

### Acknowledgements

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## Isolation and characterization of dinucleotide microsatellite loci in the Great White Shark, *Carcharodon carcharias*

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The great white shark, *Carcharodon carcharias* is considered to be a threatened species. The suspected decrease in numbers (Bruce 1992) has prompted concern with several countries, including South Africa, Australia and North America (Wintner & Cliff 1999), instituting protective legislation. However, these measures are considered pre-emptive largely due to the lack of essential population dynamics data either at a local or global level (Wintner & Cliff 1999). White sharks occur worldwide preferring cold and warm temperate waters off continental and insular shelves (Compagno 1984). Within this distribution the extent of long range movements

between disjunct populations, for example Australia and South Africa, is unknown. As a result white shark conservation status remains uncertain, which is evident by the IUCN Red List classification of white sharks as Category K 'suspected but not definitely known to be threatened' (Compagno *et al.* 1997).

Future conservation management decisions need to be underpinned by information on the dynamics of local movements and interactions, and the extent to which these are affected by larger scale movements. These data have proven difficult to obtain through traditional methods of tracking, tagging and observation largely due to the inherent difficulties of working in a marine environment with an intractable, elusive species, such as the great white shark. In this paper we describe the isolation and characterization of the first dinucleotide microsatellite loci in *Carcharodon carcharias*.

Microsatellite loci were isolated using a modified enrichment protocol (Armour *et al.* 1994) following Pierny *et al.* (1998). Total genomic DNA was extracted from muscle tissue of eight individuals sampled in the region of Dyer Island, South Africa, using standard proteinase K digestion and phenol-chloroform extraction procedures following Sambrook *et al.* (1989). Size selected (300–1000 bp) *Sau3AI* digested DNA fragments were ligated to a SAU linker molecule (made by annealing equimolar amounts of SAU-L-A [5'-GCCGTAC-CCGGAAGCTTGG-3'] and SAU-L-B [5'-GATCCCAAGCT-TCCCGGTACCGC-3'] oligonucleotides). The resultant fraction was denatured and hybridized to a 1 cm<sup>2</sup> piece of Hybond N membrane (Amersham) saturated with (CA)<sub>n</sub> and (GT)<sub>n</sub> polymers in 2× SSC, 0.1% SDS at 65 °C. These polymers had a ddATP ligated on the end using Terminal Dinucleotidyl Transferase according to the manufacturer's instructions (Promega) to prevent probe polymerization in subsequent amplifications. Filters were washed several times to remove non repetitive DNA. The enriched microsatellite fragments were removed by chemical stripping by incubating at room temperature first with 50 mM NaOH, 0.01% SDS and followed by 50 mM Tris-HCl (pH 7.5), 0.01% SDS. This fraction was ethanol precipitated from the combined wash solutions. The complementary strands were reformed using the SAU-L-A linker as a primer in a polymerase chain reaction (PCR) with 30 cycles of 90 °C denaturation for 1 minute, 55 °C annealing for 1 minute, 72 °C extension for 5 s and a final extension at 72 °C for 5 min. The PCR product was ligated into a pGEM®-T vector (Promega), transformed into JM109 high efficiency competent cells (Promega) and grown overnight at 37 °C on Luria-Bertani (LB) medium containing 50 mg/mL ampicillin and surface streaked with 40 µL of 50 mg/mL X-gal and 10 µL 20% IPTG. Recombinant colonies were gridded out onto duplicate, fresh LB plates and regrown overnight. The colony streaks were lifted onto Hybond-N membranes and fixed by UV cross-linking. Recombinant colonies were screened for microsatellite repeats with α-<sup>32</sup>P labelled CA and GT oligonucleotide repeats using random priming (Sambrook *et al.* 1989). DNA from positive clones were sequenced using an ABI 377 automated sequencer (cycle sequenced using Big Dye Terminator Kit according to the manufacturer's protocols). Primers were designed using the program OLIGO™ Macintosh version 4.1 (National Biosciences Inc., USA) from the unique sequence flanking the microsatellite repeats.

**Table 1** Dinucleotide microsatellite loci from *Carcharodon carcharias*. GenBank accession nos for these sequenced clones are AF184087, AF184089, AF216864–AF216866

Locus	Primer sequence 5'–3'	Repeat motif	T °C	Size (bp)*	No. of alleles	H <sub>E</sub>	H <sub>O</sub>
Ccar1	F GCAGAGGTTGGGAAAGAGTT R GCTATTCAGTGACACTCTCC	(AC) <sub>22</sub>	65–55	170	5	0.65	0.68
Ccar3.1	F CTTGTGCTCGCTGCTCTAC R GGTGGTTTGTGATTCTGTG	(AC) <sub>7</sub>	65–60	149	2	0.51	0.63
Ccar9	F AATGGGTTGTGATGGGAGTTT R CAAGTGGAGTCAAGCAGGTT	(TG) <sub>23</sub>	65–55	218	10	0.83	0.77
Ccar13	F GCTGAGTGTGCTGGCTGACCT R TATCCAGTTACCATCTCCAAAAA	(TG) <sub>4</sub> TT(TG) <sub>9</sub> TT(TG) <sub>3</sub> TTTT(TG) <sub>23</sub>	65–55	288	7	0.79	0.95
Ccar19	F GCCAGACCGACACATCAGTAA R GCAACGCCACATCCATAA	(TG) <sub>16</sub> CG(TG) <sub>3</sub>	65–55	200	3	0.51	0.45

\*Determined from sequenced clone.

T °C is the optimal (touchdown) annealing temperature; H<sub>E</sub>, expected heterozygosity; H<sub>O</sub>, observed heterozygosity.

Species (n)	Ccar1	Ccar3.1	Ccar9	Ccar13	Ccar19
<i>Scyliorhinus canicula</i> (2)	–	–	–	•	+
<i>Cetorhinus maximus</i> (2)	+	•	•	•	+
<i>Holohalealurus regani</i> (1)	–	–	–	–	•
<i>Squalus mitsukurii</i> (1)	–	–	–	–	•
<i>Squalus megalops</i> (1)	–	–	–	–	•
<i>Notorynchus cepedianus</i> (1)	–	–	–	–	–
<i>Galeorhinus galeus</i> (1)	–	•	–	–	•
<i>Raja springeri</i> (1)	–	–	–	–	•
<i>Raja caudospinosa</i> (1)	–	–	–	–	•
<i>Curiraja parcomaculata</i> (1)	–	–	–	–	•
<i>Heptranchias perlo</i> (1)	•	•	–	–	–
<i>Galeocerdo cuvier</i> (1)	+	•	–	–	•
<i>Carcharinus limbatus</i> (1)	–	•	–	–	•
<i>Lamna nasus</i> (8)	•	•	+	+	•
<i>Isurus oxyrinchus</i> (1)	+	•	–	•	•

**Table 2** Success amplifying loci across other elasmobranch species (determined on 6% acrylamide gels)

+, indicates presence of scorable alleles.  
•, alleles present but requires further optimization.  
–, multiple bands, smear or no product.

DNA was extracted from muscle tissue of 20 white shark samples (Dyer Island, South Africa) and from a variety of other elasmobranch species (Table 2). Individuals were screened for each microsatellite locus separately in 10 µL PCR reaction volumes containing 20 ng template DNA, 1–2.5 mM MgCl<sub>2</sub>, 0.2 mM of each nucleotide, 2.5–5 pmoles of primer (forward primer end-labelled with [ $\gamma$ -<sup>32</sup>P] – ATP), 0.5 units of *Taq* DNA polymerase (Bioline Inc.), and 1× NH4 buffer (Bioline Inc.). PCR amplifications were performed on a Hybaid PCR express using the 'touchdown' protocol (Don *et al.* 1991). Amplified products were resolved on 6% denaturing polyacrylamide gels and product sizes determined by comparison with a M13 mp8 DNA sequence visualized using autoradiography.

Of the six primer pairs tested on this single population, five were polymorphic (Table 1), with number of alleles ranging from 2 to 10 and observed heterozygosities from 0.45 to 0.95. To our knowledge the only other published report (Heist & Gold 1999) on microsatellite loci in elasmobranchs

(sandbar sharks *Carcharinus plumbeus*) found an overall lower number of repeats per locus and low heterozygosity values. Hence, preliminary results suggest our loci may prove useful population genetic markers.

The utility of the polymorphic microsatellite primers was examined in other elasmobranchs (Table 2). PCR conditions used were the same as those given above except for a lower touchdown temperature range of 60–50 °C. The presence of microsatellites varied across the range of species tested, suggesting amplification is species-dependent.

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## Characterization of microsatellite loci in *Coffea arabica* and related coffee species

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Coffee trees (family Rubiaceae) are classified in two genera, *Coffea* and *Psilanthus*. Particular attention has been paid to the genus *Coffea* which includes two cultivated species of economic importance, *C. arabica* L. and *C. canephora* Pierre. *C. arabica* ( $2n = 4 \times = 44$ ) is an amphidiploid (Lashermes *et al.* 1999) while other *Coffea* species are diploid ( $2n = 2 \times = 22$ ). Molecular phylogenies of *Coffea* species have been successfully established (Cros *et al.* 1998). Those analyses suggest several major clades,

which present a strong geographical correspondence (i.e. west Africa, central Africa, east Africa and Madagascar). Further steps would be to study the genetic structure of populations and gene flow between species.

The aim of this present paper is to develop a set of molecular genetic markers, known as simple sequence repeats (SSRs) or microsatellites (Weber & May 1989), suitable for genetic studies of coffee species. Eleven primer pairs that reliably detect microsatellite loci are described. We assessed their potential as genetic markers in the discrimination of *C. arabica* and *C. canephora* genotypes, and examined cross-amplification in various coffee species.

The plant material (55 individuals) resulted from several collecting missions in Africa and Madagascar. The species *C. arabica* are represented by 32 individuals sampled from different locations in Ethiopia and Yemen, while *C. canephora* are represented by 10 individuals collected in the Central African Republic, Congo and Côte-d'Ivoire. A total of 13 *Coffea* taxa were surveyed. The closely related genus *Psilanthus* was also represented by two species, *P. ebracteolatus* and *P. travencorensis*. DNA was isolated from lyophilized leaves through a nuclei isolation step as described by Agwanda *et al.* (1997).

DNA clones from a partial genomic library (*C. arabica* var. Caturra) enriched for (TG)<sub>13</sub> motifs (Vascotto *et al.* 1999) were sequenced using automated fluorescent technology (ABI sequencer). Oligonucleotide primers complementary to flanking regions of identified repeats were designed for 11 sequences using the computer program Primer3 (Whitehead Institute for Biomedical Research). SSR assays were carried out by means of the polymerase chain reaction (PCR). Reaction mixtures for the PCR amplification of SSR loci contained 25 ng of genomic DNA, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM of MgCl<sub>2</sub>, 0.2 pmol of each primer, 0.2 mM of dCTP, dGTP, dTTP, 0.01 mM of dATP, 0.8 mCi of [<sup>33</sup>P]-dATP (Amersham Pharmacia), and 0.5 U of *Taq* DNA polymerase (Promega) in a 25- $\mu$ l final volume. Reactions were performed in a PTC-200 thermocycler (MJ Research). The amplification cycle consisted of an initial 2 min denaturation at 94 °C, followed by 5 cycles of denaturation at 94 °C for 45 s, 1 min primer annealing at 60 °C with decreasing temperature of one degree at each cycle, and 1 min 30 s elongation at 72 °C. Then, 30 cycles of 45 s at 90 °C, 1 min at 55 °C and 1 min 30 s at 72 °C were performed and followed by a final 8 min elongation at 72 °C. Amplification products were electrophoresed on 6% denaturing polyacrylamide gel with 8 M urea and 1  $\times$  TBE. Radioactively labelled 10-bp ladder DNA was used as a size standard.

The 11 primer pairs were successful in amplification of variable-length fragments (Table 1). Only five of the 11 microsatellite loci appeared to be polymorphic in *C. arabica*. This result illustrated the very low genetic diversity present in *C. arabica* as a consequence of its origin, reproductive biology, and evolution (Lashermes *et al.* 1999). On the other hand, the microsatellite loci showed a broad range of genetic diversity across the accessions of *C. canephora*. The mean heterozygosity values were 0.04 and 0.47 in the predominantly autogamous *C. arabica* and the self-incompatible species *C. canephora*, respectively.

Results of cross-species amplifications with the 11 primer pairs are reported in Table 2. Although designed from sequences

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