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# Isolation and characterization of 20 microsatellite markers from *Carcharhinus leucas* (bull shark) and cross-amplification in *Galeocerdo cuvier* (tiger shark), *Carcharhinus obscurus* (dusky shark) and *Carcharhinus plumbeus* (sandbar shark)

**Agathe Pirog · Antonin Blaison · Sébastien Jaquemet · Marc Soria · Hélène Magalon**

**Abstract** With the development of genetics methods, it becomes possible to study the population structure and some aspects of the reproductive behaviour of endangered sharks. Here we describe the isolation of 20 polymorphic microsatellite markers in the bull shark *Carcharhinus leucas* (Carcharhinidae) and their characteristics. Two to 10 alleles per locus were detected. Observed and expected heterozygosities ranged from 0.00 to 0.78 and from 0.05 to 0.80, respectively. Four markers showed deviations from Hardy–Weinberg equilibrium; among them, three showed presence of null alleles. No linkage disequilibrium was detected among any of the loci. Moreover, four, 11 and 19 of these 20 markers successfully cross-amplified in the tiger shark *Galeocerdo cuvier,* the sandbar shark *Carcharhinus plumbeus* and the dusky shark *Carcharhinus obscurus*, respectively.

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Bull sharks (*Carcharhinus leucas*) are apex predators in tropical and subtropical seas and are unique among Elasmobranchs for their capacity to inhabit coastal freshwater systems for prolonged periods of time (Compagno 1990). They are classified as Near Threatened in the International Union of Conservation for Nature Red List (IUCN 2013) and are submitted to recreational and commercial pressures (Karl et al. 2011).

Samples of muscle and fin tissues from 11 adults (both female and male) of *C. leucas* caught from Reunion Island were used for DNA extraction. Total genomic DNA was isolated using Qiagen DNeasy Blood and Tissue Kit and sent to GenoScreen, Lille, France (www.genoscreen.fr). One µg was used for the development of microsatellites libraries through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries as described in Malausa et al. (2011). A total of 7,556 sequences containing microsatellite motif were identified. Sequences were analysed using the software QDD (Meglecz et al. 2010) and 20 microsatellite markers were finally selected and characterized for *C. leucas*.

Thus genotyping of these 20 markers was conducted on 41 adult individuals of Reunion Island population. Each amplification reaction contained 10 µL of PCR product. Microsatellite loci developed in this study were directly fluorochrome labelled, and the reaction mixture contained 5 µL of MasterMix Applied 2x (Applied Biosystems), 1.5 µL of demineralised water, 0.5 µL of each primer (10 µM) and 2.5 µL of genomic DNA (10 ng/µL). The thermocycling program was as follow: an initial denaturing step at 94 °C for 5 min, seven cycles including a step at 94 °C for 30 s, a step at 62 °C (−1 °C at each cycle) for 30 s and a step at 72 °C for 30 s. These seven cycles are followed by 35 cycles including a step at 94 °C for 30 s, a step at 55 °C for 30 s and a step at 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min. Allelic sizes

**Table 1** Microsatellite loci developed for *Carcharhinus leucas* and their primers sequences (F Forward, R Reverse)

| Name | Primer sequences (5′-3′) | GenBank accession number | Repeat motif | Size (bp) | $N_a$ | $N$ | $H_O$ | $H_E$ | $F_{IS}$ | $r$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Cl01 | F: GACACAATGCACAGGCAATC<br>R: GCGCACAGACATATAATCTGAA | KJ916104 | $(AC)_6$ | 114–116 | 2 | 41 | 0.000 | 0.347 | 1.000*** | 0.352<br>yes |
| Cl02 | F: ACAGGCAAAGACAATTGCAAAC<br>R: GCTTCCTGTGTCTGGAGGGT | KJ916105 | $(AC)_6$ | 141–143 | 2 | 41 | 0.195 | 0.253 | 0.231 $^{NS}$ | 0.090<br>no |
| Cl03 | F: GAGGCAGTTGCTGGCTGTAT<br>R: AAAGCTCCCACACCAGACAC | KJ916106 | $(TC)_7$ | 106–108 | 2 | 41 | 0.610 | 0.505 | −0.211 $^{NS}$ | −0.118<br>no |
| Cl04 | F: GTGCAGAAAACAGTCGGACA<br>R: GGCTGCTCTAGTCCCAGCTT | KJ916107 | $(GA)_7$ | 124–130 | 2 | 41 | 0.683 | 0.455 | −0.510** | −0.437<br>no |
| Cl05 | F:AGGCCCTATCCCCATAACC<br>R:TGTGTGAGTGAAAGTGAGCG | KJ916108 | $(CA)_7$ | 137–141 | 3 | 41 | 0.415 | 0.409 | −0.015 $^{NS}$ | −0.014<br>no |
| Cl06 | F: ATGGGGATAGGCAGATGG<br>R: TTGTTTTATCTCTGTGTATGTTCATGT | KJ916109 | $(AC)_7$ | 144–150 | 3 | 41 | 0.049 | 0.048 | −0.013 $^{NS}$ | −0.025<br>no |
| Cl07 | F: TTACCAGCTGATTATCTACTCACACTC<br>R: CTCACTGCCTGCACGATTC | KJ916110 | $(CA)_7$ | 143–151 | 5 | 41 | 0.463 | 0.409 | −0.136 $^{NS}$ | −0.081<br>no |
| Cl08 | F: GAAAGGAAAACTCGAAGAATTCAC<br>R: AGTACTGGAGCAGACCGCAT | KJ916111 | $(AG)_7$ | 144–154 | 6 | 41 | 0.683 | 0.671 | −0.018 $^{NS}$ | −0.015<br>no |
| Cl09 | F: GGCTGGTGACATTATTGAGTG<br>R: TGAGGAGCTCAATCAGATCTTT | KJ916112 | $(AG)_8$ | 107–115 | 4 | 41 | 0.415 | 0.369 | −0.127 $^{NS}$ | −0.079<br>no |
| Cl10 | F: GCATCAGCTTGGAACAACAC<br>R: TTCAAAGACTTCCAAGTTGGGT | KJ916113 | $(CT)_8$ | 123–129 | 3 | 40 | 0.075 | 0.162 | 0.539* | 0.159<br>yes |
| Cl11 | F: AGATTACTGTGACTTTTGTTCTGTTTT<br>R: TCTCTATTTCTTGCTGCAGAGGT | KJ916114 | $(AG)_8$ | 232–238 | 3 | 41 | 0.341 | 0.301 | −0.135 $^{NS}$ | −0.179<br>no |
| Cl12 | F: TGCTCTTTATCACATTTCTTCACA<br>R: ACCATGGTGCAGTCTACCAA | KJ916115 | $(TC)_9$ | 105–111 | 3 | 41 | 0.171 | 0.158 | −0.081 $^{NS}$ | −0.089<br>no |
| Cl13 | F: CCCAAAACCACTCGAGTAACA<br>R: CGTTGGAGTTTGTACAAGGTGA | KJ916116 | $(AC)_9$ | 94–126 | 10 | 41 | 0.683 | 0.633 | −0.080 $^{NS}$ | −0.070<br>no |
| Cl14 | F: TGCTCACGGATGTGTAGTGC<br>R: GAGGATAGCTGCCCACGATA | KJ916117 | $(GT)_9$ | 211–219 | 6 | 41 | 0.220 | 0.203 | −0.081 $^{NS}$ | −0.113<br>no |
| Cl15 | F: TGACCTGCAATATTTTCCTGC<br>R: TCGCTGACAGGCAAGGTTAT | KJ916118 | $(GT)_9$ | 290–300 | 4 | 41 | 0.098 | 0.095 | −0.029 $^{NS}$ | −0.050<br>no |
| Cl16 | F: TCTTTGCAAACATCCCAGC<br>R: TTGAAAATACTCAGAGACGCAC | KJ916119 | $(TC)_{12}$ | 103–115 | 6 | 41 | 0.780 | 0.732 | −0.033 $^{NS}$ | −0.026<br>no |
| Cl17 | F: TGCAGACGCTATTACTTCCG<br>R: TTGTTCTCTCACTCAACGCAA | KJ916120 | $(TG)_{12}$ | 175–183 | 5 | 41 | 0.561 | 0.568 | 0.013 $^{NS}$ | −0.003<br>no |
| Cl18 | F: TCTTCGGAGTGACTAACGCA<br>R: AGTCGTGGTGCTTCTAACGA | KJ916121 | $(CA)_{12}$ | 209–219 | 6 | 41 | 0.390 | 0.432 | 0.097 $^{NS}$ | 0.039<br>no |

**Table 1** continued

| Name | Primer sequences (5′-3′) | GenBank accession number | Repeat motif | Size (bp) | $N_a$ | $N$ | $H_O$ | $H_E$ | $F_{IS}$ | $r$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Cl19 | F: ACAAGCTGCAAAGATCCTCAA | KJ916122 | $(CCA)_7$ | 198–216 | 6 | 41 | 0.415 | 0.408 | −0.016 $^{NS}$ | −0.058 |
| | R: TGGGGTTACCTGGACACTTT | | | | | | | | | no |
| Cl20 | F: ACGAGGATGACAACAAACTGG | KJ916123 | $(AGG)_9$ | 134–167 | 10 | 41 | 0.634 | 0.797 | 0.207* | 0.092 |
| | R: CTTTTCCCTCCTTTCCCATC | | | | | | | | | yes |

Accessibility numbers in GenBank are indicated

$N_a$ number of alleles per locus, $N$ number of individuals, $H_O$ observed heterozygosity, $H_E$ expected heterozygosity, $F_{IS}$ inbreeding coefficient

Concerning the $F_{IS}$, is indicated the significance of the P-values for deviation to Hardy-Weinberg equilibrium. *NS*: non significant,* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Null allele frequencies ($r$) are issued from MicroChecker v 2.2.3 (Van Oosterhout et al. 2004), and presence or absence is indicated below (yes or no)

Annealing temperature $T_a = 55$ °C

**Table 2** Cross-amplification for 20 microsatellite markers designed for *Carcharhinus leucas* across three carcharhinidae species: *Carcharhinus obscurus* (n = 2), *Carcharhinus plumbeus* (n = 3) and *Galeocerdo cuvier* (n = 41) (+, amplified, +P, polymorphic; −, no amplification). Size ranges in base pairs and number of alleles (in brackets) were also indicated

| Locus Name | *C. obscurus* (n = 2) | *C. plumbeus* (n = 3) | *G. cuvier* (n = 41) |
|---|---|---|---|
| Cl01 | + 114 (1) | +P 114–134 (2) | – |
| Cl02 | + 141 (1) | – | – |
| Cl03 | + 106 (1) | + 106 (1) | – |
| Cl04 | + 124 (1) | +P 122–126 (3) | – |
| Cl05 | + 141 (1) | – | – |
| Cl06 | + 144 (1) | – | – |
| Cl07 | + 143 (1) | + 143(1) | – |
| Cl08 | + 144 (1) | +P 144–146 (2) | – |
| Cl09 | +P 111–113 (2) | – | + 107 (1) |
| Cl10 | + 123 (1) | – | – |
| Cl11 | +P 232–238 (3) | +P 106–116 (5) | – |
| Cl12 | + 111 (1) | +P 103–105 (2) | +P 105–115 (2) |
| Cl13 | +P 94–102 (2) | + 96 (1) | – |
| Cl14 | +P 209–215 (2) | + 216 (1) | +P 184–210 (2) |
| Cl15 | + 300 (1) | – | – |
| Cl16 | +P 103–111 (3) | – | – |
| Cl17 | + 179 (1) | +P 171–179 (3) | +P 167–169 (2) |
| Cl18 | – | – | – |
| Cl19 | + 198 (1) | – | – |
| Cl20 | + 143 (1) | + 149 (1) | – |

were determined using Genemapper v 4.0 (Applied Biosystems).

Diversity indices, Hardy–Weinberg equilibrium and linkage disequilibrium were assessed using Arlequin v 3.5.1.2. All loci were polymorphic, the number of alleles ranged from 2 to 10, observed heterozygosities ranged from 0.00 to 0.78 and expected heterozygosities from 0.05 to 0.80 (Table 1).

Linkage disequilibrium was observed for some loci at a significance level of 0.05 but none were still significant after Bonferroni correction for multiple testing. Four loci were found to deviate from Hardy–Weinberg equilibrium: three of them probably due to the presence of null alleles assessed using MicroChecker v 2.2.3 (Van Oosterhout et al. 2004) (Table 1).

The markers developed were also tested on *Galeocerdo cuvier, Carcharhinus plumbeus* and *Carcharhinus obscurus*: 4, 11 and 19 markers successfully cross-amplified, respectively (Table 2).

The development of these markers will be very useful in studying sharks ecology, which remains poorly documented, above all in assessing population structure, effective population size and phylopatry.

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