

PRIMER NOTE

Polymorphic microsatellite loci for the zebra shark *Stegostoma fasciatum*

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Abstract

We report on the development and characterization of 14 polymorphic microsatellite loci in the zebra shark (*Stegostoma fasciatum*). Five tetranucleotide and nine dinucleotide loci were polymorphic with heterozygosities ranging from 0.400 to 0.967 and from three to 22 alleles per locus. Cross-species amplification of these zebra shark primers on four other species of orectolobid sharks was not successful.

Keywords: microsatellite loci, *Stegostoma fasciatum*, zebra shark

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The zebra shark *Stegostoma fasciatum* occurs in shallow coastal waters of the western Pacific and Indian Oceans (Compagno 2002). Zebra sharks are known to form temporal aggregations and along with their shallow habitat preference, they are highly susceptible to capture from many fishing methods. This potential reduction in numbers from both targeted and nontargeted fishing, and lack of information about their biology in the wild, has led to the classification of zebra sharks outside Australian waters as 'Vulnerable' on the IUCN Red List (Pillans & Simpfendorfer 2003). Zebra sharks are not targeted for fishing in Australia though few animals are removed for aquaria internationally where they are a popular species due to their ease of domestication and attractive appearance.

In this note, we describe the development and characterization of microsatellite loci isolated from zebra sharks. These loci will be used for investigating population structure and kinship relatedness within wild and captive populations.

Fin clips for microsatellite development were taken from captive *S. fasciatum* (Reef HQ, Australia and Shedd Aquarium, Chicago, USA) and preserved in 20% DMSO solution saturated with NaCl. Microsatellite loci for zebra sharks were prepared from two enriched libraries. The first library development, done at the Southern Fisheries Centre, followed

the protocol of Fischer & Bachman (1998). Genomic DNA, extracted from 100 mg fin using a DNeasy kit (QIAGEN) was digested and ligated to adapter-primer oligos, hybridized with 10 µM of biotin-labelled oligo probes [(AC)₁₅, (TC)₁₅, (AAT)₁₀, (AAG)₁₀, (ATG)₁₀, (TAGA)₁₀], enriched via polymerase chain reaction (PCR) amplification and then cloned into plasmid vectors (pGEM-T Easy Vector; Promega). Bacteria colonies with inserts were amplified overnight in LB broth (Sambrook *et al.* 1989) and the plasmid rescued using a QIAprep Spin Miniprep Kit (QIAGEN). The plasmid was sequenced with the vector primers SP6 and T7 using BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems), and resolved on a 3730xl Genetic Analyser (Applied Biosystems). The second library development, done at The Field Museum, followed the enrichment protocol of Glenn & Schable (2005). This technique also employs biotin-labelled probes and streptavidin-coated magnetic beads. Genomic DNA was extracted following a salting out protocol (Sunnucks & Hales 1996). We screened for microsatellite loci using the following probes: (AAC)₆, (AAG)₈, (AAT)₁₂, (ACT)₁₂, (ATC)₈, (AAAT)₈, (AACT)₈, (AAGT)₈, (ACAT)₈ and (AGAT)₈. Positive bacteria colonies were used as a template for direct PCR in a 25 µL reaction containing 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM MgCl₂, 10 × BSA, 0.12 mM of each dNTP, 0.25 µM of M13 primers and 1 U *Taq* DNA polymerase. Thermal cycling was as follows: an initial denaturing step at

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Table 1 Characterization of 14 polymorphic microsatellite loci for *Stegostoma fasciatum*. Reported are: locus name and GenBank Accession no.; repeat motif; sequences for forward (F) and reverse (R) primers (note that forward primers were modified with an M13 sequence – see text); optimal annealing temperature (T_a); allele size range in base pairs; number of scored individuals (N_i); number of alleles (N_a); observed heterozygosity (H_o); expected heterozygosity (H_e); and probability of Hardy–Weinberg equilibrium (HWE)

Locus/GenBank Accession no.	Repeat motif	Primer sequence (5'–3')	T_a (°C)	Allele size range (bp)	N_i/N_a	H_o	H_e	P -value for HWE test
Sf2 DQ436481	(TG) ₁₂ (AG) ₆	F-GACTTCACTTCCTCCATCAG R-ACACCCCATACTTGTCTACAG	60	189–193	30/3	0.500	0.584	0.5907
Sf38 DQ436482	(CA) ₃₀	F-ACTCAATGCTGATGTCTTCC R-GTCGACTAGTGGTAATTTGGA	60	220–260	22/10	0.773	0.817	0.4046
Sf41 DQ436483	(TG) ₁₈	F-AGGCATACCTGCAATGCT R-ACTCCACAAAGGACCACAG	60	199–237	24/10	0.833	0.788	0.7064
Sf72 DQ436484	(CA) ₃₂	F-GATAGCCTCAACCAGGATCA R-GCTTCTGAACAAGATGGAA	60	216–286	26/22	0.923	0.913	0.2750
Sf138 DQ436485	(TG) ₁₆ TATGTTCT	F-AGGCAACTGCTAATCCTCACA R-TCACCTTTGATGTCCAGGGT	57	177–183	25/4	0.400	0.338	1.0000
Sfa205 DQ436486	(TAGA) ₁₆	F-TGGGCCAAAGTCTTATTTA R-AAATAACATTCAGTTATAGGAAATGA	52	355–391	28/8	0.821	0.776	0.9983
Sfa221 DQ436487	(AC) ₁₄	F-AAACAGATGCGATCATTAGCA R-AGGATCATCTCAGCACTGGAA	60	231–247	28/7	0.786	0.684	0.4347
Sfa236 DQ436488	(TAGA) ₁₆	F-AGACAGGCAGACATAGACAGA R-GAGGGAATAATGCTGCCTCA	60	235–279	28/9	0.857	0.789	0.9651
Sfa248 DQ436489	(TATC) ₂₃ TATT	F-CAITCAGCTTTTTCCTTAAAGTTGTCA R-GCAGAAATAGATGCATAGACAGCA	60	262–346	25/16	0.960	0.908	0.8947
Sfa335 DQ436490	(AGAT) ₁₃ GGAT	F-GATGGGCATGAAACAAGATTT R-GTGGCCTGCCTTCTTGATT	57	355–411	31/13	0.839	0.880	0.4953
Sfa382 DQ436491	(TAGA) ₁₄ (CAGA) ₁₀	F-GTTTCCAATCGCACACAGAG R-CGACAGCTTATTGCCCTCCAT	57	161–189	29/6	0.862	0.684	0.6267
Sfa387 DQ436492	(TG) ₂₄ CGTT(TG) ₈	F-CGCCCTCCCCTAAAATAGAC R-ACATCCTCGTTGCCCTTTGAT	57	225–245	28/8	0.786	0.776	0.3853
Sfa418 DQ436493	(TG) ₅ TA(TG) ₁₀	F-TGGAAGTTGCATGTCTGAAG R-GCACCATCAGTTTTCCAGGT	57	226–232	27/4	0.444	0.529	0.0231
Sfa454 DQ436494	(AC) ₂₅	F-TGAAGGTGCAGCAAGAATTG R-ATGTGCATGCATGTTTTGGT	57	172–220	30/18	0.967	0.917	0.6904

95 °C for 7 min, 35 cycles at 95 °C for 20 s, 50 °C for 20 s and 72 °C for 90 s. These PCR products were cleaned using MultiScreen-PCR Filter Plates following the manufacturer's protocol (Millipore). DNA sequencing was performed using the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and products resolved on a 3730 DNA Analyser (Applied Biosystems). We sequenced 138 clones yielding 13 potential loci and 466 clones yielding 40 potential loci from the first and second libraries, respectively. Primers flanking core microsatellite repeats were developed using PRIMER 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_http://www.cgi).

We tested loci from other shark species that had been shown to amplify in zebra sharks (Ovenden *et al.* 2006). These included Cli108 (Keeney & Heist 2003); Cs01, Cs08, Ct03, Ct04 (Ovenden *et al.* in press); Gc010 (Heist *et al.* 2003); Iox12, Iox30 (Schrey & Heist 2002).

All forward primers had M13 tails (5'-GAGCGGAT-ACAATTTACACAGG-3') attached to the 5' end to allow

for labelling with fluorescent M13 primers. Initial PCR trials on samples from a population of wild zebra sharks in southern Queensland, Australia, were performed in a 9700 PerkinElmer thermocycler using approximately 10 ng of genomic DNA, 0.25 µM of reverse primer and M13 primer, 0.025 µM of forward primer, 200 µM of each dNTP, 0.05 U of *Taq* DNA polymerase (QIAGEN) and 1.5 mM of MgCl₂ in a 10 µL reaction volume. After an initial denaturation step at 95 °C for 5 min, PCR was performed for 35 cycles of 15 s denaturing at 95 °C, 30 s annealing at 52 °C, 57 °C or 60 °C, 30 s extension at 72 °C, and a 45 min final extension at 72 °C. Products were resolved by capillary electrophoresis on a 3130xl Genetic Analyser and sized with GENEMAPPER software (Applied Biosystems).

Fourteen primer pairs developed for *S. fasciatum* microsatellite loci consistently amplified DNA from zebra shark tissue and were polymorphic. Five of these loci were developed from the first enrichment library (prefix Sf, see Table 1) and consisted entirely of dinucleotide repeats.

The remaining loci, developed from the second enrichment library (prefix Sfa), consisted of five tetranucleotide repeats and four dinucleotide repeats. The eight primer pairs from other shark species amplified zebra shark DNA but were not polymorphic. Observed and expected heterozygosities for *S. fasciatum* loci were calculated using GENALEX version 6 (Peakall and Smouse 2006). The nine dinucleotide loci had wide ranging heterozygosities (0.400–0.967) while the five tetranucleotide loci showed consistent high levels of heterozygosities (0.821–0.960). Exact tests implemented in GENEPOP version 3.4 (Raymond & Rousset 1995) found deviations from Hardy–Weinberg equilibrium in one of the 14 loci (Sfa418, $P = 0.0231$). Lack of equilibrium at this locus is most likely due to null alleles. Linkage disequilibrium tests (GENEPOP 3.4) revealed significant linkage between two pairs of loci (Sfa236 & Sfa205, $P = 0.000$; Sfa236 & Sfa221, $P = 0.000$) following Bonferroni adjustments.

We tested PCR amplification of these loci on two individuals from four other carpet shark species (*Orectolobus ornatus*, *Orectolobus maculatus*, *Chiloscyllium punctatum* and *Rhincodon typus*). Only *C. punctatum* had successful amplification at three loci (Sfa225, Sfa382 and Sfa418), although these were monomorphic. This lack of cross-species utility of microsatellite loci between zebra sharks and other shark species may be indicative of the taxonomic divergence of zebra sharks. Zebra sharks are currently classified as the sole species within the family Stegostomatidae though they have previously been included within other Orectolobiform families (Compagno 2002).

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References

- Compagno LJV (2002) *Sharks of the World. An Annotated and Illustrated Catalogue of Shark Species Known to Date*, Volume 2. FAO Species Catalogue for Fishery Purposes No. 1. FAO, Rome, 269pp.
- Fischer D, Bachman K (1998) Microsatellite enrichment in organisms with large genomes (*Allium cepa* L.). *BioTechniques*, **24**, 796–800.
- Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. *Methods in Enzymology*, **395**, 202–222.
- Heist EJ, Jenkot JL, Keeney DB *et al.* (2003) Isolation and characterization of polymorphic microsatellite loci in nurse shark (*Ginglymostoma cirratum*). *Molecular Ecology Notes*, **3**, 59–61.
- Keeney DB, Heist EJ (2003) Characterization of microsatellite loci isolated from the blacktip shark and their utility in requiem and hammerhead sharks. *Molecular Ecology Notes*, **3**, 501–504.
- Ovenden JR, Street R, Broderick D (2006) New microsatellite loci for Carcharhinid sharks (*Carcharhinus tilstoni* and *C. sorrah*) and their cross-amplification in other shark species. *Molecular Ecology Notes*, **6**, 415–418.
- Peakall R, Smouse PE (in press) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.
- Pillans RD, Simpfendorfer CA (2003) Zebra shark, *Stegostoma fasciatum* (Hermann, 1783). In: *The Conservation Status of Australasian Chondrichthyans* (eds Cavanagh RD, Kyne PM, Fowler SL, Musick JA, Bennet MB), p. 170. The University of Queensland, School of Biomedical Sciences, Brisbane, Australia.
- Raymond M, Rousset F (1995) GENEPOP version 1.2: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.
- Schrey AW, Heist EJ (2002) Microsatellite markers for the shortfin mako and cross-species amplification in lamniformes. *Conservation Genetics*, **3**, 459–461.
- Sunnucks P, Hales DF (1996) Numerous transposed sequences of mitochondrial cytochrome oxidase I–II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). *Molecular Biology and Evolution*, **13**, 510–524.