Development of microsatellite markers in the toxic dinoflagellate *Alexandrium minutum* (Dinophyceae)

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Received:

Keywords: *Alexandrium minutum*, microsatellite, paralytic shellfish poisoning, phytoplankton, SSR, toxic dinoflagellate

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Running title: Microsatellite markers of *A.minutum*

Abstract

Outbreaks of paralytic shellfish poisoning caused by the toxic dinoflagellate *Alexandrium minutum* (Dinophyceae) are a worldwide concern from both the economic and human health points of view. For population genetic studies of *A. minutum* distribution and dispersal, highly polymorphic genetic markers are of great value. We isolated 12 polymorphic microsatellites from this cosmopolitan, toxic dinoflagellate species. These loci provide one class of highly variable genetic markers, as the number of alleles ranged from 4 to 12, and the estimate of gene diversity was from 0.560 to 0.862 across the 12 microsatellites; these loci have the potential to reveal genetic structure and gene flow among *A. minutum* populations.

The toxic dinoflagellate *Alexandrium minutum*, which is haploid except for the sexual stage of the planozygote and the cyst, produces potent neurotoxins such as saxitoxin. *A. minutum* is one of several globally distributed *Alexandrium* species that are responsible for paralytic shellfish poisoning (Taylor *et al.* 1995). The geographic range of poisonings due to *Alexandrium* species appears to be increasing on both regional and global scales (Anderson 1989; Hallegraeff 1995). We believe that an assessment of genetic relationships among *A. minutum* populations with highly polymorphic genetic markers provides the most promising approach to elucidate their mixing and dispersal. As the first step to tackle this topic, we characterize here 12 polymorphic microsatellite markers developed for *A. minutum*.

Total genomic DNA was extracted from vegetative cells grown in modified f/2 medium without silicate (Guillard 1975, Anderson *et al*. 1994) using a modified CTAB method (Lian *et al.* 2001). About 5.0×10^5 cells were homogenized in 750 µl of 2 x CTAB solution with a vortex mixer (Iwaki, Japan) and incubated at 65℃ for 1h. DNA was isolated using choloroform-isoamyl alcohol (24:1) extraction, precipitation in

isopropanol, and washing in 80% ethanol. Extracted DNA was dissolved in 30 μl Tris-EDTA (TE) buffer. Microsatellite regions were isolated following a dual-suppression-PCR technique (Lian & Hogetsu 2002; Nagai *et al.* 2004). The DNA was separately digested with *Afa*I, *Alu*I and *Hae*III blunt-end restriction enzymes. The DNA fragments were then ligated with a blunt adaptor, consisting of a 48-mer (5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3') and an 8-mer with the 3' end capped by an amino residue (5'-ACCAGCCC-NH_2-3'), using a DNA Ligation Kit (TakaraBio, Shiga, Japan). To completely block polymerase catalysed extension of the 8-mer adaptor strand, the ligated fragments were further treated with ddGTP by Ampli *Taq* Gold Kit (Applied Biosystems, Foster City, CA, USA).

To extract the microsatellite regions, fragments flanked by a microsatellite at one end were amplified from the constructed libraries by the $(AC)_{10}$ or $(GA)_{10}$ primers and an adaptor primer designed from the longer strand of the adaptor. The amplified fragments were cloned and sequenced. Of 480 sequenced clones, 87 fragments containing the $(AC)_n$ or $(GA)_n$ microsatellite sequences at one end were obtained. Next, a nested primer IP1 designed from the sequenced region flanking the microsatellite and another primer IP2 based on the sequence between IP1 and the microsatellite were prepared. As adaptor-primers for nested PCR, AP1 (5'-CCATCCTAATACGACTCACTATAGGGC-3') and AP2 (5'-CTATAGGGCACGCGTGGT-3') were also prepared. From 63 of 87 fragments IP1 and IP2 primers were successfully designed. The primary PCR reaction was conducted with each constructed DNA library using IP1 and AP1 primers. The secondary PCR reaction was conducted with a 100-fold dilution of the primary PCR products using IP2 and AP2 primers. The single-banded fragments were sub-cloned and sequenced. Primer IP3 was designed for each locus from the newly identified sequence between

the AP2 binding site and the microsatellite. Primer pairs IP1/IP3 or IP2/IP3 were used as microsatellite markers. The sequences flanking the microsatellite were successfully sequenced for 17 of the 63 fragments obtained in the first step.

To examine the PCR amplification effectiveness of the 17 primer pairs developed, we performed PCR in a reaction mixture (10 µl) containing 5 ng of template DNA, 0.2 mM of each dNTP, 0.5 µM of each designed primer pair, with one primer labelled with 6FAM, NED, PET, or VIC, 1× PCR buffer (10mM Tris-HCl, pH 8.3, 500mM KCl, 15 mM $MgCl_2$, 0.01% w/v gelatin, Applied Biosystems, Foster City, CA, USA), and 0.25 U of Ampli *Taq* Gold (Applied Biosystems) on a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems). The PCR cycling conditions were as follows: 10 min at 94°C, 38 cycles of 30 s at 94°C, 30 s at 60°C for the first 10 cycles then at the primer-specific annealing temperature for the last 28 cycles, and 1 min at 72°C, and a final elongation for 5 min at 72°C.

To characterize the developed microsatellites, we screened DNA samples of 24 *A. minutum* individuals, which were collected from Australia, England, France, Ireland, Italy, South Africa and Spain. PCR products were diluted 3-5 x with sterile water of which 1 µl was added to 0.25 µl 500 LIZ Size Standard (Applied Biosystems) and 8.75 µl Hi-Di Formamide (Applied Biosystems), and then analyzed using the 3730xl DNA Analyzer (Applied Biosystems). Allele sizes were determined using GeneMapper Software Version 3.7 (Applied Biosystems).

Of the 17 loci tested, characteristics of 12 polymorphic microsatellites are shown in Table 1. Of the remaining 5 regions, 2 contained monomorphic microsatellites and 3 had poor amplification rates (<50%). Each of the 12 loci presented showed a single band in each individual, consistent with the haploid genome of this species. All but 3 loci (*Aminu11, Aminu22* and *Aminu39*) occasionally yielded no visible PCR band in some individuals, possibly due to the presence of null alleles at these loci. Linkage

disequilibrium for all pairs of loci was tested with GENEPOP version 3.4 on the web (Raymond & Rousset 1995). After Bonferroni correction, seventeen pairs of loci were found to be significantly linked: *Aminu39* with *Aminu11* and *Aminu22*; *Aminu41* with *Aminu10* and *Aminu39*; *Aminu43* with *Aminu39* and *Aminu41*; *Aminu44* with *Aminu11*, *Aminu22*, *Aminu39*, *Aminu41* and *Aminu43*; *Aminu48* with *Aminu10*, *Aminu11*, *Aminu39*, *Aminu41*, *Aminu43* and *Aminu44* ($P<0.001$). This may be explained by proximity of loci, sampling errors due to limited sample size or possibly population sub-structuring. The number of alleles at the 12 loci ranged from 4 to 12 with an average of 7.4, and the estimate of gene diversity (Nei 1987) varied between 0.560 and 0.862, suggesting that these microsatellites have the potential to reveal *A. minutum* genetic structure.

Acknowledgements

We thank Dr. Y. Watanabe, National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency of Japan, for his critical reading of the manuscript and his encouragement during this study. We also thank Dr. CL. Lian, Asian Natural Environmental Science Center, the University of Tokyo, for her technical advice and fruitful discussions on the manuscript. Support for this research provided in part (to DMA) by U.S. National Science Foundation grants OCE-0136861 and OCE-0430724, and the National Institute of Environmental Health Sciences Grant 1 P50 ES012742-01.

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Table 1 Primer pairs for amplification of 12 polymorphic microsatellite regions in the toxic dinoflagellate *Alexandrium minutum* and some characteristics of the microsatellite loci. T_a indicates annealing temperature; Gene diversity was calculated after Nei (1987). The number of clonal strains screened was twenty-four at each locus.

Locus	Repeat motif	Primer sequence	T_a (°C)	No. of non-amplifying samples	No. of alleles	Size range (bp)	Gene diversity	Genbank accession number
Aminu08	$(CT)_7GTC_3(CT)_3$	F: AGCCTCCTTGTCTCACTTCGTTTC R: PET- GTTATGCTATGCCATGCCTTGCC	52	8	4	189-223	0.560	AB242303
Aminu10	$(GT)_5G_{11}CA_5$	F: 6FAM- GCTTGAGATGGAGTGGATAACGG R: GATACAATTTCGGGGGTAGAAGACTGG	52	2	6	148-162	0.748	AB242304
Aminu11	$(CT)_{13}$	F: AGGAGAAATCACAAGCGGTGG R: VIC- GCAAACAAACAGGACTCTGAGAGC	52	0	9	226-256	0.767	AB242305
Aminu15	$(CT)_{14}$	F: 6FAM- CTTTACATACGCCTGTCTAGATCCCTT R: CCACASACAGTCTGACAGGAAGG	52	4	4	209-215	0.720	AB242306
Aminu20	$(CT)_5C_3(CT)_{13}$	F: VIC- ACCTTGACAATGCTCCTGTTGGG R: CSYTGCTCTTGACATCACCATCTTG	55	8	5	245-283	0.727	AB242307
Aminu22	$(CT)_{19}$	F: ATTTGGTCAACTGTCTCTCACCCTCAC R: 6FAM- GTAGCCATCACTATCCTCATTCGC	55	0	9	182-204	0.844	AB242308
Aminu29	$(CT)_4C_3(CT)_{13}$	F: NED- GCAAACTGGATTCTGGCGAAAGG R: CTGAACAACTGTATTCGCCATCGC	52	1	6	232-250	0.647	AB242309
Aminu39	$(CT)_{10}T_6GAG_7$	F: TCCTTTTTCTTTGAGGCGCTCG R: 6FAM- CAAGGTGTGATGGCCATCATG	53	0	6	142-156	0.719	AB242310
Aminu41	$(CT)_{13}$	F: CTCCTGAGAAATGTGATTAGTGTTCG R: VIC- CAAGGCACGTGTGTTTGAAGTC	55	3	12	165-247	0.862	AB242311
Aminu43	$(CTA)_2T(CT)_{14}GAG_5$	F: CACAAGGTTGCATCAGTAGG R: VIC- GAAAGAATTGCTTCCTCGACTG	52	1	10	182-224	0.843	AB242312
Aminu44	$(CT)_{17}(CA)_3$	F: CCTTGAACGTAGTAAGTAGCAACC R: 6FAM- GTCTACCCTTTTCTTTCTCAGAGCC	52	1	9	259-285	0.813	AB242313
Aminu48	$(GT)_2CT(GT)_4N_4(GT)_6(GC)_5$	F: 6FAM- GCAGCTGGCAAAGTGATCCGTT R: CAAGGGTCTGGTTGATTCGG	55	1	9	234-252	0.847	AB242314