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TECHNICAL NOTE

Isolation and characterization of fifteen microsatellite loci from the endangered pampas deer (*Ozotoceros bezoarticus*, Cervidae)

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Abstract *Ozotoceros bezoarticus* is categorized as Threatened in Argentina. In this study, we report 15 microsatellite loci—in the Argentinian populations, 12 are polymorphic and 3 monomorphic—isolated from a dinucleotide enriched genomic library. Among 59 individuals sampled from the four remaining populations in Argentina, the mean number of alleles was 7.58, the expected heterozygosity ranged from 0.33 to 0.88 and the observed heterozygosity from 0.08 to 0.83. Three loci showed deviation from Hardy–Weinberg equilibrium. Some of the loci showed linkage disequilibrium, although there was no coincident pattern for this parameter in all populations. The primers reported here constitute the first specific set for the species, and their use will be invaluable to generate the necessary information on genetic diversity in order to help conservation plans.

Keywords *Ozotoceros bezoarticus* · Enriched genomic library · Polymorphic loci · Conservation

Ozotoceros bezoarticus is distributed throughout Bolivia, Brazil, Paraguay, Uruguay and Argentina. This cervidae was a widespread and abundant species occupying a long range of open habitats, but towards the beginning of the twentieth century a decrease in the number and extension of populations started to be evident. Currently it is considered Near Threatened in the global IUCN red list assessment of the species (IUCN 2011), whereas in Argentina it is considered Threatened. In this country, there are only four isolated populations distributed in the Provinces of Corrientes, San Luis, Buenos Aires and Santa Fe, these last two with population numbers not exceeding a few hundred (Dellafiore et al. 2001; Jackson and Langguth 1987; Pautasso and Peña 2002; González et al. 2010).

Actually, there are no microsatellite markers developed for this species. Although 15 microsatellites designed for ungulates were tested for cross-species amplification, only five were useful for research on Pampas deer (Cosse et al. 2007).

For microsatellite loci isolation, we developed a genomic SSR-enriched library according to Billotte et al. (1999). Genomic DNA was extracted from blood samples obtained from *O. bezoarticus* from Corrientes province using Quiagen DNeasy Blood and Tissue Kit according to the manufacturer protocol. Five micrograms of DNA were digested with *Afa*I and ligated to RSA21 (5′-CTCTTGCTTACGCGTGG-ACTA-3′) and RSA25 (5′-TAGTCCACGCGTAAGCAA-GAGCACA-3′) adaptors. The library was enriched by the hybridization of DNA fragments containing microsatellites with $(GT)_8$ and $(CT)_8$ biotin-linked probes and recovered with streptavidin magnetic-coated beads (Streptavidin MagneSphere Paramagnetic Particles). Selected fragments were ligated to vectors (pGEM-T vector System) and transformed into *Escherichia coli* XL1-Blue competent cells.

Fifty-two clones were double-sequenced with T7 and SP6 promoter primers using Big Dye Terminator Cycle Sequencing Kit (v.3.1) in an ABI377 Automated Sequencer

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(Applied Biosystems). Sequences were aligned, edited and excluded, if redundant, with SeqMan (DNAStar). We used MICROSAT (A.M. Risterucci, pers. comm.) to remove adaptors and restriction sites. Microsatellites were identified using the Simple Sequence Repeat Identification Tool (SSRIT) (Temnykj et al. 2001). We considered dinucleotides with more than five repeats, and tri-, tetra-, penta- and hexanucleotides with more than four repeats. Microsatellite primer pairs were designed using PrimerSelect (DNAStar) and Primer3Plus (Untergasser et al. 2007). Every forward primer was manufactured with a 5′-M13 tail (5′-CACGACGTTGTAAAACGA-3′, Boutin-Ganache et al. 2001) for use following a universal dye-labelling method (Schuelke 2000). Polymerase chain reactions were carried out in a final volume of 20 μL containing 2 ng of template DNA, 1× PCR buffer, 2.5 mM Magnesium chloride, 0.2 μM of each dNTP, 0.2 μM of each primer, 0.015 μM of M13 primer labeled with different fluorochromes (FAM, TMR, or HEX) and 1U *Taq* DNA polymerase (Fermentas). PCR reactions were performed in a Bio-Rad thermocycler, under the

Table 1 Population statistics of 15 microsatellite loci tested in 4 populations of Argentinian pampas deer

Locus	Primer sequence (5′–3′)	Repeat motif	No A	Size range (bp)	T_a (°C)	H_e	H_o	PIC value
Ob1	F:CAGCTTTGGCTTATGGATGTTT	$c(CA)_4$ $(CA)_{15}$ $(AC)_4$	8	18–315	62–55[a]	0.7113	0.5278	0.6754
	R:CTCAAAGGTTTTCTAAGGCAGTAAT							
Ob2	F:GGCTGTGGTCCATAAGGTT	$(AC)_{19}$	12	17–158	65–55[a]	0.8774	0.3393	0.8561
	R:AGGAGCAATAAGGAAGAAGAGAG							
Ob5	F:GAAAGCATGTATAGCAGTCTCAGG	$(GT)_7(GA)_4$	4	189–346	65–55[a]	0.3287	0.0784	0.2889
	R:CCAACTAATAAAAATAAAT GGAAAAAA							
Ob7	F:AAATGCCAACCTGCTGAAC	$(AC)_7$	6	35–285	62–57[a]	0.5246	0.3137	0.476
	R:GATGGCCAATCTAAGTAAATCC							
Ob9	F:GTGTCAGGGAGCTTTTCAGAT	$(TG)_7$	5	20–239	65–55[a]	0.6153	0.375	0.5562
	R:GTCACTCCCCACTTCACCTC							
Ob10	F:CAGGTGGGACTTGCTTCTCT	$(CA)_{15}$	9	175–375	65–55[a]	0.806	0.5918	0.7671
	R:GACTCCCTTCCCTTTGCTG							
Ob11	F:CTGCTGTAATAAAATCTATCACTTG	$(TG)_8$ $(GT)_{11}$	6	514–732	65–55[a]	0.7491	0.8333	0.6896
	R:CAGAATCCCCATGAACAGAG							
Ob12	F:GCTTGATGTTTACTTCATCAGG	$(AC)_7$	11	22–201	65–55[a]	0.5541	0.2	0.5364
	R:AAAGGAGGGAAGAAGGAAAG							
Ob13	F:GTATCTTCACGCAAAATCATCAC	$(AC)_{18}$ $(TC)_4$	10	54–297	65–55[a]	0.8438	0.5897	0.8142
	R:CTGAAAGTAGTCCTAGAGAG AGAAAAC							
Ob15	F:GTTCTTCCTTTTTTTCTCCCTT	$(TG)_7$	7	67–224	55–53[a]	0.5592	0.125	0.4719
	R:ACTTTGCAAATTTATTTTACCTGG							
Ob18	F:AATAGAGATTCCTAGAATAAATG TTTTCA	$(TG)_8$	10	7–202	62–57[a]	0.5494	0.551	0.5236
	R:CCACTCCAGTATTCTTGCCAG							
Ob19	F:TATCCCACCCCCTATCCTC	$(CA)_7$	3	24–270	65–55[a]	0.4754	0.4074	0.424
	R:GCCTTCTTGTTTGTGTTTGTGA							
Ob6	F:TGATACCCTGGTTCTGATGACA	$(TG)_5$ $(TG)_7$	Monomorphic	15–279	65–55[a]			
	R:CACCCTATGGATGACCTCTGAC							
Ob8	F:GGGCAGAGTCCAGGATAGGTCA	$(GT)_{24}$ $ct(GC)_8$ $gc(CT)_5$	Monomorphic	11–335	65–55[a]			
	R:TGGGAAAGAACACGGGAGAAAC							
Ob16	F:GTTCTAACCTACCCACTCAAGG	$(AC)_6$	Monomorphic	53–331	65–55[a]			
	R:AAGAAGACAAAGGCAAATCAAG							

[a] Starting from the first indicated temperature (*left*) and ending with the second indicated temperature

T_a Annealing temperature; H_e expected heterozygote frequency; H_o observed heterozygote frequency. *PIC* polymorphism information content

following conditions: 94 °C for 4 min, 10 cycles of 94 °C for 1 min, with the annealing temperature decreasing by 1 °C per cycle, and 72 °C for 1 min; followed by 35 additional cycles at the final annealing temperature. The amplification ended with an extension step at 72 °C for 15 min. Table 1 shows the starting and ending annealing temperatures for each locus. Sequences were deposited in GenBank under accession numbers JX310349–JX310363.

Amplification products were first visualized in a 2 % agarose gel stained with ethidium bromide prior to its genotyping. Two microlitres of the amplicon were added to a 10 µl mix of TWIN ET-ROX (GE Healthcare). The labeled PCR products were genotyped in a MegaBACE1000 automated sequencer (GE Healthcare), with ET-ROX-550 as internal size standard. Microsatellite patterns were examined using the software Fragment Profiler 1.2 (GE Healthcare).

To characterize the designed microsatellites, we used fresh tissue samples from 59 individuals collected in all four Argentinian populations (Santa Fe = 5, Corrientes = 28, Buenos Aires = 12 and San Luis = 14). They consisted in blood, muscle and skin. Blood samples were extracted with Quiagen DNeasy Blood and Tissue Kit and tissue samples with sodium dodecyl sulphate (SDS)-proteinase K-NaCl-alcohol precipitation (modified from Miller et al. 1988).

Using Arlequin v3.11 (Excoffier et al. 2006), we calculated observed and expected heterozygosity and tested for Hardy–Weinberg Equilibrium (HWE) and linkage disequilibrium for each locus. Using MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004) we tested scoring errors due to stuttering, large allele dropout, or null alleles. Null alleles were also checked with Cervus 3.0 (Kalinowski et al. 2007) and polymorphic information content (PIC) was estimated with Microsatellite-Toolkit for Excel.

Three loci were monomorphic, while 12 loci were found to be polymorphic with number of alleles ranging from 3 to 12, their expected and observed heterozygosities from 0.33 to 0.88 and 0.08 to 0.83 respectively, and a PIC mean value of 0.59 (Table 1). Out of the 12 microsatellites, three loci—Ob2, Ob12 and Ob15—showed deviation from Hardy–Weinberg equilibrium ($P < 0.00416$) once we applied the Bonferroni correction for multiple comparisons (Rice 1989). We found evidence for scoring errors due to stuttering and null alleles in all populations for these same loci. Although some pairwise comparisons between the loci showed departure from independence, the pattern was not consistent in all populations and the loci were considered to be at linkage equilibrium.

We isolated specific microsatellites to investigate genetic diversity and help conservation efforts focusing on population structure, connectivity, viability and the effects of recent habitat fragmentation. The future theoretical background that could be obtained from research with these markers will help management decisions in order to decrease the species risk of extinction.

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