Reference: *Biol. Bull.* **185:** 329–330. (October, 1993)

RFLP Analysis of a Fragment of the Large-Subunit Ribosomal RNA Gene of Globally Distributed Populations of the Toxic Dinoflagellate *Alexandrium*

Bryan S. Judge (Michigan State University), Christopher A. Scholin (Monterey Bay Aquarium Research Institute), and Donald M. Anderson (Woods Hole Oceanographic Institution)

The dinoflagellate genus *Alexandrium* includes several species that produce potent neurotoxins responsible for paralytic shellfish poisoning (PSP). Commonly called "red tides," these events occur when shellfish and other marine animals consume the toxic phytoplankton as food, concentrating their toxins to levels that can cause illness and even death in consumers. PSP has been a problem for centuries in many of the world's coastal regions, but in recent years, the number of countries and areas affected has increased. This increase has been attributed in part to natural and human-assisted dispersal (1, 2). The evaluation of dispersal hypotheses depends on an understanding of the global biogeography of *Alexandrium* species, yet virtually nothing is known of their biogeography.

At the regional level, the inter- and intraspecific relationships of toxic *Alexandrium* have been assessed by morphological comparisons, isozyme electrophoresis, and toxin composition analysis (*e.g.*, 3, 4, 5). Unfortunately, these methodologies often gave conflicting information about the relatedness of isolates of *Alexandrium* from different regions. Recently, sequence analysis of a fragment of the large-subunit ribosomal RNA gene (LsrDNA) from globally distributed *Alexandrium* isolates revealed eight distinctive classes of sequences, or "ribotypes" (6). For morphologically distinct species, such as *A. affine, A. minutum,* and *A. andersoni,* separate ribotypes are identified, confirming the validity of species designations based on morphology. However, for morphologically similar species within the so-called *A. tamarense/catenella* species complex (the "tamarensis group"), ribotypes appear to delineate regional populations, not morphospecies. Regarding the latter observation, isolates from the same geographic region have ribotypes that are similar, regardless of morphotype, whereas isolates from geographically distant populations display more divergent ribotypes even when the same morphospecies are compared (6).

Sequencing of LsrDNA has proven useful for assessing the inter- and intraspecific relationships of toxic *Alexandrium* strains, but it is also time consuming, tedious, and expensive. We therefore sought to develop a restriction-fragment-length polymorphism (RFLP) assay for each ribotype, to reduce analytical costs of our program of biogeographic surveys. To accomplish this goal, sequences representing each of the tamarensis group's ribotypes ("North American," "Western European," "Temperate Asian," "Tropical Asian," and "Tasmanian") and those of *A. affine, A. minutum,* and *A. andersoni* ("affine," "minutum," and "andersoni," respectively) were examined for restriction site polymorphisms. Based on this analysis, five restriction enzymes were selected that theoretically should distinguish each of these ribotypes, as well as "subribotypes" within the North American and Temperate Asian clusters (6).

To test the predictions, 11 isolates previously compared by LsrDNA sequencing were selected for trial RFLP analysis: (1) North American ribotype—PWO6 (*A. tamarense*); AFNFA4 (*A. fundyense*); OFO41 (*A. tamarense*); (2) Western European ribotype—WKS-1 (*A. tamarense*); (3) Temperate Asian ribotype—OF1O1 (*A. catenella*); G. HOPE 1 (*A. tamarense*); (4) Tropical Asian ribotype—CU-13 (*A. tamarense*); (5) Tasmanian ribotype—ATTBO1 (*A. tamarense*); (6) "affine" ribotype—CU-1 (*A. affine*); (7) "minutum" ribotype—AMADO6 (*A. minutum*); (8) "andersoni" ribotype—TCO2 (*A. andersoni*).

Nucleic acid extractions and LsrDNA polymerase chain reaction (PCR) amplifications were performed as previously described (6). PCR-amplified LsrDNA from each isolate was then digested separately with *Nsp*-I, *Hinc*-II, *ApaL*-I, *Mse*-I, and *Afl*-III, following enzyme manufacturers' recommendations. Digestion products were resolved on 2.5% NuSieve (FMC Corp.), 1x

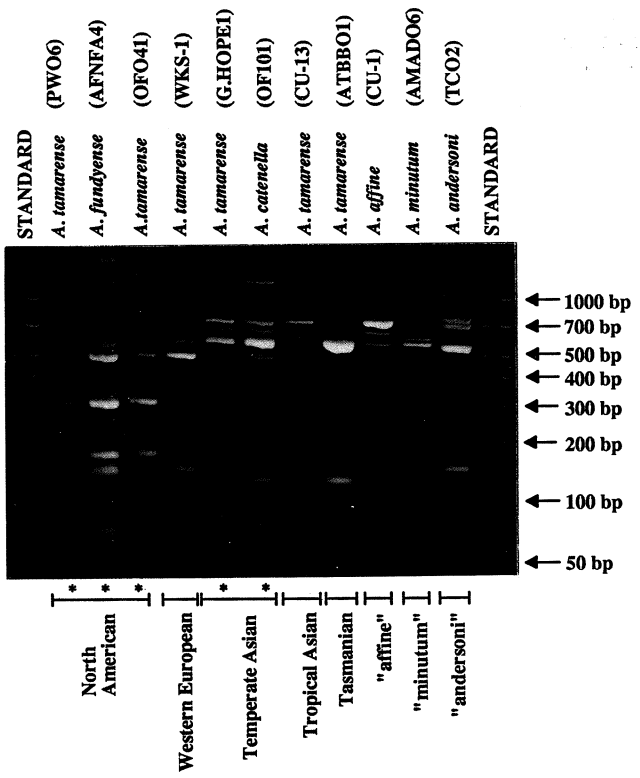

Figure 1. *Agarose gel showing LsrDNA fragments from RFLP analysis of eleven isolates of* Alexandrium *that were digested with the restriction enzyme* Nsp-*I. Species designations are listed at the top of the figure followed by the isolate designation. Ribotype classifications, as determined from sequence analysis, are listed along the bottom of the figure. The * represents subribotype classifications within the North American and Temperate Asian ribotypes (6). Ten restriction patterns were resolved with* Nsp-*I: one pattern each for the Western European, Tasmanian, Tropical Asian, "affine," "minutum," and "andersoni" ribotypes, and two patterns for the North American and Temperate Asian ribotypes. DNA standards are in the left and right lanes, with fragment sizes as indicated. Variability in the intensity of bands among lanes reflects differences in the amount of PCR product digested. With image enhancement, these differences were easily adjusted with the GelReader program.*

TBE gels stained with ethidium bromide (7), and compared to size standards. Gel photographs were scanned, and then the National Center for Supercomputing Applications' GelReader computer program was used to identify bands indicative of restriction fragments.

Results to date demonstrate that patterns of restriction fragments delineate the major ribotypes, as well as subribotypes, represented by the 11 *Alexandrium* isolates used in this study. The most informative enzyme is *Nsp*-1, which yields 10 distinct patterns (Fig. 1). The remaining enzymes also yield restriction patterns that are useful in defining an isolate's ribotype, and in some cases subribotypes as well (data not shown). As an application of this concept, the RFLP assay employing five restriction enzymes was used to screen two uncharacterized *A. tamarense* and *A. catenella* isolates that matched two established ribotype patterns, coinciding with our predictions based on the isolates' geographic origins (data not shown). Thus, a suite of five enzymes appears to provide sufficient information to confidently assign ribotype designations to previously uncharacterized *Alexandrium* isolates.

In conclusion, implementation of the LsrDNA RFLP assay as a "front line" screening procedure of *Alexandrium* cultures will expedite their sorting and genetic characterization, and at the same time greatly reduce analytical costs per isolate. We expect this to facilitate a better understanding of the global biogeography of toxic *Alexandrium* species, hence providing a stronger foundation from which to evaluate dispersal hypotheses (1, 2). Finally, as more isolates are examined with the RFLP technique, new ribotypes may be discovered. If so, the LsrDNA of these isolates will be sequenced, leading to a better definition of their specific characteristics, and the RFLP assay will be revised accordingly to incorporate these findings.

The technical assistance of D. M. Kulis is gratefully acknowledged. This study has been supported by funds from the Woods Hole Marine Sciences Consortium, Michigan State University, and a grant from the National Science Foundation (OCE89-11226).

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