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PRIMER NOTE

Six new microsatellite markers for the toxic marine dinoflagellate *Alexandrium tamarense*

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Abstract

We report the characterization of six new microsatellite loci for the toxic marine dinoflagellate *Alexandrium tamarense* (North American ribotype), using 56 isolates from a range of locations. The numbers of alleles per locus ranged from five to nine and gene diversities ranged from 0.041 to 0.722. We tested primers for these six loci on other *A. tamarense* ribotypes and on other *Alexandrium* species; the results suggest that the primers are specific to *A. tamarense* isolates belonging to the North American ribotype.

Keywords: *Alexandrium tamarense* 'species complex', dinoflagellate, harmful algal blooms, microsatellites

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Alexandrium is a marine, planktonic dinoflagellate genus, with haploid vegetative cells and diploid motile and nonmotile zygotes. Paralytic shellfish poisoning (PSP) neurotoxins are produced by nine of the 29 species so far described (Balech 1995). Three of these toxic *Alexandrium* species (*A. tamarense*, *A. fundyense* and *A. catenella*) form part of a taxonomically unresolved group, the *A. tamarense* 'species complex'. Phylogenetic studies indicate that ribotypes cluster depending on geographical origin rather than morphological similarity (Scholin *et al*. 1994). So far, six different ribotypes have been identified (John *et al*. 2003). The North American (NA), temperate Asian and tropical Asian ribotypes are exclusively toxic, whereas the Western European, Tasmanian and Mediterranean ribotypes are exclusively nontoxic. The application of microsatellite markers in population studies will help to understand better the development and dynamics of *Alexandrium* blooms. Microsatellite markers have been developed for Japanese strains of *A. tamarense* (Nagai *et al*. 2004). Here we report the development of six new microsatellite markers for Scottish strains of *A. tamarense* (NA ribotype) and the results of cross-amplifiability tests in other ribotypes and species.

Genomic DNA was extracted from an exponentially growing culture of *A. tamarense* (NA ribotype) strain BAHME 182 using a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle 1990). The DNA was enriched for CA repeats following Edwards *et al*. (1996). In brief, the DNA was digested with *Alu*I (New England Biolabs), ligated to a *Mlu*I adapter, amplified using one of the adapter oligonucleotides and hybridized to CA dinucleotide repeat oligonucleotides immobilized on Hybond N^+ filter paper (Amersham Biosciences). The enriched microsatellites were re-amplified as above and cloned into pJV1 vector (Edwards *et al*. 1996). Cloned fragments were amplified using M13 forward and reverse primers (MWG Biotech) and sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit and a MegaBACE 1000 sequencer (Amersham Biosciences).

Ninety-five clones (47%) contained microsatellites. PRIMER 3 (Rozen & Skaletsky 2000) was used to design primer pairs flanking 13 cloned CA-repeats. Their ability to amplify the corresponding microsatellite loci was tested using a Mastercycler gradient thermal cycler (Eppendorf). The 20-μL reactions contained 10–20 ng of DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 or 0.5 μM of each primer and 1 U of HotMaster™ *Taq* DNA polymerase (Eppendorf). After 5 min at 94 °C, 40 or 45 cycles were performed: 20 s at 94 °C, 10 s at 51.5–68.5 °C, and 30 s at 70 °C. The annealing temperature was either constant for 40 cycles, or was reduced by 0.3 °C per cycle for 25 cycles, followed by 20 cycles at a constant annealing temperature (Table 1).

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Table 1 Attributes of *Alexandrium tamarense* strain BAHME 182 (NA ribotype) microsatellite loci: starting annealing temperature during touchdown PCR cycles (TD-T_a, °C), annealing temperature during PCR cycles with constant annealing temperature (T_a, °C), allele size range (bp), number of alleles (N_A) and number of non-amplifying samples (N_O). The numbers of alleles exclusive to the North Sea and culture collection isolates are given as $N_{A(NS)}$ and $N_{A(CC)}$, respectively. All loci were tested in a total number of 56 isolates

Locus (GenBank Accession no.)	Core sequence	Primer sequence (5′–3′)	TD-T_a	T_a	Primer conc. (final)	Size range (bp)	N_A	N_O	$N_{A(NS)}$	$N_{A(CC)}$	Gene diversity (North Sea population)
ATB1	$(GT)_4GC(GT)_7GC(GT)_3GC(GT)_4(GC)_2$	F: CGCCTGCTCGAGAAAAGA	N/A	53	0.5 µM	260–292	5	0	1	3	0.041
(DQ396619)	$(GT)_4GC((GT)_2GC)_9CTG_3(TCT(G)_3)_{13}$	R: TTGGGGACAGTTGAGTTTC									
ATB8	$(GT)_4(CT(GT)_3)_{15}CT(GT)_2(CT)_2G(TA)_2$	F: CAGGGTAGCCGATCAAACAC	61	54	0.1 µM	377–415	8	26	7	0	0.685
(DQ396620)	$T_2(GC)_2GTGC(GT)_4(GCGT)_4GT(GC(GT)_2)_4$ $GT(GC(GT)_2)_3GCGT(GTGC)_3GC(GT)_3$	R: CTTCCATCGCCTTGCATACT									
ATD8	$(CA)_5GA(CA)_2GA((CA)_3GA)_6(CAGG)_3$	F: CAACACTGGAAGCGTGCTAA	61	54	0.1 µM	263–278	6	2	2	2	0.669
(DQ396621)	$CA_2GC(A)_3CAG_2(CAGA)_3(CA)_3$	R: CCCATGCGCTACCTCTTACA									
ATF1	$(GT)_{12}(GCGT)_5(GC)_2$	F: CATTTAGGTTGCGGTGCATA	61	54	0.1 µM	163–197	9	0	3	2	0.722
(DQ396622)		R: TGAGCGACCAACATGCTTAC									
ATF11	$(GT)_3(GC)_7(GT)_3$	F: AGCAGCGCGGCGGGAGATT	68.5	61.5	0.1 µM	258–315	7	5	3	2	0.248
(DQ396623)		R: ACCTGCGGCTGCGACACGACT									
ATG6	$(GT)_{22}$	F: GGTATGCATGTGTGCAGGTG	62.7	55.7	0.1 µM	168–204	7	12	3	2	0.383
(DQ396624)		R: CCGATCGCAAGTCCTCTTAG									

Six microsatellite primer pairs (Table 1) generated amplification products of the expected size; 5′-fluorescently labelled forward primers (6-FAM, HEX or NED; Applied Biosystems) were used in repeat reactions using 74 isolates belonging to different *A. tamarense* 'species complex' ribotypes and *Alexandrium* species (*A. minutum*, *A. lusitanicum*, *A. insuetum*, *A. ostenfeldii* and *A. taylori*; list of strains on request from authors). Fifty-six NA ribotype isolates were genotyped: 48 were collected in 2004 from one eastern Scottish North Sea water sample and eight came from culture collections. Amplification of glutamate synthase (GOGAT; Röttgers 2002) confirmed the amplifiability of each DNA template. One microlitre of the PCR generated amplicons in 15 µL of Hi-Di formamide (Applied Biosystems) and 0.5 µL of the size marker GENESCAN-500 [ROX] (Applied Biosystems) were sized using a 3130xl Genetic Analyser (Applied Biosystems). Microsatellite alleles were scored using GENEMAPPER version 3.5 software (Applied Biosystems). The six primer-pairs yielded 42 scorable microsatellite alleles with a minimum of five and a maximum of nine per locus (Table 1). ARLEQUIN version 2.000 (Schneider *et al.* 2000) was used to test pairs of loci for linkage disequilibrium (Slatkin 1994). Using the data derived from the 48 North Sea individuals, none of the pairs of loci was found to be in significant linkage disequilibrium ($P = 0.05$).

Only NA ribotype isolates of the *A. tamarense* 'species complex' yielded amplification products, indicating that cross-amplification of the microsatellite loci does not occur between ribotypes and species. The numbers of alleles and gene diversities (Nei 1987) in the North Sea isolates suggest that the loci are sufficiently polymorphic for use in population studies. Eleven and 19 alleles were unique to the culture collection strains and North Sea isolates, respectively, suggesting a high degree of molecular diversity within the NA ribotype.

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