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PRIMER NOTE

Microsatellite DNA markers for *Podocnemis unifilis*, the endangered yellow-spotted Amazon River turtle

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Abstract

We developed specific primers for microsatellite DNA regions of *Podocnemis unifilis* and tested their utility in population genetic and paternity studies on the species and other closely related Amazonian chelonians. Seventeen microsatellite loci were polymorphic in *P. unifilis* and all, plus two monomorphic microsatellites in *P. unifilis*, were polymorphic in at least one additional chelonian species, including *Peltocephalus dumeriliana*.

Keywords: chelonian, microsatellite, *Podocnemis unifilis*, tracaja

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Many chelonian species are threatened with extinction as a consequence of the excessive harvesting of eggs and adults, as well as the destruction of their natural habitat. In South America, six species of the *Podocnemis* genus are found: *Podocnemis vogli*, *P. lewyana*, *P. expansa*, *P. unifilis*, *P. sextuberculata*, and *P. erythrocephala*, of which the last four are found in Brazil. At present, molecular genetic analyses have been carried out only for the largest species of the genus, *P. expansa* (Sites *et al*. 1999; Pearse *et al*. 2006a, b). *Podocnemis unifilis*, the yellow-spotted Amazon River turtle, popularly known in the Brazilian Amazon as 'tracajá', is classified as 'Vulnerable' in the Red List of the International Union for the Conservation of Nature and Natural Resources and is listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora. It is the second largest and the second most popularly consumed species after *P. expansa*, its increase in popularity coinciding with the drastic decrease in census sizes and local extinctions of *P. expansa*. *Podocnemis unifilis* is more of an ecological generalist than *P. expansa*, and thus appears better suited to captivity and ranching schemes (Fachín-Terán *et al*. 1997). As with most Amazonian vertebrates, no genetic profile exists for this species, and we have little knowledge of its system of mating. Therefore, the main aim of this study is to develop primers for DNA microsatellite loci of *P. unifilis* and to test their utility for population and paternity analyses of this species. We also test the applicability of these markers in other *Podocnemis* species (*P. sextuberculata*, *P. expansa*, *P. erythrocephala*, *P. vogli*) and a closely related genus represented by *Peltocephalus dumeriliana*.

Total genomic DNA was extracted from blood using GFX DNA extraction kit (GE Healthcare). Microsatellite loci were isolated and identified from a partial genomic library enriched for CT_{12} repeats following the protocol of Farias *et al*. (2003, 2006). Enriched DNA was ligated into an Invitrogen pCR 2.1 TOPO cloning vector and transformed into chemically competent *Escherichia coli* (TOPO TA Cloning kit) following the manufacturer's recommendations. The transformed cells were grown overnight on a 1 × LB/Amp (Sambrook *et al*. 1989) agar plate. Individual colonies were picked, and regrown for 16 h in a 96-well culture plate containing 150 μL of liquid 1 × LB/Amp (100 μg/mL) solution. Polymerase chain reaction (PCR) amplification using M13 forward (–20) and M13 reverse primers was performed directly on the bacterial cultures following the manufacturer's protocol. PCR products were purified using GFX spin-columns (GE Healthcare), and sequencing reactions were performed with the M13 primers using ET

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Table 1 Characterization of 18 microsatellite loci of *Podocnemis unifilis*

Locus/GenBank Accession no.	Primer (5′–3′)	Repeat motif	Size range (bp)	T (°C)	No. of individuals/ alleles	H_O	H_E	I	Q
Puni_1A5	F-5′-TCTTCAGCGTTGTGGTTCTG-3′	$(GA)_{22}$	156–206	62	21/11	0.762	0.892	0.0290	0.7518
EF455680	R-5′-GAGGGGAAAGACGACAATGA-3′								
Puni_1B2	F-5′-GTAGTGGCACTGCGACAAAT-3′	$(GA)_{17}$	343–369	55	24/10	0.792	0.858	0.0433	0.7009
EF455681	R-5′-CCACTGTCACATCTCCTGAAAC-3′								
Puni_1B10	F-5′-CCAAACTAGGTTCATGTCCAAA-3′	$(GA)_8$	238	60	Monomorphic*				
EF455685	R-5′-GAAGCGTCAGGAAGGAAAGA-3′								
Puni_1B11	F-5′-CCAGACCTCTCCTGTTTTGG-3′	$(GA)_7$gg$(GA)_9$	265–287	60	24/7	0.750	0.666	0. 1772	0.4267
EF455682	R-5′-GGTTCTGGGCTCCTTACACA-3′								
Puni_1C3	F-5′-CCCTACCGAAACAGCTTGAG-3′	$(GA)_8$	182–218	62	11/8	0.364§	0.785	0.0720	0.6160
EF455683	R-5′-ATCTGGCTTGGAGCTGTGTT-3′								
Puni_1C9	F-5′-GCTGCAACAAGGAGAAGTGG-3′	$(GA)_{17}$	113–141	64	23/5	0.565‡	0.513	0.2890	0.2990
EF455684	R-5′-CCCTTCGGAATTCAGTGGA-3′								
Puni_1D9	F-5′-GCTGGGGAACTGACTACCT-3′	$(GA)_{12}$	133–157	62	24/8	0.458	0.664	0.1572	0.4510
EF455686	R-5′-CACGAGGTAGGAATGCCTGT-3′								
Puni_1D11	F-5′-CACGAACCTCTTCCATCCAG-3′	$(GA)_{19}$	125	64	Monomorphic†				
EF455687	R-5′-CTGACACTCTCGCTGCACTC-3′								
Puni_1D12	F-5′-AGGAGCTGCAGGTGCAAAC-3′	$(GA)_{10}$	174–180	55	24/3	0.875§	0.595	0.3199	0.2672
EF455688	R-5′-GATCACCCAGATGCTGACCT-3′								
Puni_1E1	F-5′-GGCCTCTACTGTCTGAAAGTCC-3′	$(CT)_9$tt$(CT)_7$	185–209	64	23/7	0.783	0.809	0.0738	0.6140
EF455689	R-5′-GAAGGAGAGCTCCAGGTGAA-3′								
Puni_1F10	F-5′-GCCTGCaGCTCCTCTCATAA-3′	$(CT)_8$	203–215	60	24/4	0.208§	0.574	0.2785	0.3072
EF455690	R-5′-CCCAGGAACTTGAGAATAGTGG-3′								
Puni_1H9	F-5′-GGGGCTACAGAGAAGGAGAA-3′	$(GA)_{12}$	171–191	60	24/7	0.458‡	0.594	0.2149	0.3767
EF455692	R-5′-ATTTATATGGGCCCCCTACC-3′								
Puni_2A9	F-5′-CTGTTCCCAACAGCTGAGAG-3′	$(GA)_{12}$	161–183	55	20/11	0.950	0.881	0.0355	0.6640
EF455693	R-5′-GGTCCTCAAGAAAGCCCAAA-3′								
Puni_2C11	F-5′-AAGGTGCCTGGAGAATAGGA-3′	$(CT)_{17}$	271–287	55	22/9	0.773	0.744	0.0964	0.5300
EF455694	R-5′-TGCACCCTTCCATTTAAGC-3′								
Puni_2D9	F-5′-CAGCATTTTCTGACAGACAGC-3′	$(GA)_7$ca$(GA)_6$	240–246	60	23/5	0.826	0.703	0.1510	0.4612
EF455695	R-5′-CCACAGCAACCATCTCAGC-3′								
Puni_2D10	F-5′-GCCAGTCcCTTAAAAACTAGGG-3′	$(GA)_8$	164–172	55	24/5	0.375	0.395	0.2148	0.3782
EF455696	R-5′-GGGATGCTTCTACTGCTGCT-3′								
Puni_2E7	F-5′-CTGGACCCATATGCAGTGAC-3′	$(GA)_5$gc$(GA)_8$	260–282	56	24/7	0.792‡	0.761	0.1159	0.5235
EF455697	R-5′-CACTTGAGCTTCTGAGGGAGA-3′								
Puni_2F6	F-5′-CTGGTCCAACCCAATTTCTG-3′	$(CT)_6$t$(CT)_{10}$	291–325	60	24/7	0.583	0.727	0.1204	0.5129
EF455698	R-5′-CCTTGACCAGGACTGCACTT-3′							$IC = 2.16 \times 10^{-15}$	$QC = 0.9999910$

*Polymorphic for *P. sextuberculata*; †polymorphic for *P. erythrocephala*.
H_O, observed heterozygosity; H_E, expected heterozygosity; ‡$P < 0.05$, §$P < 0.01$; I, probability of genetic identity at an individual locus; *IC*, probability of genetic identity for all loci; *Q*, probability of paternity exclusion at an individual locus; *QC*, probability of paternity exclusion for all loci.

Table 2 Cross-species amplification test. Codes: +/+ polymorphic; +/– monomorphic; –/– PCR or genotyping did not work

Locus	*Podocnemis sextuberculata* (*N* = 4)	*Podocnemis erythrocephala* (*N* = 12)	*Podocnemis expansa* (*N* = 5)	*Podocnemis vogli* (*N* = 8)	*Peltocephalus dumerilianus* (*N* = 4)
Puni_1A5	+/+	–/–	+/+	–/–	–/–
Puni_1B2	+/+	+/–	+/+	+/+	–/–
Puni_1B10	+/+	–/–	–/–	+/–	–/–
Puni_1B11	+/+	+/+	+/+	+/+	–/–
Puni_1C3	+/–	–/–	–/–	+/+	–/–
Puni_1C9	+/+	+/+	–/–	–/–	–/–
Puni_1D9	+/+	+/+	+/–	+/+	–/–
Puni_1D11	–/–	+/+	–/–	–/–	+/–
Puni_1D12	+/+	+/+	+/+	+/–	–/–
Puni_1E1	+/–	+/+	+/+	+/+	+/+
Puni_1F10	+/–	+/+	+/+	+/–	–/–
Puni_1H9	+/+	+/–	+/–	+/+	+/+
Puni_2A9	+/+	+/–	–/–	+/+	–/–
Puni_2C11	–/–	+/+	–/–	+/–	–/–
Puni_2D9	+/+	–/–	+/+	–/–	–/–
Puni_2D10	+/+	+/+	–/–	–/–	–/–
Puni_2E7	+/+	+/+	+/+	–/–	–/–
Puni_2F6	+/–	–/–	+/+	+/–	–/–

terminator sequencing chemistry. Products were visualized on a MegaBACE 1000 (GE Healthcare).

Good quality microsatellite repeats — perfect or nearly perfect repeat motif of at least 10 repeats units — were found in 80 out of 192 clones sequenced. Primer pairs complementary to regions flanking the microsatellite repeats were designed for 34 clones using the program PRIMER 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Each forward primer had an M13 sequence tail added to its 5′ end to allow for dynamic fluorescent labelling (TET-labelled M13 primer) following the economic protocol of Schuelke (2000). PCR was performed in 10 μL reaction volumes. The final volume was 5 mM KCl, 1 mM Tris-HCl, 1.75 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM reverse primer, 0.1 μM forward primer, 0.1 μM TET-labelled M13 label primer, 0.05 U/mL LGC Biotecnologia *Taq* DNA Polymerase, and about 1 ng/mL genomic DNA. PCRs were run in a Thermo Hybaid thermocycler and had two main steps: an initial denaturation (94 °C, 1 min) followed by 25 cycles of 50 s at 94 °C, 50 s at primer-specific annealing temperature (Table 1), 1 min at 72 °C; followed by 20 cycles of 40 s at 92 °C, 35 s at 50 °C, 40 s at 68 °C, and a extension for 20 min at 72 °C. Microsatellite clones were deposited in GenBank (Table 1). PCR products were visualized on a MEGABACE 1000 Fragment Profiler version 1.2 software (GE Healthcare). Allele sizes were scored against the size standard ET-400 ROX (GE Healthcare).

We genotyped on average three individuals per eight different nests of *P. unifilis* (between 20 and 24 individuals successfully assayed). The nest sites were located in the area of Barreirinha (2°43′59″S, 57°07′30″W), Amazonas state, Brazil. Out of 34 primer pairs developed, 19 amplified a single band, three were monomorphic, and 16 were highly polymorphic, with number of alleles ranging from four to 11 (Table 1). The observed per-locus heterozygosities ranged from 0.208 to 0.950 (ARLEQUIN 3.1, Excoffier *et al*. 2005). We also tested the statistical power of the 16 loci for inferring genetic identity (*I*) (Paetkau *et al*. 1995) and paternity exclusion (*Q*) (Weir 1996). Hardy–Weinberg equilibrium was observed in all loci but Puni_1F10 and Puni_1H9 (heterozygosity deficiency), and Puni_1D12 and Puni_2E7 (heterozygosity excess). Across all loci-pairs, we found less than 5% of pairs with linkage disequilibrium after Bonferroni corrections ($P < 0.01$) which involved comparison between loci 1D12 × 1B2, and 1D12 × 1B11. The locus Puni_1H8 (not listed in Table 1) was monomorphic for *P. unifilis* and did not amplify for the other species; additional samples should be tested at a population level to confirm the monomorphism of this locus. Due to amplification failures, only 11 individuals could be genotyped for the locus Puni_1C3; additional testing will be necessary to confirm if this result indicates the presence of null alleles, considering that allele frequencies at this locus deviated from Hardy–Weinberg equilibrium (heterozygote deficit, $P < 0.01$).

For the cross-species amplification test, we used four to 12 individuals of other *Podocnemis* species (Table 2) and the closely related *Peltocephalus dumeriliana*. Surprisingly, the two monomorphic loci in *P. unifilis* (Puni_1B10 and Puni_1D11) were polymorphic in at least one of the other species. The microsatellite loci developed for *P. unifilis* appear

to be informative for the genus as a whole (all species but *P. lewyana* from the Magdalena River of Colombia were tested), and thus provide a powerful molecular toolkit for conservation, management and molecular ecological studies of these highly threatened turtles.

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