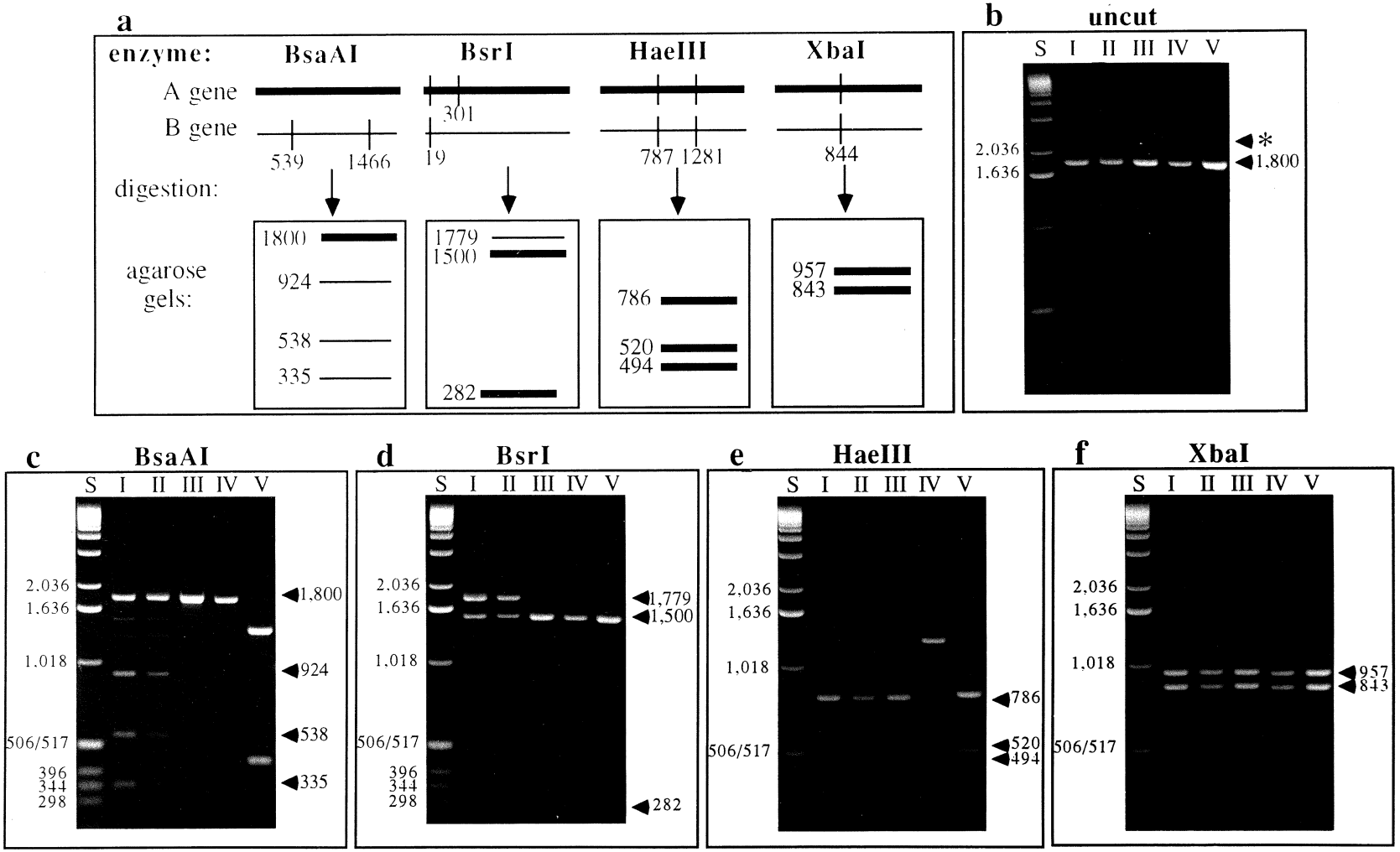

FIG. 1. SSU rDNA A/B gene restriction test. a) Schematic of the assay. Restriction maps of the A and B genes for *Bsa*AI (B cutter), *Bsr*I (A cutter), and *Hae*III and *Xba*I (A and B cutters) are shown on top; numbers refer to the nucleotide distal to the cleavage and are relative to the position in the A gene (Scholin et al. 1993). Schematized agarose gels of predicted digestion products are shown below the restriction maps; numbers refer to length of the digestion products in base pairs. b–f) Ethidium bromide-stained agarose gels showing representative PCR amplification (b) and restriction patterns (c–f) of identified *Alexandrium* SSU rDNA RFLP groups. Lane designations are as follows: S = size standards (length in base pairs is indicated on the left); I = Group I *A. fundyense* (strain GtCA29); II = Group II *A. fundyense* (strain PI32); III = Group III *A. tamarense* (strain Pgt183); IV = Group IV *A. affine* (strain PA5V); V = Group V *A. minutum* (strain AMAD06). Arrowheads point to b) SSU rDNA PCR products (~1800 base pairs) and larger products (*) found in Group I and c–f) A/B gene digestion fragments (in base pairs) predicted for each enzyme. Observed digestion fragments are congruent with those predicted for Groups I and II. Exceptions to the predictions are as follows: minor *Bsa*AI products (c, lanes I and II), and the "Hae (1)" (e, lane IV), "Bsa (1)" and "Hae (2)" (c and e, lane V) RFLPs (see Table 2). Restriction patterns of Groups III–V do not indicate presence of the B gene, nor SSU rDNAs analogous to the B gene. There was some variation in the total amount of DNA loaded into each lane; this is especially noticeable in (c), lanes I and II.

TABLE 1. *Strain numbers, species designations, isolation locales, and toxicity of* Alexandrium *cultures screened using the SSU rDNA A/B gene restriction test. PCR amplification characteristics and results of the RFLP assays for each culture are indicated (+).*

Geographic block	Strain[a]	Species designation[b]	Isolation locale	Clonal culture?	Toxic?[c]	Larger SSU PCR products[d]	SSU rDNA restriction sites[e]				
							"A"	"B"	Polymorphisms[f] Bsa(1)	Hae(1)	Hae(2)
North America											
West Coast	PW05	*A. tamarense* (EB)	Port Benny, AK	Yes	Yes		+	+			
	PW06	*A. tamarense* (EB)	Port Benny, AK	Yes	Yes		+	+			
	PI32	*A. fundyense* (EB)	Porpoise Island, AK	Yes	Yes		+	+			
	IP02	*A. fundyense* (EB)	AK	Yes	Yes		+	+			
	ACQH01	*A. catenella* (EB)	Puget Sound, WA	Yes	Yes		+	+			
	ACQH02	*A. catenella* (EB)	Puget Sound, WA	Yes	Yes		+	+			
	BGt1	*A. catenella* (EB)	Russian River, CA	No	Yes		+	+			
East Coast	ATSL01	*A. fundyense* (EB)	Gulf St. Lawrence	Yes	Yes	+	+	+			
	AFNFA3	*A. fundyense* (EB)	Newfoundland	Yes	Yes	+	+	+			
	AFNFA4	*A. fundyense* (EB)	Newfoundland	Yes	Yes	+	+	+			
	Gony.#7	*A. fundyense* (EB)	Bay of Fundy	Yes	Yes	+	+	+			
	Gt429	*A. fundyense* (EB)	Ipswitch Bay, MA	Yes	Yes	+	+	+			
	GtCA29	*A. fundyense* (EB)	Cape Ann, MA	Yes	Yes	+	+	+			
	GtMP	*A. fundyense* (EB)	Orleans, MA	No	Yes	+	+	+			
	GtPP01	*A. tamarense* (EB)	Falmouth, MA	Yes	Yes	+	+	+			
	GtPP06	*A. tamarense* (EB)	Falmouth, MA	No	Yes	+	+	+			
	GtCN1	*A. tamarense* (EB)	Groton, CN	No	Yes	+	+	+			
	GtCN16	*A. tamarense* (EB)	Groton, CN	Yes	Yes	+	+	+			
	GtLI21	*A. tamarense* (EB)	Babylon, NY	Yes	Yes	+	+	+			
	TC02	*A. andersoni* (EB)	Eastham, MA	Yes	No				+		+
Western Europe											
U.K.	Pgt183	*A. tamarense* (MT)	Plymouth	Yes	No		+				
Spain	PE1V	*A. tamarense* (EB)	Galicia	No	No*		+				
	PE2V	*A. tamarense* (EB)	Galicia	No	No		+				
	PA5V	*A. affine* (IB)	Galicia	Yes	No					+	
	AL2V	*A. lusitanicum* (IB)	Ria de Vigo	Yes	Yes				+		+
Portugal	GtPort	*A. lusitanicum* (EB)		Yes	Yes			+		+	
France	AM2	*A. minutum* (ED)	Morlaix Bay	Yes	Yes				+		+
	AM3	*A. minutum* (ED)	Morlaix Bay	Yes	Yes				+		+
Japan											
North	N520	*A. catenella* (YF)	Hachinobe Hbr.	Yes	Yes		+				
	ND-1	*A. tamarense* (YF)	Noda Bay	Yes	Yes	+	+	+			
	OK875-1	*A. tamarense* (YF)	Okkirai Bay	Yes	Yes	+	+	+			
	OF875-8	*A. tamarense* (YF)	Ofunato Bay	Yes	nd		+	+			
	OF84423D	*A. tamarense* (YF)	Ofunato Bay	Yes	Yes		+	+			
	OF041	*A. tamarense* (YF)	Ofunato Bay	Yes	Yes		+	+			
	OF051	*A. tamarense* (YF)	Ofunato Bay	Yes	Yes		+	+			
	OF101	*A. catenella* (YF)	Ofunato Bay	Yes	Yes		+				

TABLE 1. *Continued.*

Geographic block	Strain[a]	Species designation[b]	Isolation locale	Clonal culture?	Toxic?[c]	Larger SSU PCR products[d]	SSU rDNA restriction sites[e]				
							"A"	"B"	Polymorphisms[f] Bsa(1)	Hae(1)	Hae(2)
South	TN-9	*A. catenella* (YF)	Tanabe Bay	Yes	Yes		+				
	WKS-1	*A. tamarense* (YF)	Tanabe Bay	Yes	No		+				
	WKS-3	*A. catenella* (YF)	Tanabe Bay	Yes	Yes		+				
	WKS-8	*A. catenella* (YF)	Tanabe Bay	Yes	Yes		+				
	N239	*A. tamarense* (YF)	Harima Nada	Yes	Yes		+	+			
Thailand	CU-1	*A. affine* (YF)	Gulf of Thailand	Yes	No					+	
	CU-13	*A. tamarense* (YF)	Gulf of Thailand	Yes	Yes		+				
Australia											
Mainland	ACPP01	*A. catenella* (GH)	Port Phillip Bay, Vic.	Yes	Yes		+				
	ACPP02	*A. catenella* (GH)	Port Phillip Bay, Vic.	Yes	Yes		+				
	ACPP03	*A. catenella* (GH)	Port Phillip Bay, Vic.	Yes	Yes		+				
	ACPP09	*A. catenella* (GH)	Port Phillip Bay, Vic.	Yes	Yes		+				
	AMAD01	*A. minutum* (GH)	Port River, S.A.	Yes	Yes				+		+
	AMAD06	*A. minutum* (GH)	Port River, S.A.	Yes	Yes				+		+
Tasmania	ATBB01	*A. tamarense* (GH)	Bell Bay	Yes	No*		+				
	AABB01/2	*A. affine* (GH)	Bell Bay	Yes	No					+	
Ballast water	I72/21#2	*A. tamarense* (GH)	Muroran, Japan[g]	Yes	Yes	+	+	+			
	I72/22#2	*A. tamarense* (GH)	Muroran, Japan[g]	Yes	Yes	+	+	+			
	I72/24#1	*A. tamarense* (GH)	Muroran, Japan[g]	Yes	Yes	+	+	+			
	ACJP03	*A. catenella* (GH)	Kashima, Japan[g]	Yes	Yes		+				
	G. Crux	*A. catenella* (GH)	Singapore[h]	No	Yes		+				
	G. Hope 1	*A. catenella* (GH)	Samchonpo, South Korea[g]	No	Yes		+				
	G. Hope 2	*A. catenella* (GH)	Samchonpo, South Korea[g]	No	Yes		+				

[a] Strain listings currently in use in the D. M. Anderson culture collection.
[b] As determined by (or using the criteria of) E. Balech (EB), F. J. R. Taylor (MT), I. Bravo (IB), E. Erard-Le Denn (ED), Y. Fukuyo (YF), and G. Hallegraeff (GH).
[c] Determined by mouse bioassay and/or HPLC analysis; nd, not determined; *, may contain trace amounts of toxin (D. Kulin, pers. commun.).
[d] Indicates presence of PCR products larger than expected (see Fig. 1b).
[e] Presence of restriction sites characteristic of the A gene (*Bsr*I digestion; "A"), the B gene (*Bsa*AI digestion; "B"), and both genes (*Hae*III and *Xba*I digestion; Fig. 1a).
[f] Deviations from predicted restriction patterns based on the sequences of the A and B genes; Bsa(1), anomalous *Bsa*I restriction pattern; Hae(1) and Hae(2), anomalous *Hae*III restriction patterns (see Fig. 1a, c, e).
[g] Origin of ballast water (Hallegraeff and Bolch 1992).
[h] Hailing port of vessel; origin of ballast water uncertain (Hallegraeff and Bolch 1992).

TABLE 2. *Summary of SSU rDNA RFLP group characteristics, representative species, known toxicity, and isolation locales of* Alexandrium *cultures screened using the A/B gene restriction test.*

SSU rDNA RFLP group	A/B restriction test characteristics[a]	Representative *Alexandrium* species	Toxic?[b]	Representative isolation locales[c]
I	A&B genes + large amplification products	*tamarense/catenella/fundyense*	Yes	Eastern North America
		tamarense	Yes	Japan
		tamarense	Yes	Ballast water (Japan)
II	A&B genes	*tamarense/catenella/fundyense*	Yes	Western North America
		tamarense	Yes	Japan
III	A gene only	*tamarense*	No	Western Europe
		tamarense	Wk	Thailand
		tamarense	No	Australia (Tasmania)
		catenella	Yes	Australia (mainland)
		tamarense/catenella	Yes	Japan
		tamarense/catenella	Yes	Ballast water (Japan, South Korea, and ?)
IV	*Hae*III RFLP [Hae(1)]	*affine*	No	Spain, Australia (Tasmania), and Thailand
V	*Bsa*AI & *Hae*III RFLPs [Bsa(1) & Hae(2)]	*minutum*	Yes	Australia (Tasmania) and France
		lusitanicum	Yes	Spain and Portugal
		andersoni	No	Eastern North America

[a] See Figure 1.
[b] Toxicity data for one Group II culture is lacking; Wk, weakly toxic (D. Kulis, pers. commun.)
[c] Some geographic regions are represented by only one or several isolates; the origin of one ballast water culture (?) is not known (see Table 1).

This holds true within both the closely related *A. tamarense/catenella/fundyense* group and the more distantly related *A. affine, A. lusitanicum,* and *A. minutum.* However, to date, all organisms found to harbor the B sequence were toxic (Table 2).

DISCUSSION

Sequencing of the A and B genes is labor-intensive and expensive because it requires analysis of multiple SSU rDNA clones to document both molecules and their respective nucleotide differences (Scholin et al. 1993). The prospects of screening a large number of isolates for these genetic markers using conventional sequencing techniques is therefore daunting, yet the A and B sequences clearly have the potential to be useful taxonomic and biogeographic markers. A compromise approach was to create theoretical restriction maps of the known A and B sequences and identify enzymes that could distinguish the genes, thereby providing a basis for their rapid detection. When both genes are present, enzymes that discriminate the two sequences should give rise to restriction fragments whose sum is approximately twice (or at least greater than) the size of the PCR-amplified SSU rDNA. In contrast, an enzyme that cleaves both genes in identical locations will produce restriction fragments whose sum is equal to that of the PCR product (Fig. 1a). The latter result is also expected when only a single class of gene is present.

Results of our study demonstrate that the A/B restriction test is a rapid and effective means of determining sequence heterogeneity among PCR-amplified SSU rDNAs from a variety of *Alexandrium* species. The RFLP patterns indicate that the *A. tamarense/catenella/fundyense* complex exists as a series of genetically distinct strains that do not strictly correspond to the three morphologically defined species. Instead, the strains appear indicative of divergent populations, each of which may harbor at least two or all three morphotypes. The RFLP analyses also indicate that this large species complex is distinct from *A. affine, A. minutum, A. lusitanicum,* and *A. andersoni,* regardless of geopgraphic origin. The A/B restriction test further subdivides the latter group of species, with *A. affine* being distinguishable from the *A. minutum/lusitanicum/andersoni* cluster. As currently defined, the RFLP screening procedure thus resolves relationships among *Alexandrium* species and strains (or populations). As additional enzymes are incorporated in the screening procedure, resolution of the assay should improve.

Specifically, three distinct groups within the *Alexandrium* tamarensis complex can now be recognized on the basis of their SSU rDNA characteristics (Groups I–III; Fig. 1a–f, lanes I–III; Table 2). All *A. tamarense, A. catenella,* and *A. fundyense* examined share the predicted A gene restriction pattern. The primary subdivision among this large group stems from those isolates that carry the B gene (Groups I and II) and those that do not (Group III). Cultures harboring the B gene are further distinguishable on the basis of whether their SSU rDNA PCR products include molecules larger than those expected (Group I) and those whose PCR products appear homogeneous and of the predicted size (Group II; Table 2). Presence or absence of the larger products was initially considered to be an artifact of the PCR process. Repeated attempts to optimize the amplification reactions failed to eliminate the apparently spurious molecules but, otherwise, resulted in highly specific

amplifications (Fig. 1b). Preliminary analysis of the larger products indicates they are a class of *Alexandrium* SSU rDNA that contains a direct repeat of a portion of the sequence in the 3′ half of the molecule (Scholin 1993). The distinction between Group I and II isolates is further supported by sequence analysis of their LSU rDNA (Scholin 1993).

A peculiar characteristic of both Groups I and II is that the B gene appears to comprise approximately half of the PCR-amplified SSU rDNA product. There is little to no variation in these proportions among all Group I and II isolates. If the ratio of the PCR products reflects the relative abundances of the genes in the extracted DNAs, then it is possible that half of the ribosomal transcription units in these organisms contain a B sequence. The mechanism responsible for maintaining such a high copy number of an apparent pseudogene remains a mystery.

Another consistent characteristic of PCR-amplified B genes is the unpredicted (but minor) *Bsa*AI digestion products. The minor bands appear to originate from molecules that contain only one of the two predicted B sequence *Bsa*AI sites. Partial digestion of the B gene is one explanation for the appearance of these minor products, but increasing enzyme concentration and time of digestion does not eliminate them (data not shown). Consequently, the minor bands likely originate from chimeric molecules; SSU rDNA, which includes a 5′ portion of the A gene and 3′ portion of the B gene, or 5′ portion of the B gene and 3′ portion of the A gene. It is possible these chimeras are generated during PCR by template strand switching (Erlich et al. 1991) or represent yet another, minor class of SSU rDNA that exists *in vivo*.

Within the tamarensis species complex, there is no strict correlation between morphospecies designations and SSU rDNA RFLP group (Table 2). For example, isolates from western North America include *A. tamarense, A. catenella,* and *A. fundyense*; nonetheless, all share Group II characteristics. Likewise, isolates from eastern North America include *A. tamarense* and *A. fundyense,* but all belong to Group I. Thus, morphospecies designations may or may not agree with groupings based on the RFLP assay. The same is true for Japanese isolates. For example, OF041 (*A. tamarense*) and OF101 (*A. catenella*) are members of Groups II and III, respectively, but WKS-1 (*A. tamarense*) and TN9 (*A. catenella*) are both members of Group III. A similar pattern is observed among ballast water isolates. I1724#1 (*A. tamarense,* Japan) and ACJP03 (*A. catenella,* Japan) belong to Groups I and III, respectively, but the former isolate and G. Hope 1 (*A. tamarense,* Korea) are also members of Groups I and III, respectively. Many other examples of this kind of inconsistency are evident in Tables 1 and 2.

A possible explanation for the disparity between RFLP patterns and morphospecies designations could be the fact that different taxonomists classified our cultures and, thus, that species designations were not consistent. This is unlikely because examples of positive and negative correlations between morphotype and RFLP patterns can be found within groups of cultures examined by the same taxonomist. In the examples cited earlier, the western and eastern North American isolates were classified by Balech, the Japanese isolates by Fukuyo, and the ballast water isolates by Hallegraeff (Table 1). Therefore, agreements or disagreements between morphology and SSU rDNA RFLP group are not a function of the taxonomist. Rather, it appears that observed relationships reflect the geographic origin of the cultures as well as the particular strains chosen for analysis. This conclusion agrees with the collective observations of Cembella and Taylor (1986). Cembella et al. (1987, 1988), Hayhome et al. (1989), and Sako et al. (1990, 1993): *A. tamarense, A. catenella,* and *A. fundyense* show no consistent correlation between morphological and biochemical characteristics.

The fact that the B gene exists in *A. tamarense, A. catenella,* and *A. fundyense,* but not in all representatives of their globally distributed populations, supports the notion that morphological features used to define the three species reflect strain-specific characters, not clearly defined species boundaries. Furthermore, different regional populations of the same morphospecies can be genetically distinct (e.g. North American and western European *A. tamarense*). Taken together, these results and those of the investigators already noted lead us to the conclusion that the *Alexandrium* tamarensis complex is composed of genetically distinct *populations,* within which can be at least two or all three of the morphotypically defined species. An evolutionary model accounting for this scenario will be presented elsewhere.

The known biogeography of Groups I–III is noteworthy: eastern North American isolates belong to Group I, western North American isolates belong to Group II, and Australian, western European, and the weakly toxic isolate from Thailand are within Group III. In contrast, isolates from Japan were found among all three groups. Presently, we are exploring the possibilities that Group I and II characteristics reflect genetic markers indicative of eastern and western North American regional populations, respectively, and that the genetic diversity of Japanese *A. tamarense* and *A. catenella* stems from multiple introductions from genetically distinct source populations (including those in North America). In this context, it is of note that paralytic shellfish poisons first became a problem in Japan in the late 1940s (Anraku 1984). The potential dispersal of North American *A. tamarense* and *A. catenella* to Japan will be addressed in greater detail at a later time.

RFLP patterns of ballast water isolates provide evidence that resting cysts of *A. tamarense* and *A.*

catenella from documented blooms in Japan and Korea have not only been transported to specific Australian ports (Hallegraeff and Bolch 1991, 1992) but have also originated from genetically distinct populations. One ship ballasted in Muroran, Japan, contained Group I *A. tamarense*; a second ballasted in Kashima, Japan, carried a Group III *A. catenella*; and a third ballasted in Shamchonpo, South Korea, contained Group III *A. tamarense*. The potential "seeding" of Australian waters with genetically diverse representatives of the tamarensis group provides a striking example of what may have occurred in Japan sometime in the recent past. In addition, this demonstrates that SSU rDNA RFLP patterns are potentially useful tools for tracing movements of particular strains throughout the globe.

Isolates of *A. affine, A. minutum, A. lusitanicum,* and *A. andersoni* were included in the A/B restriction tests because they are considered to be taxonomically distinct from the *A. tamarense/catenella/fundyense* group. Given the significant morphological differences between these groups (Balech 1985, Balech and Tangen 1985), it is not surprising that their SSU rDNA sequences differ as well. The unique *Hae*III and *Bsa*AI restriction patterns identified are a reflection of this divergence and fortuitously made it possible to subdivide *A. affine, A. minutum, A. lusitanicum,* and *A. andersoni* into two distinct clusters (Groups IV and V), with *A. affine* being separate from the other species (Fig. 1, Table 2). To date, no evidence indicates multiple classes of SSU rDNA within *A. affine, A. minutum, A. lusitanicum,* and *A. andersoni* since the sum of the restriction products for each individual digest roughly equals that of the PCR products.

SSU rDNA from *A. minutum, A. lusitanicum,* and *A. andersoni* all showed the Hae(2) and Bsa(1) RFLP patterns that constitute the Group V designation. These species have previously been separated on the basis of fine-scale morphological variations (Balech 1985), but Hallegraeff (pers. commun.) suggested that these may be variants of the "same" species. The restriction enzymes employed in the A/B restriction tests support Hallegraeff's contention: *A. minutum, A. lusitanicum,* and *A. andersoni* share common restriction patterns and thus are more closely related to each other than to *A. affine* or members of the tamarensis complex. Further analysis using sequences of LSU rDNA has resolved linkages within the *A. minutum/lusitanicum/andersoni* cluster and indicates that *A. andersoni* is distinct from *A. minutum/lusitanicum* (Scholin 1993).

The differentiation of those organisms in Groups I–III from those in Groups IV and V is consistent with current taxonomic designations. That is, results of the RFLP assay agree that the *A. tamarense/catenella/fundyense* group as a whole is distinct from *A. affine, A. minutum, A. lusitanicum,* and *A. andersoni.* The further delineation between *A. affine* (Group IV) and *A. minutum, A. lusitanicum,* and *A. andersoni* (Group V) also agrees with current morphotaxonomic designations.

CONCLUSIONS

This study clearly demonstrates that SSU rDNAs are sufficiently variable to separate closely related *Alexandrium* species or populations and that the A/B gene restriction test is a technically simple way to reveal these genetic differences. It should be possible to move beyond the work presented here to devise highly specific tests for defined groups of *Alexandrium* species and strains of single species by increasing the number of enzymes or by obtaining complete SSU rDNA sequences. The growing RFLP pattern and sequence data bases could thus provide genetic criteria for characterizing isolates and predicting their potential toxicity or geographic origins. In addition, the identificaiton of genetically distinct populations of *A. tamarense, A. catenella,* and *A. fundyense* begins to shed light on the long-standing controversy concerning correlations (or the lack thereof) between morphological and biochemical characteristics.

A limitiation of A/B gene restriction test as currently defined is that it samples only 3 of the 40 known nucleotide differences between the two sequences. If the B gene is no longer under selective pressure, it may be undergoing rapid evolution; consequently, further resolution of sexually isolated populations that carry this sequence ("B gene subgroups") is possible. It is also possible that isolates within Groups III–V carry "B-like genes" (i.e. other SSU rRNA pseudogenes) that were not detected by the RFLP assay. Establishing the existence of "B gene subgroups" and "B-like sequences" are important areas of future research that must be pursued prior to making rigorous conclusions based on the "uniqueness" of the B gene.

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Amann, R. I., Krumholz, L. & Stahl, D. A. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* 172:762–70.

Anderson, D. M. 1989. Toxic algal blooms and red tides: a global perspective. *In* Okaichi, T., Anderson, D. M. & Nemoto, T. [Eds.] *Red Tides: Biology, Environmental Science and Toxicology.* Elsevier, New York, pp. 11–20.

Anderson, D. M., Kulis, D. M. & Binder, B. J. 1984. Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamarensis*: cyst yield in batch cultures. *J. Phycol.* 20:418–25.

Anraku, M. 1984. Shellfish poisoning in Japanese waters. *In* White, A. W., Anraku, M., Hooi, K-K. [Eds.] *Toxic Red Tides and Shellfish Toxicity in Southeast Asia.* Southeast Asian Fisheries Development Center, Singapore, and the International Development Research Centre, Ottawa, Canada, pp. 105–9.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Siedman, J. G., Smith, J. A. & Struhl, K., [Eds.] 1987. *Current Protocols in Molecular Biology,* Vols. 1, 2. Wiley Interscience, New York.

Balech, E. 1985. The genus *Alexandrium* or *Gonyaulax* of the tamarensis group. *In* Anderson, D. M., White, A. W. & Baden, D. G. [Eds.] *Toxic Dinoflagellates.* Elsevier, New York, pp. 33–8.

Balech, E. & Tangen, K. 1985. Morphology and taxonomy of toxic species in the *tarmarensis* group (Dinophyceae): *Alexandrium excavatum* (Braarud) comb. nov. and *Alexandrium ostenfeldii* (Paulsen) comb. nov. *Sarsia* 70:333–43.

Cembella, A. D., Sullivan, J. J., Boyer, G. L., Taylor, F. J. R. & Andersen, R. J. 1987. Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis/catenella* species complex: red tide dinoflagellates. *Biochem. Syst. Ecol.* 15:171–86.

Cembella, A. D. & Taylor, F. J. R. 1986. Electrophoretic variability within the *Protogonyaulax tamarensis/catenella* species complex: pyridine linked dehydrogenases. *Biochem. Syst. Ecol.* 14:311–23.

Cembella, A. D., Taylor, F. J. R. & Therriault, J-C. 1988. Cladistic analysis of electrophoretic variants within the toxic dinoflagellate genus *Protogonyaulax. Bot. Mar.* 31:39–51.

Curran, J., Baillie, D. L. & Webster, J. M. 1985. Use of genomic DNA restriction fragment length differences to identify nematode species. *Parasitology* 90:137–44.

Destombe, C., Cembella, A. D., Murphy, C. A. & Ragan, M. A. 1992. Nucleotide sequence of the 18S ribosomal RNA genes from the marine dinoflagellate *Alexandrium tamarense* (Gonyaulacales, Dinophyta). *Phycologia* 31:121–4.

Distel, D. L., DeLong, E. F. & Waterbury, J. B. 1991. Phylogenetic characterization and in situ localization of the bacterial symbiont of shipworms (Teredinidae: Bivalvia) by using 16S rRNA sequence analysis and oligonucleotide probe hybridization. *Appl. Environ. Microbiol.* 57:2376–82.

De Buyser, M-L., Morvan, A., Aubert, S., Dilasser, F. & El Solh, N. 1992. Evaluation of a ribosomal RNA gene probe for the identification of species and subspecies within the genus *Staphylococcus. J. Gen. Microbiol.* 138:889–99.

Erlich, H. A., Gelfland, D. & Sninsky, J. J. 1991. Recent advances in the polymerase chain reaction. *Science (Wash. D.C.)* 252: 1643–51.

Field, K. G., Olsen, G. J., Lane, D. J., Giovannoni, S. J., Ghiselin, M. T., Raff, E. C., Pace, N. R. & Raff, R. A. 1988. Molecular phylogeny of the animal kingdom. *Science (Wash. D.C.)* 239: 748–53.

Fukuyo, Y. 1985. Morphology of *Protogonyaulax tamarensis* (Lebour) Taylor and *Protogonyaulax* catenella (Whedon and Kofoid) Taylor from Japanese coastal waters. *Bull. Mar. Sci.* 37: 529–37.

Gobel, U. B., Geiser, A. & Stanbridge, E. J. 1987. Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* species. *J. Gen. Microbiol.* 133:1969–74.

Goff, L. J. & Coleman, A. W. 1988. The use of plastid DNA restriction endonuclease patterns in delineating red algal species and populations. *J. Phycol.* 24:357–68.

Hallegraeff, G. & Bolch, C. J. 1991. Transport of toxic dinoflagellate cysts via ship's ballast water. *Mar. Poll. Bull.* 22:27–30.

——— 1992. Transport of toxic dinoflagellate cysts via ship's ballast water: implications for plankton biogeography and aquaculture. *J. Plankton Res.* 14:1067–84.

Hallegraeff, G., Bolch, C. J., Blackburn, S. I. & Oshima, Y. 1991. Species of the toxigenic dinoflagellate genus *Alexandrium* in southeastern Australian waters. *Bot. Mar.* 34:575–87.

Hayhome, B. A., Anderson, D. M., Kulis, D. M. & Whitten, D. J. 1989. Variation among congeneric dinoflagellates from the northeastern United States and Canada. I. Enzyme electrophoresis. *Mar. Biol. (Berl.)* 101:427–35.

Lenaers, G., Scholin, C. A., Bhaud, Y., Saint-Hilaire, D. & Herzog, M. 1991. A molecular phylogeny of dinoflagellate protists (Pyrrhophyta) inferred from the sequence of the 24S rRNA divergent domains D1 and D8. *J. Mol. Evol.* 32:53–63.

Levy, M., Romao, J., Marchetti, M. A. & Hamer, J. E. 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. *Plant Cell* 3:95–102.

Maeda, M., Uryu, N., Murayama, N., Ishi, H., Ota, M., Tsuji, K. & Inoko, H. 1991. A simple and rapid method for HLA-DRB and DQB typing by digestion of PCR amplified DNA with allele specific restriction endonucleases. *Tissue Antigens* 38:60–71.

McCutchan, T. F., de la Cruz, V. F., Lal, A. A., Gunderson, J. H., Elwood, H. J. & Sogin, M. L. 1988. Primary sequences of two small subunit ribosomal RNA genes from *Plasmodium falciparum. Mol. Biochem. Parasitol.* 28:63–8.

Moody, M. D. 1989. DNA analysis in forensic science. *BioScience* 39:31–6.

Olsen, G. J., Lane, D. J., Giovannoni, S. J., Pace, N. R. & Stahl, D. A. 1986. Microbial evolution: a ribosomal RNA approach. *Annu. Rev. Microbiol.* 40:337–65.

Prakash, A., Medcof, J. C. & Tennant, A. D. 1971. Paralytic shellfish poisoning in eastern Canada. *Bull. Fish. Res. Board. Can.* 177:1–87.

Rowan, R. & Powers, D. A. 1991. A molecular genetic classification of zooxanthellae and the evolution of animal–algal symbioses. *Science (Wash. D.C.)* 251:1348–51.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. D.C.)* 239:487–91.

Sako, Y., Adachi, M. & Ishida, Y. 1993. Preparation and characterization of monoclonal antibodies to *Alexandrium* species. *In* Smayda, T. J. & Shimizu, Y. [Eds.] *Toxic Phytoplanton Blooms in the Sea.* Elsevier, New York, pp. 87–93.

Sako, Y., Kim, C. H., Ninomiya, H., Adachi, M. & Ishida, Y. 1990. Isozyme and cross analysis of mating populations in the *Alexandrium catenella/tamarense* species complex. *In* Graneli, E., Sundstrom, B., Edler, L. & Anderson, D. M. [Eds.] *Toxic Marine Phytoplankton.* Elsevier, New York, pp. 320–3.

Schlegel, M., Steinbruck, G., Kramer, M. & Brockmeyer, V. 1991. Restriction patterns as molecular markers for species identification and phylogenetic analysis in the genus *Enchytraeus* (Oligochaeta). *Z. Zool. Syst. Evolut.-Forsch.* 29:362–72.

Scholin, C. A. 1993. Analysis of toxic and non-toxic *Alexandrium* (Dinophyaceae) species using ribosomal RNA gene sequences. Ph.D. thesis, Massachusetts Institute of Technology/ Woods Hole Oceanographic Institution, WHOI93-08, 251 pp.

Scholin, C. A. & Anderson, D. M. 1993. Population analysis of toxic and nontoxic *Alexandrium* species using ribosomal RNA signature sequences. *In* Smayda, T. J. & Shimizu, Y. [Eds.] *Toxic Phytoplankton Blooms in the Sea.* Elsevier, New York, pp. 95–102.

Scholin, C. A., Anderson, D. M. & Sogin, M. 1993. The existence of two distinct small-subunit rRNA genes in the toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae). *J. Phycol.* 29:209–16.

Sogin, M. L. 1990. Amplification of ribosomal RNA genes for molecular evolution studies. *In* Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. [Eds.] *PCR Protocols: A Guide to Methods and Applications.* Academic Press, San Diego, pp. 307–14.

Sogin, M. L., Elwood, H. J. & Gunderson, J. H. 1986. Evolutionary diversity of eukaryotic small-subunit rRNA genes. *Proc. Nat. Acad. Sci. U.S.A.* 83:1383–7.

Stahl, D. A., Flesher, B., Mansfield, H. R. & Montgomery, L. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol.* 54:1079–84.

Steidinger, K. A. 1990. Species of the *tamarensis/catenella* group of *Gonyaulax* and the fucoxanthin derivative-containing gymnodiniods. *In* Graneli, E., Sundstrom, B., Edler, L. & Anderson, D. M. [Eds.] *Toxic Marine Phytoplankton.* Elsevier, New York, pp. 11–6.

Steidinger, K. A. & Moestrup, Ø. 1990. The taxonomy of *Gonyaulax, Pyrodinium, Alexandrium, Gessnerium, Protogonyaulax* and *Goniodoma. In* Graneli, E., Sundstrom, B., Edler, L. & Anderson, D. M. [Eds.] *Toxic Marine Phytoplankton.* Elsevier, New York, pp. 522–3.

Taylor, F. J. R. 1984. Toxic dinoflagellates: taxonomic and biogeographic aspects with emphasis on *Protogonyaulax. In* Ragelis, E. P. [Ed.] *Seafood Toxins.* Am. Chem. Soc. Symp. Series No. 262, Washington, D.C., pp. 77–97.

——— 1985. The taxonomy and relationships of red tide dinoflagellates. *In* Anderson, D. M., White, A. W. & Baden, D. G. [Eds] *Toxic Dinoflagellates.* Elsevier, New York, pp. 11–26.

——— 1990. Red tides, brown tides and other harmful algal blooms: a view into the 1990's. *In* Graneli, E., Sundstrom, B., Edler, L. & Anderson, D. M. [Eds.] *Toxic Marine Phytoplankton.* Elsevier, New York, pp. 527–33.

Wetton, J. H., Carter, R. E., Parkin, D. T. & Walters, D. 1987. Demographic study of a wild house sparrow population by DNA fingerprinting. *Nature (Lond.)* 327:147–9.