

PRIMER NOTE

Microsatellite DNA markers for population genetic studies in the dinoflagellate *Karenia brevis*

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Abstract

Nine nuclear-encoded microsatellites from an enriched genomic DNA library of the HAB (harmful algal bloom) dinoflagellate *Karenia brevis* were isolated and characterized. The microsatellites include five perfect (three dinucleotide and two trinucleotide) and four imperfect (two dinucleotide and two trinucleotide) repeat motifs. Gene (haplotype) diversity ranged from 0.153 to 0.750 among a sample of 13 isolates; the number of alleles among the isolates ranged from two to six and pairwise tests of genotypic disequilibria were nonsignificant. The microsatellites developed in this study will provide insight into the genetic diversity of this HAB species and tools that may prove useful in predicting source populations and physiological parameters of individual *K. brevis* blooms.

Keywords: genomic library, harmful algal species, *Karenia brevis*, microsatellites

Received 25 March 2006; revision accepted 24 May 2006

Karenia brevis (formerly *Gymnodinium breve*) is an unarmored, photosynthetic dinoflagellate that produces a suite of potent neurotoxins (brevetoxins) and is responsible for harmful algal blooms (HABs) in the Gulf of Mexico (Steidinger *et al.* 1998). Blooms of *K. brevis* can result in the death of large numbers of fish and wildlife, and threats to human health can occur from consumption of shellfish that have accumulated the toxins or from exposure to aerosolized brevetoxin (Cheng *et al.* 2005). Although clonally reproducing, haploid organisms such as *K. brevis* often display little or no physiological or genetic variation (Hughes 1989), a number of studies (e.g. Loret *et al.* 2002) have reported physiological variation in both growth rates and levels of toxin production among different *K. brevis* isolates. In this note, we report development from an enriched genomic library of *K. brevis* DNA of polymerase chain reaction (PCR) primers for nine nuclear-encoded microsatellites. Hyper-variable genetic markers such as microsatellites may provide insight into the genetic diversity of this species.

Whole genomic DNA from a clonal culture (SP2 isolate) of *K. brevis* was extracted using the cetyltrimethyl

ammonium bromide (CTAB) DNA isolation protocol described by Doyle & Doyle (1990). An enriched microsatellite library was constructed via the generation of adaptor-ligated template with biotinylated oligonucleotide probing as outlined in Prochazka (1996). Whole genomic *K. brevis* DNA was digested with *Hae*III, *Rsa*I and *Dra*I (New England BioLabs) and fragments ranging from 300 to 500 base pairs (bp) were size selected by extraction from a 1% agarose gel and purified using a gel extraction kit (QIAGEN). Fragments were ligated with the double-stranded adaptor molecule AP11/12 (5'-CTCTTGCTTAGATCTGGACTA-3' and 5'-TAGTCCAGATCTAAGCAAGAGCACA-3', respectively) for 16 h at 14 °C. The adaptor-ligated products were pre-amplified using the single AP11 primer, denatured and annealed in the presence of 5'-biotinylated oligonucleotides [(TA)₃₀ (CA)₂₀ (GA)₂₀ (TGA)₁₅ (ACA)₁₅], and bound to streptavidin-coated paramagnetic beads (Promega). The beads were exposed to several washes prior to elution of target DNA via addition of 60 °C 0.1 M NaOH. The eluted genomic DNA was neutralized, desalted and finally amplified via PCR, using the AP11 primer. Approximately 60 ng of amplified DNA was then cloned into the TA-cloning vector pCR4-TOPO through topoisomerase-mediated ligation

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Table 1 Summary data for microsatellites developed from *Karenia brevis*

Microsatellite	Primer sequence (5'–3')†	Repeat sequence‡	T_A	N/N_A	Size range	D
<i>Kbr 1</i>	GGACGGAGGTTTGTGTGTC GCTTTTGACTAGCCGTTCTGC	Complex repeat*	52	12/6	249–261	0.750
<i>Kbr 3</i>	CGTGACTCAGAGTGGCAATGG AACATGGCTGATCAACTCAACACC	(GTT) ₉	52	12/4	229–244	0.417
<i>Kbr 4</i>	TACATGCTCAAGGTCACCTG ACAGGCAGAGCAGCAGAAAA	(TC) ₁₅	52	13/5	254–270	0.746
<i>Kbr 5</i>	GACATAAACTCAGGACAATGGATGC CACTTCACGCACAAAACCAAGG	(CT) ₁₁	52	13/5	182–190	0.734
<i>Kbr 6</i>	GAGGGTTCAAAAGTGTAACTCTGC CATGGTGGCAAGAATAGCAT	(CT) ₈ TC(CT) ₁₀	52	13/4	219–225	0.639
<i>Kbr 7</i>	GGATCTGAAAGACACCGTGA CTGTCCAGTGGCATTTCAC	(GAT) ₁₃	52	12/2	259–262	0.153
<i>Kbr 8</i>	GTCCACACAAACCAACGCAAGG CAATCATTGTGGCATGGAGG	(CA) ₇ CT(CA) ₃ TA(CA) ₂ 4 bp(CA) ₆	52	13/4	129–135	0.722
<i>Kbr 9</i>	CAAACAGTTTCAGTGGGAGA CAAGAGCGTGAGAGAGTG	(GAT) ₇ GGT(GAT) ₈ 6 bp(GAT) ₆	52	13/2	161–164	0.426
<i>Kbr 10</i>	CTTTCGCTTACTGATGTC GGTTAGGATGACGGTAGT	(GT) ₉	52	12/4	172–178	0.708

* (CTT)₈16 bp(CTT)₈32 bp(CTT)₆12 bp(CTT)₅.

†Primer sequences are forward (top) and reverse (bottom); ‡Repeat sequence indicates repeat motif of cloned sequence; T_A is annealing temperature in °C; N is the number of isolates assayed; N_A is the number of alleles detected; Size range refers to alleles thus far uncovered; D is Nei's (1973) gene diversity. The fluorescently labelled primer is in bold. Sequences of clones are listed in GenBank (Accession nos DQ400700–DQ400704, DQ400706–DQ400709).

(Invitrogen) and transformed into chemically competent TOP 10 *Escherichia coli*.

Transformed cells were plated on Luria–Bertani (LB) agar with 50 µg of ampicillin and grown overnight at 37 °C. A total of 480 microsatellite-enriched clones were picked and inoculated into 96-well plates that contained 50 µL of LB freezing media [36 mM K₂HPO₄, 13.2 mM KH₂PO₄, 1.7 mM sodium citrate, 0.4 mM MgSO₄, 6.8 mM (NH₄)₂SO₄, 4.4% (v/v) glycerol, 50 µg/mL ampicillin, LB], and incubated overnight at 37 °C before freezing at –80 °C. A total of 384 clones were screened as follows. Frozen glycerol stocks were used to inoculate 1 mL cultures of Luria broth selective media (ampicillin) and incubated overnight at 37 °C. Plasmid DNA was amplified using the 555 (forward) (GGCGATTAAGTTGGGTAACG) and 837 (reverse) (GGAATTGTGAGCGGATAACA) primers (Makova & Patton 1998) and visualized on a 2% agarose gel; the target DNA fragment was selected by extraction and purified using a gel extraction kit (QIAGEN). Purified DNA was quantified, normalized and both strands sequenced using the 555 and 837 primers and ABI PRISM BigDye Terminator version 3.1. Products were electrophoresed on an ABI 3100 automated DNA sequencer (Applied Biosystems). Sequences were edited and vector trimmed with SEQUENCHER 3.0 (Gene Codes). A total of 25 primer pairs were developed using AMPLIFY 1.2 (Engels 1993) and NETPRIMER (<http://www.premierbiosoft.com/netprimer>).

Unlabelled PCR primers were purchased from Invitrogen and tested for amplification by initially screening DNA extracted from two different, clonal isolates. PCR amplifications were performed in 20 µL reaction volumes containing 2 µL (~20 ng) DNA, 2 µL 10× reaction buffer (500 mM KCl, 100 mM Tris, 10% Triton-X 100), 0.2 U of *Taq* DNA polymerase (Gibco-BRL), 1 µM of each primer, 400 µM of each dNTP, and 2 mM MgCl₂. Gradient PCR conditions consisted of an initial denaturation at 94 °C for 3 min, followed by 38 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C–65 °C for 45 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized with UV light. Once an appropriate annealing temperature was established for each primer, microsatellite arrays were further tested with genomic DNA extracted from nine additional clonal isolates and visualized on a 2% agarose gel; 10 microsatellite repeats were initially chosen for further screening. One of these was dropped after further scrutiny, leaving the nine primer pairs presented in Table 1. These microsatellites included five perfect (three dinucleotide and two trinucleotide) and four imperfect (two dinucleotide and two trinucleotide) repeat motifs. Lengths of cloned alleles ranged from 134 to 264 bp and all nine primer pairs amplified at an annealing temperature of 52 °C.

A total of 13 *K. brevis* clonal isolates generated from samples obtained from the Atlantic coast of Florida (JAXC5)

and the Gulf coasts of both Florida (CCMP718, CCMP2228, CCMP2229, CCMP2281 and C4) and Texas (C5, NBK, SP-1, SP-2, SP-3, TXB3 and TXB4) were screened for allelic variation at the nine microsatellites. One primer from each PCR primer pair was labelled with the fluorescent label of set D (Applied Biosystems) and run on an automated ABI PRISM 377 automated DNA sequencer. Alleles were sized using GENESCAN 3.1.2 and GENOTYPER version 2.5 software. Genetic variability of the markers was measured by the number of alleles and gene (haplotype) diversity (Nei 1973) as implemented in POPGENE version 1.31 (<http://www.ualberta.ca/~fyeh/>). Genotypic disequilibrium between pairs of microsatellites was tested by exact tests as implemented in GENEPop (Raymond & Rousset 1995) and using 5000 dememorizations, 500 batches and 5000 iterations per batch.

Summary data for each microsatellite are presented in Table 1. The number of alleles detected per microsatellite ranged from two to six; gene diversity ranged from 0.153 to 0.750. All pairwise tests of genotypic disequilibrium were nonsignificant. The nine microsatellites developed in this work will prove useful for evaluating genetic variation and diversity both within and among blooms of *K. brevis* from different localities of the Gulf of Mexico. Comparisons between genotypic and phenotypic (e.g. toxicity) variation and diversity of isolates may provide insight for predicting bloom dynamics and potential toxicity.

Acknowledgements

We thank A. Pepper for advice on development of the microsatellite library and G. Doucette for DNA from the JAXC5 strain. Funding

was provided by the EPA Science to Achieve Results (STAR) program (Award R83041301-0) and by the Texas Agricultural Experiment Station (Project H-5703). This paper is number 143 of the Center for Biosystematics and Biodiversity at Texas A & M University.

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