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PRIMER NOTE

Isolation and characterization of five dinucleotide microsatellite loci in the sandbar shark, *Carcharhinus plumbeus*

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Abstract

Five dinucleotide markers were isolated and optimized from a microsatellite-enriched genomic library obtained from the sandbar shark, *Carcharhinus plumbeus*. Genotypic distributions of all markers were found to be in conformance with the expectations of Hardy–Weinberg equilibrium with four to 39 alleles present per locus. We amplified these loci in two female sharks and their litters. A maternal allele was recovered at each locus in all progeny indicating reliable amplification. More than two paternal alleles were recovered across both litters indicating genetic polyandry. Additionally, these markers were amplified across 10 carcharhiniform species to examine their utility in other studies.

Keywords: *Carcharhinus plumbeus*, microsatellites, population structure, Sandbar shark

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Sandbar sharks are large coastal carcharhinids with a cosmopolitan distribution. They have a 30-year lifespan and take 15 years to reach maturity (Sminkey & Musick 1995). In the western North Atlantic, sandbar sharks are a major component of the commercial shark fishery and are caught recreationally (NMFS 2001). Due to the species' slow growth and late maturity, along with the pattern of exploitation, it is listed as 'conservation dependent' (IUCN 2004). Previous studies based on mitochondrial DNA (mtDNA) and microsatellites suggest a single western North Atlantic stock (Heist *et al*. 1995; Heist & Gold 1999). Information essential for conservation and management, such as the level of female philopatry to nursery grounds and the magnitude of gene flow between disjunct populations, has not been acquired because markers lacked sufficient variability. Here, we characterize five highly polymorphic dinucleotide microsatellite loci.

Sandbar shark muscle was powdered by grinding in liquid nitrogen, and high molecular weight DNA was extracted following the protocols of Sambrook & Russell (2001). Microsatellites were isolated following the protocols of Hamilton *et al*. (1999) with minor modifications. Briefly, genomic DNA was digested using *Rsa*I, *Bst*UI and *Xmn*I (New England Biolabs) simultaneously, dephosphorylated, and resulting fragments were ligated to SNX (Invitrogen) linkers in the presence of *Xmn*I. Biotinylated $(GT)_{12}$ (Invitrogen) was used to perform subtractive hybridization reactions at 75 °C overnight following Kijas *et al*. (1994). After hybridization, Streptavidin MagneSphere Paramagnetic Particles (Promega) were added at a concentration of 1 mg/mL and the solution was agitated for several hours in a shaker bath at 43 °C. Beads were washed twice with 200 μL of 2× SSC, 0.1% SDS and four times with 200 μL of 1X SSC, 0.1% SDS for 5 min per wash. Beads were separated from the solution between washes using a MagneSphere Magnetic Separation Stand (Promega). Microsatellite-enriched DNA was eluted by adding 60 μL of preheated T.E. (10 mM Tris, 0.1 mM EDTA), incubating at 95 °C for 10 min and separating the solute from the beads. The recovered single-stranded DNA was amplified using a forward SNX linker as a primer. The resulting double-stranded products were ligated into PCR 2.1 vector (Invitrogen) and transformed into Top10 One Shot (Invitrogen) competent *Escherichia coli* cells. Colonies containing inserts were selected following manufacturer protocols and suspended in 100 μL of sterile water. Suspensions were boiled for 5 min and centrifuged for 2 min at 16 000 *g* to extract plasmids.

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Table 1 Five microsatellite loci developed for sandbar sharks includes: locus name, GenBank Accession no., primer sequence, repeat motif, annealing temperature (T_a), dye label (DL), observed size range, number of alleles observed, observed heterozygosity (H_O) vs. expected heterozygosity (H_E), conformance to Hardy–Weinberg equilibrium (Phw) and number of individual genotyped (No.)

Locus	GenBank Accession no.	Primer sequence 5′–3′	Repeat motif	T_a (°C)	DL	Size range (bp)	Alleles	H_O (H_E)	Phw	No.
Cpl 53	DQ191806	F-CAAGCAGGCAGCTAAGAG R-CATTTTGTCTGTATAGAGCATAAG	$(TG)_{18}$	(62–60) 57	IR-800	166–186	4	0.63(.57)	0.862	50
Cpl 90	DQ191807	F-GTTGTTGCCTTGTCTTTCAATCG R-TGTGTCACTGTGTCTCTGTGTGCC	$(AC)_{24}$	56	IR-700	214–278	26	0.88(.93)	0.109	51
Cpl 128	DQ191805	F-GCTGTGATCTTTGCTGATTGAGC R-GGATGGTGGATTGTGGATTTTG	$(CA)_{13}TA(CA)_{13}$	65	IR-800	216–254	15	0.90(.87)	0.636	50
Cpl 166	DQ191809	F-TGGACATGACAATTACAGCACAGG R-CTGTTTACAACTTCCCTGGAGTGC	$(GT)_{17}$	63	IR-800	223–325	39	1(.96)	0.958	47
Cpl 169	DQ191810	F-TGACACAACCATTTATTCCCACG R-GGTTTCCTTGAGTGAAAGAGAGAGC	$(TG)_{42}$	64	IR-700	107–209	29	0.92(.93)	0.650	55

Ten microlitres PCRs using M13F and M13R primers were used to screen the library for microsatellite inserts. All PCRs were run on a PJC-200 thermocycler (MJ Research). Reaction conditions consisted of a denaturation at 95 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Recombinant plasmids containing inserts of at least 100 bp were re-amplified at a volume of 50 µL as above and used as template for sequencing reactions with the Thermosequence Primer Cycle Sequencing Kit (Amersham). Reactions were electrophoresed on a 3.7% polyacrylamide gel using a LiCor global IR2 system with either IRD-700-labelled M13R or IRD-800-labelled M13F primers (LiCor). Locus-specific primers were designed using the 'find PCR primer pairs' option in the analysis menu of MACVECTOR 8.0 (Accelrys).

One hundred eighty-nine inserts were sequenced, 35 contained repeats and 27 primer pairs were ordered. Five primer pairs (Cpl-53, Cpl-90, Cpl-128, Cpl-166 and Cpl-169) reliably amplified a single locus; no more than two bands were present on polyacrylamide gels with labelled primers. Products resulting from these five primer pairs were subsequently cloned and resequenced for validation purposes. These five loci were tested on 47–55 sandbar sharks. Five microlitre reactions contained 20 mM Tris-HCL (pH 8.4), 1.2–1.5 mM $MgCl_2$, 0.001 mg/µL BSA, 0.2 mM dNTP mix, 20 pmol of primer (except Cpl 128, which contained 10 pmol of primer), 0.2 µL of template and 0.025 U/µL of *Taq* polymerase (Invitrogen). Forward primers were labelled with IRD-800 or IRD-700 fluorescent dye (LiCor). Reaction conditions consisted of a denaturation at 95 °C for 4 min followed by 25–40 cycles at 94 °C for 1 min, the appropriate annealing temperature (Table 1) for 0.5–1 min, 72 °C for 1 min, followed by 72 °C for 10 min. The locus Cpl-53 was amplified with a touchdown protocol at 95 °C for 1 min followed by three iterations of five cycles at 94 °C for 1 min, annealing (62, 61, 60 °C) for 1 min, 1 min at 72 °C, followed by 25 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, followed by an extension at 72 °C for 10 min. Products were separated on 25 cm 6.5% polyacrylamide gels using a LiCor 4200 Global IR^2 system. A 50–350 bp size standard was run in the first, middle and last lanes of each gel and with locus-specific standards in every 8th lane. Alleles were scored using GENE IMAGIR 4.05 (Scanalytics). GENEPOP 3.4 (Raymond & Rousset 1995) was used to analyse conformance to Hardy–Weinberg equilibrium (HWE) and test for linkage disequilibrium. All loci were cross-amplified in 10 other species of carcharhiniform sharks using a gradient thermocycler with annealing temperatures between 52 and 65 °C. Products were electrophoresed on 2% agarose gels to assess amplification success (Table 2).

All loci were unlinked and polymorphic, with between four and 39 alleles present, and conformed to the expectations of HWE (Table 1). Microsatellite loci were used to genotype two female sandbar sharks and their respective litters. Maternal alleles were recovered in every pup in both litters, indicating reliable amplification for all primer pairs. More than two paternal alleles were noted within each litter at all loci with the exception of Cpl-53 in one of the litters. This demonstrates genetic polyandry in *Carcharhinus plumbeus*. Further analyses are necessary to determine the prevalence of this reproductive behaviour. Using the sandbar-specific primer pairs, all loci could be amplified in at least one other carcharhiniform species (Table 2). Since products were separated on agarose gels and inspected by eye, accurate determination of allele size or number was not possible.

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Table 2 Results of cross-amplification for other carcharhiniform sharks *Carcharhinus longimanus* (C. lon), *Carcharhinus limbatus* (C. lim), *Carcharhinus brevipinna* (C. bre), *Carcharhinus falciformis* (C. fal), *Carcharhinus obscurus* (C. obs), *Galeocerdo cuvier* (G. cuv), *Rhizoprionodon terraenovae* (R. ter), *Prionace glauca* (P. gla), *Mustelus canis* (M. can) and *Sphyrna lewini* (S. lew). Numbers next to species designation indicate number of individuals

Marker	C. lon(7)	C. lim(3)	C. bre(3)	C. fal(4)	C. obs(17)	G. cuv(2)	R. ter(1)	P. gla(10)	M. can(2)	S. lew(3)
cpl-53	*(52–56)	/	/	/	/	0	0	*(52–55)	*(55–59)	*(56–58)
cpl-90	*(56–62)	*(60–63)	*(59–63)	*(59–63)	*(60–63)	/	0	*(56–60)	/	0
cpl-128	*(52–56)	0	0	*(52–56)	*(52–56)	0	/	*(56–60)	0	*(52–56)
cpl-166	*(56–62)	0	*(52–55)	0	/	0	0	0	0	/
cpl-169	*(54–56)	/	/	*(59–62)	*(54–56)	0	0	*(53–56)	0	0

*(#), indicates temperature range over which appropriately sized amplicons appeared.
/, indicates some nonspecific amplification requiring further optimization.
0, indicates smear or no product.

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