IDENTIFICATION OF *ALEXANDRIUM* SPECIES AND STRAINS USING RFLP ANALYSIS OF PCR-AMPLIFIED LSU rDNA

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ABSTRACT

A variety of toxic and non-toxic *Alexandrium* (Halim) Balech species collected from many regions of the world were compared previously on the basis of their large-subunit ribosomal RNA gene sequences (LSU rDNA), revealing 11 genetically distinct groups termed "ribotypes" and "subribotypes." Collection and analysis of these sequences is labor intensive, restricting broader-scale comparisons of rDNA from existing as well as novel cultures. Here, we describe the development of a restriction fragment length polymorphism (RFLP) assay that speeds and eases LSU rDNA characterization. Using this technique, one can identify all of *Alexandrium* ribotypes and subribotypes definied thus far at a fraction of the time, labor and cost required for sequencing. Applications of this technique include mapping the biogeographic distribution of ribotypes, species and populations of *Alexandrium* to infer routes of natural and human-assisted dispersal, and testing the apparent positive correlation between an organism's evolutionary lineage and its ability to produce toxin.

INTRODUCTION

In a previous study, Scholin et al. [1] compared 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 to assess inter- and intraspecific relationships. Cultures examined were from North America, western Europe, Thailand, Japan, Australia and the ballast water of several cargo vessels, and included both toxic and non-toxic isolates. Toxic isolates are those that produce saxitoxin and its derivatives, potent neurotoxins responsible for paralytic shellfish poisoning [2]. Parsimony analyses revealed eight major sequence types, or "ribotypes," indicative of both species and strain-specific genetic markers. Each ribotype was given a name as shown in Fig. 1 and Table 1. The North American and temperate Asian ribotypes also contain subgroups, termed "subribotypes." A total of 11 unique *Alexandrium* rDNA genotypes were thus identified [see 1]. The objective of the work reported here was to determine if signature sequences denoting ribotypes and

subribotypes could be visualized independently using restriction fragment length polymorphism (RFLP) analysis.

Globally distributed populations of *A. tamarense, A. catenella* and *A. fundyense* (the "*tamarensis* complex") are comprised of at least five major evolutionary lineages. These lineages do not reflect the development of different morphotypes, but instead reveal the independent evolution of geographically isolated regional populations. Members of the *tamarensis* complex can be genetically similar or distinct, regardless of their expressed morphotype. Elsewhere, we proposed that this pattern reflects a monophyletic radiation and global dispersal that began many millions of years ago [3]. *Alexandrium affine, A. minutum* and *A. andersoni* also exhibit unique LSU rDNA "signatures" that serve to distinguish them from one another, as well as from those of the *tamarensis* group as a whole. *Alexandrium lusitanicum* and *A. minutum* appear identical with respect to their LSU rDNA sequence. Franco et al. [4] have proposed that the latter two species are synonomous on the basis of morphology, a result that agrees with our genetic analyses. There is a positive correlation between terminal groups defined phylogenetically and those organisms considered toxic (Fig. 1).

Collection and analysis of rDNA sequences from a variety of *Alexandrium* species has proven labor intensive and difficult. In many cases, clonal, unialgal isolates were found to contain multiple classes of rDNA not attributable to culture contaminants [1]. This severely restricts broader-scale comparisons of rDNA from existing as well as novel cultures, because multiple rDNA clones must be sequenced individually to document different classes of molecules present in a single culture [5]. Furthermore, clonal biasing (i.e., the random selection of one sequence variant over another from a common pool of PCR products) affects the appearance of sequences generated for a given culture [1]. Errors may thus be propogated in the sequence data base, in turn affecting further applications of acquired data [8].

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 sequence analyses also offer fine-

A. tamarense ✶ (PW06)
A. fundyense ✶ (PI32)   **Western**
A. catenella ✶ (BGt)   **1**

A. fundyense ✶ (AFNFA3.1)

A. fundyense ✶ (CA29)
A. fundyense ✶ (AFNFA3.2)   **Eastern**
A. fundyense ✶ (AFNFA4)   **2**
A. fundyense ✶ (GtMP)
A. tamarense ✶ (I72/24#1)

SSU
B Genes

North
American
1-3

. A. tamarense ✶ (OF041)   **Alternate**
A. tamarense ✶ (OF051)   **3**

A. tamarense (183)
A. tamarense (PE1V)   **Western** **4**
A. tamarense (PE2V)   **European**
A. tamarense (WKS-1)

SSU
A Genes

A. catenella ✶ (OF101)
A. catenella ✶ (TN9)
A. catenella ✶ (WKS-8)
A. catenella ✶ (ACPP01)   **Japanese**
A. catenella ✶ (ACPP02)   **5**
A. catenella ✶ (ACJP03)
A. catenella ✶ (G. Crux)
A. tamarense ✶ (G.Hope 1)   **Korean**
A. tamarense ✶ (G. Hope 2)   **6**

Temperate
Asian
5-6

A. tamarense (ATBB02) **Tasmanian 7**
A. tamarense ✶ (CU-13) **Tropical Asian 8**

SSU
Hae(1) RFLP

A. affine (CU-1)
A. affine (AABB01/2)   *affine* **9**
A. affine (PA5V)

SSU
Hae(2) & Bsa(1) RFLPs

A. minutum ✶ (AMAD01)
A. minutum ✶ (AMAD06)   *minutum* **10**
A. lusitanicum ✶ (GtPort)

A. andersoni (TC02) *andersoni* **11**
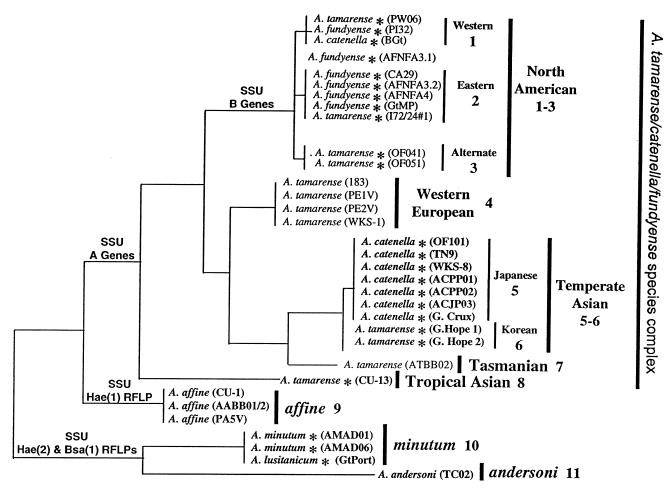
A. tamarense/catenella/fundyense species complex

Fig.1. Phylogentic tree showing the relationships of a variety of *Alexandrium* species and populations based on small and large-subunit rDNA sequences [1, 5, 6]; * denotes those isolates considered toxic [1]. Numbers 1-11 refer to distinct ribotypes and subribotypes (terminal groups) as discussed elsewhere [1].

scale resolution of distinct groups contained within larger regional populations. The latter is relevant to reconstructing the organisms' evolutionary history and routes of natural and human-assisted dispersals [1, 3]. 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 employed.

In this article we report on efforts to test the accuracy of our sequence data base. To this end we pose two questions, both of which rely on RFLP analysis: (a) Are *Alexandrium* LSU rDNA sequences acquired thus far consistent with major classes of sequence present in corresponding pools of PCR-amplified molecules? and, (b) can we apply knowledge gained from (a) to speed characterization of LSU rDNA from additional *Alexandrium* isolates and thereby facilitate studies of the organisms' evolution, biogeography, dispersal, and toxicity?

METHODS

Cultures used in this investigation are shown in Table 1. Each isolate represents a unique terminal group from Fig. 1. Details of culture sources, culture maintenance, nucleic acid extraction, LSU rDNA sequencing, and designation of terminal taxa are noted elsewhere [1, 6]. Reference sequences were scanned for restriction sites using MacDNASIS Pro (v 1.0) software, then compared to determine which enzymes would delineate one or more ribotypes; *Afl* III (New England Biolabs; NEB), *Apa* L (NEB), *Hinc* II (NEB), *Mse* I (NEB), and *Nsp* I (United States Biochemical Corp.) were chosen for further testing. Two fragments of LSU rDNA, termed D1R/D2C and D1R/D3Ca, were digested with the above enzymes. The former fragment is the same as that analyzed by cloning and sequencing as described earlier [1]. The latter is a longer fragment that includes the D1/D2C region as well as ~ 200 base pairs (bp) of sequence distal to the D2C primer target [7]. PCR amplification and restriction digests followed general guidelines as discussed by Scholin and Anderson [6] and Scholin et

Table 1. *Alexandrium* cultures used as standards for RFLP analysis; each represents a terminal group in Fig. 1. Also listed are their associated ribotype, subribotype, culture code, and species designation. See [1] for additional details and acknowledgments for culture source.

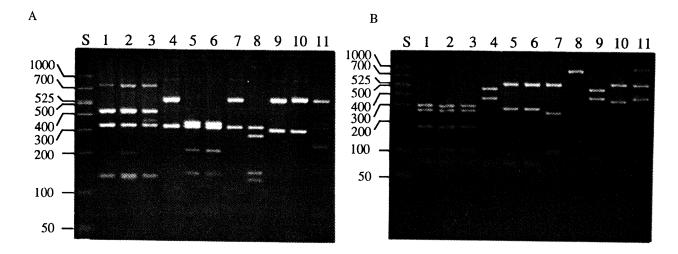| Terminal Group	Ribotype	Subribotype	Culture Code	Species Designation
1	North American	western	PW06	*A. tamarense*
2	North American	eastern	AFNFA4	*A. fundyense*
3	North American	alternate	OF041	*A. tamarense*
4	Western European		WKS-1	*A. tamarense*
5	Temperate Asian	Japanese	OF101	*A. catenella*
6	Temperate Asian	Korean	G. Hope1	*A. tamarense*
7	Tasmanian		AtBB03	*A. tamarense*
8	Tropical Asian		CU13	*A. tamarense*
9	*affine*		CU1	*A. affine*
10	*minutum*		AMAD06	*A. minutum*
11	*andersoni*		TC02	*A. andersoni*

Fig. 2. Agarose gels showing *Mse* I (A) and *Nsp* I (B) digests of the DIR/D3Ca LSU rDNA fragment from isolates representing terminal groups shown in Fig. 1 and Table 1. Gel lane numbers correspond to ribotypes and subribotypes in Table 1; S = size standards (bp).

al. [1, 7]; details will be given elsewhere [8]. Products of digestions were resolved by standard gel elcetrophoresis using 1x TAE buffer [9] and a 3:1 mixture of Nusieve: SeaPlaque agaroses (FMC Corp.).

RESULTS AND DISCUSSION

There is excellent agreement between predicted and observed RFLP patterns for the D1R/D2C-primed LSU rDNA fragment [8]. All ribotypes and subribotypes defined by sequencing and phylogenetic analysis (Fig. 1, Table 1) were also revealed independently and repeatedly by restriction digests of PCR-amplified material. Five sites of fine-scale heterogeneity noted by sequencing were confirmed by restriction digests. These sites represent signature sequences indicative of

multiple classes of LSU rDNA that define subribotypes of the North American and temperate Asian groups [1]. RFLP analysis of the D1R/D3Ca-primed fragment gave results equivalent to those above. Examples of *Mse*I and *Nsp*I digests of the D1R/D3Ca fragment are shown in Fig. 2. These two treatments serve to identify 10 of the 11 known *Alexandrium* ribotypes; application of additional enzymes confirms these designations independently and also reveal differences between the Japanese and Korean subribotypes of the temperate Asian cluster [8], resolving 11 of 11.

The fact that many classes of rDNA are evident by both sequencing and RFLP analyses suggests that the tree branch lengths shown in Fig. 1 may not accurately describe the divergence of terminal taxa. Clonal biasing has undoubtedly introduced some error into the

sequence data base and phylogenetic tree [1], but the extent of this error is not clear, especially when one considers the likelihood of additional heterogeneity not yet recorded. Nevertheless, results of RFLP analyses substantiate the overall tree topology as shown in Fig. 1. These results also provide further evidence that fine-scale sequence differences such as those discussed by Scholin et al. [1, 5] are valid genetic markers which should not be ignored. Although multiple classes of rDNA from a single isolate complicate sequence analyses, such "complications" also provide a basis for improving resolution of genetically distinct species and populations [1, 3, 5, 6]. Similar findings were reported for a number of *Pseudo-nitzschia* H. Peragallo species, some of which are associated with the production of domoic acid [7].

CONCLUSIONS

We have devised RFLP assays that define 11 *Alexandrium* LSU rDNA ribotypes and subribotypes, each of which serves as a species and/or population-specific marker. Novel clones are now being screened with relative ease to define their rDNA evolutionary lineage at a fraction of the time, cost and labor required for sequencing. This information can be crossed-referenced with a wide range of other data [e.g., 10-15]. By applying the RFLP assay to define an organism's rDNA evolutionary lineage, it may be possible to delineate that isolate's mating group affinity and, perhaps, to predict its ability to produce toxins (etc.). Mapping the biogeographic distributions of known ribotypes will also be easier with the advent of this technique. This will speed tests of evolutionary concepts and dispersal hypotheses as proposed elsewhere [3]. The RFLP assay is also a rapid means to identify novel ribotypes that can then be characterized by sequence analysis.

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