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Cross-amplification tests of ungulate primers in the endangered Neotropical pampas deer (*Ozotoceros bezoarticus*)

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ABSTRACT. In cross-species amplification tests of 15 ungulate primers in pampas deer, five were retained to form a small panel of highly polymorphic loci that could be used to efficiently screen populations of this endangered species. The polymerase chain reactions were performed incorporating the universal fluorescent labeled M13 (-21) primer. In 69 pampas deer, average allelic diversity was 15, expected heterozygosity was 0.869 and the mean polymorphic information content value was 0.847. Paternity exclusion probabilities over loci were NE-1P = 0.01336 and NE-2P = 0.00135, and combined non-exclusion probability of identity was $P_{(ID)} = 3 \times 10^{-8}$.

Key words: Bovidae, Cervidae, Cross-species amplification, Microsatellite, Pampas deer

INTRODUCTION

At the beginning of the 20th century, the pampas deer (*Ozotoceros bezoarticus*) had large populations that occupied a wide range of open habitats throughout South America. However, today a few small isolated populations remain, and the pampas deer is considered one of the most endangered Neotropical species of deer (González et al., 1998). The fast development of cost-efficient tools to measure genetic diversity in populations of endangered species such as the pampas deer is needed for conservation management efforts.

Microsatellite markers are useful in assessing population structure and trends, patterns of dispersal, social organization, levels of inbreeding, population relationships, and in planning translocations (Beja-Pereira et al., 2004).

Although cloning is the most accurate technique for developing species-specific primers, the time and effort required are substantial (Galan et al., 2003). For this reason, several studies have taken advantage of the relatively highly conserved microsatellite flanking regions of domestic species. A large number of polymorphic loci have been reported and these have been applied in wild populations of phylogenetically closely related species (Galan et al., 2003; Vial et al., 2003; Maudet et al., 2004).

The objectives of the present study were: i) to test in pampas deer 15 microsatellite loci isolated from cattle, sheep and goat, that have been demonstrated to be polymorphic across other Cervids (Table 1), and ii) to characterize a small set of highly polymorphic microsatellite loci across six pampas deer populations.

Table 1. List of 15 microsatellite loci isolated from domestic species and tested in pampas deer.

Marker	Cloned on	Chromosome	*Ovis aries*	*Cervus elaphus*	*Cervus nippon*	*Gazella dorcas*	*Ammotragus lervia*	*Capreolus capreolus*	Reference
BM1818	*Bos* sp	23	-	Polymorphic	-	4	6	-	Beja-Pereira et al., 2004; Kuehn et al., 2003
BM203	*Bos* sp	27	6	8	3	4	4	-	Beja-Pereira et al., 2004; Slate et al., 1998
BM4513	*Bos* sp	14	1	12	3	-	-	Nonspecific amplification	Slate et al., 1998; Galan et al., 2003
BM757	*Bos* sp	9	3	7	2	-	-	Polymorphic	Slate et al., 1998; Galan et al., 2003
CSSM41	*Bos* sp	22	3	6	4	-	-	Polymorphic	Slate et al., 1998; Galan et al., 2003
CSSM43	*Bos* sp	27	3	11	4	-	-	Polymorphic	Slate et al., 1998; Galan et al., 2003
ETH225	*Bos* sp	9	-	Polymorphic	-	4	5	-	Beja-Pereira et al., 2004; Kuehn et al., 2003
HUJ175	*Bos* sp	22	3	5	3	-	-	-	Slate et al., 1998
INRA05	*Bos* sp	12	-	-	-	6	6	-	Beja-Pereira et al., 2004
INRA40	*Ovis* sp	2	-	-	-	6	7	-	Beja-Pereira et al., 2004
OarVH98	*Bos* sp	17	3	6	2	-	-	-	Slate et al., 1998
SR12	*Capra* sp	-	-	-	-	4	7	-	Beja-Pereira et al., 2004
SR24	*Capra* sp	Unassigned	-	-	-	4	7	-	Beja-Pereira et al., 2004
TGLA10	*Bos* sp	8	3	6	3	-	-	-	Slate et al., 1998
TGLA378	*Bos* sp	10	6	6	4	-	-	Low polymorphism	Slate et al., 1998; Galan et al., 2003

Data are reported as number of alleles found in other species with a reference to the original study.

MATERIAL AND METHODS

DNA was isolated from tissue samples according to González et al. (1998). Primers were first tested in three pampas deer samples. A single polymerase chain reaction per locus was performed with the universal fluorescent labeled M13 primer (5'[6-FAM] TGTAAAACGGCCAGT-3') following the procedure described by Schuelke (2000). We used a reaction volume of 20 µL, containing 50 ng DNA, 5 pmol of specific forward primer with its M13 (-21) sequence, 20 pmol of the universal M13 (-21) primer, 20 pmol of specific reverse primer, 1X Taq buffer with KCl, 3.25 mmol $MgCl_2$, 0.5 µg/µL BSA, 2 mM dNTPs, and 1.25 unit Taq polymerase (Fermentas).

The thermo-cycling conditions included two stages; during the first 29 cycles, the forward primer was incorporated. This stage consisted of denaturation at 94°C for 30 s, annealing at 56°C (for all of our specific primers) for 45 s, and extension at 72°C for 45 s. This was followed by 9 cycles where the universal M13 (-21) primer was incorporated in the annealing step at 53°C, and a final extension phase of 10 min at 72°C. To confirm amplification, 5 µL of each product was electrophoresed on a 2% agarose gel (Invitrogen) for 180 min. Afterward, 1 µL polymerase chain reaction product was added to 9 µL formamide/ROX solution (Applied Biosystems), electrophoresed and detected on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). Migration was performed in a 22-cm length capillary array using POP4 polymer (Applied Biosystems) with the following parameters: 15 kV, 100 amp, and 11 mW at 60°C for 35 min. Fragment size analysis was performed using the GeneScan® and Genotyper® software (Applied Biosystems).

From the 15 microsatellites tested, we discarded 10 that gave poor amplification or low levels of polymorphism and chose five loci that showed the highest levels of polymorphism and that were consistently amplified and easily scored.

In addition, one homozygote sample from each of the five loci was sequenced to verify that these loci were orthologous to the domestic species loci. Sequence data have been submitted to GenBank (accession numbers: EF571647-EF571651).

These loci were then tested in 69 DNA samples of pampas deer from six populations. We calculated heterozygosity and tested for Hardy-Weinberg equilibrium and linkage disequilibrium using GENEPOP (Raymond and Rousset, 1995). Polymorphic information content (PIC), paternity non-exclusion probability (NE-1P, NE-2P) and combined non-exclusion probability of identity ($P_{(ID)}$) were estimated with Cervus 3.0 (Kalinowski et al., 2007).

RESULTS

Tests for linkage disequilibrium between pairs of loci revealed no significant results for multiple comparisons. Hence, we conclude that the microsatellites selected can be considered independent and neutral. Of the five microsatellites, one (BM203), showed a slight deviation from Hardy-Weinberg equilibrium once we applied Bonferroni corrections for multiple comparisons (Rice, 1989). Allelic diversity of the five microsatellites across loci ranged from 13 to 20 alleles/locus (mean = 15; Table 2). Average expected heterozygosity over loci was 0.869 and the mean PIC value was 0.847. Paternity non-exclusion probability

Table 2. Characteristics of five microsatellite loci selected to screen six populations of pampas deer (*Ozotoceros bezoarticus*).

Marker	Primer sequence	N	S (bp)	Na	H(O)	H(E)	PIC	NE-1P	NE-2P
BM1818	AGCTGGGAATATAACCAAAGG AGTGCTTTCAAGGTCCATGC	50	251-279	13	0.620	0.886	0.865	0.397	0.247
BM203	GGGTGTGACATTTTGTTCCC CTGCTCGCCACTAGTCCTTC	61	241-265	13	0.885*	0.871*	0.849	0.429	0.271
BM757	TGGAAACAATGTAAACCTGGG TTGAGCCACCAAGGAACC	57	195-227	15	0.877	0.904	0.887	0.347	0.209
CSSM41	AATTTCAAAGAACCGTTACACAGC AAGGGACTTGCAGGGACTAAAACA	53	117-203	20	0.604	0.869	0.848	0.425	0.269
INRA40	TCAGTCTCCAGGAGAGAAAAC CTCTGCCCTGGGGATGATTG	55	165-197	14	0.527	0.815	0.786	0.531	0.359

*Significant deviation from Hardy-Weinberg equilibrium. N = number of individuals tested. Size (S) = allele length range. Na = number of alleles. H(O) = observed heterozygosity. H(E) = unbiased expected heterozygosity. PIC = polymorphism information content. NE-1P = non-exclusion probability (first parent). NE-2P = non-exclusion probability (second parent).

over loci were NE-1P = 0.0133 and NE-2P = 0.0013 for all populations. Combined non-exclusion $P_{(ID)}$ was 3 x 10^{-8}.

DISCUSSION

These high non-exclusion probabilities and combined non-exclusion probability provide a high confidence level when assigning paternity or individual identification with our five selected microsatellites. This set of primers proved to have sufficient levels of polymorphism and heterozygosity to provide enough power to analyze pampas deer population structure, to make inferences regarding group structure, mating strategies and evolutionary relationships among pampas deer populations. The combination of the universal fluorescent labeled M13 primer method and this small set of highly polymorphic markers proved to be a reliable, fast and cost-effective molecular ecology tool, useful in resolving conservation problems related to management programs for the endangered pampas deer.

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