Cross-species tests of 45 microsatellite loci isolated from different species of ungulates in the Iberian red deer (*Cervus elaphus hispanicus*) to generate a multiplex panel

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Abstract

The Iberian red deer (*Cervus elaphus hispanicus*) is an emblematic game species in Spain. To generate a battery of polymorphic markers for multiplex polymerase chain reactions for the Spanish red deer, 45 loci isolated in different species of ungulates were tested. Of the primers tested, 27 amplified but only 21 were polymorphic. Eleven of these markers were subsequently optimized for multiplex in four polymerase chain reactions. This allows analysing several molecular markers jointly to substantially reduce costs. Finally, we report descriptive summary statistics such as number of alleles for the former and also test of disequilibria and heterozygosity for the latter.

Keywords: *Cervus elaphus hispanicus*, cross-species amplification, microsatellite, multiplex PCRs, polymorphism

During decades, natural red deer (*Cervus elaphus L)* populations have been affected by anthropogenic activities which have caused changes in their genetic structure.

Factors such as the selective hunting for trophies, habitat fragmentation, translocations, introduction of deer from genetically distinct populations, and the keeping of isolated populations in enclosures are known to produce depletion of genetic variability within a population, increase of genetic diversity among populations, as well as introgression and changes in allele frequencies (Hart & Zachos 2003).

The Iberian red deer (*Cervus elaphus hispanicus*) is one of the most abundant and emblematic big game species of Spain. An understanding of the population genetics of the natural red deer populations is essential to achieve a sustainable management, since they provide information about their levels of diversity, integrity and effective size. Microsatellites are the most widely used molecular markers in population genetic studies because of their high polymorphism, codominance and abundance throughout the genome (Vial *et al.* 2003). In ungulates, microsatellite flanking sequences are often conserved across close species and even across families, allowing a cross-species amplification of different loci (Roed & Midthjell 1998). For studies requiring genetic characterization of many individuals at a set microsatellite loci, time and cost become serious limiting factors and thus, the development of a multiplex panel which gather the total number of markers to use in a few polymerase chain reactions (PCR) is almost essential.

The aim of this study was to get a battery of polymorphic microsatellite loci with a high-quality amplification in the Spanish red deer and to gather different markers in as few as possible PCRs. Furthermore, we aimed at loading all the selected markers (a total of 11) in a single gel lane.

Tissue samples were obtained from animals shot over three hunting season (2003–2006) from different points in southern Iberian Peninsula. Eight individuals from a natural Andalusian population were used to test a set of 45 microsatellite markers developed in either cattle, sheep, reindeer of goats, initially using a single PCR per locus (Table 1).

DNA was extracted from tongue tissue through a Hot Sodium and Tris (HotSHOT) protocol (Truett *et al.* 2000). PCR amplifications were performed in a reaction volume of 20 μL, containing 50 ng of DNA, 0.25 μm of each primer, 250 μm of dNTP, 2 mm of $MgCl_2$, 1× PCR buffer (Bioline) and 0.5 U of *Taq* polymerase (Bioline). The amplification of

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Table 1 Characteristics of 45 primer pairs tested in a sample of eight individuals to develop multiplex PCRs in the Iberian red deer. Twenty-one microsatellites were polymorphic and 11 of them were chosen because of their high polymorphism and size range

Locus (Ref)	GenBank Accession no.	Primers (5′–3′)	Amplification	Size range	N_a
BM864¶	U06764	TGGTAGAGCAATATGAAGGCC GGAAATCCAAGAAAGAGGGG	No amplification		
BL41¶	NC007301	CCTCGCCATCTTTATTCCT AAGATCAACTTATTCCTCACAGTGG	No amplification		
BL42¶	NC007311	CAAGGTCAAGTCCAAATGCC GCATTTTTGTGTTAATTTCATGC	No amplification		
BM6437¶	G18436	GAGGAAATACAGAACTCAGCCG TCAAACAGCATCTAGGCGG	No amplification		
BP1¶	NC007303	AAAATCCCTTCATAACAGTGCC CATCGTGAATTCCAGGGTTC	No amplification		
BP7¶	NC007304	GACCTTTTCACTGCCCTCTG TTTATTTCTGAGTGTTTGGGGC	Monomorphic	289	1
BM415¶	G18413	GCTACAGCCCTTCTGGTTTG GAGCTAATCACCAACAGCAAG	Polymorphic	136–168	4
BM143¶	G18387	ACCTGGGAAGCCTCCATATC CTGCAGGCAGATTCTTATCG	No amplification		
BM888¶	G18484	AGGCCATATAGGAGGCAAGCTT CTCGGTGAGCTCAAAACGAG	Polymorphic	189–219	6
BM4513¶	G18507	GCGCAAGTTTCCTCATGC TCAGCAATTCAGTACATCACCC	Polymorphic	132–146	3
BM302¶	G18774	GAATTCCCATCACTCTCTCAGC GTTCTCCATTGAACCAACTTCA	Polymorphic	140–148	4
BM848¶	G18511	TGGTTGGAAGGAAAACTTGG CCTCTGCTCCTCAAGACAC	Polymorphic	289–321	2
BM1443¶	G18438	AATAAAGAGACATGGTCACCGG TCGAGGTGTGGGAGGAAG	No amplification		
BM4505¶	G18511	TTATCTTGGCTTCTGGGTGC ATCTTCACTTGGGATGCAGG	Polymorphic	231–233	2
BM757¶	G18473	TGGAAACAATGTAAACCTGGG TTGAGCCACCAAGGAACC	Polymorphic	160–170	2
BM203¶	G18500	GGGTGTGACATTTTGTTCCC CTGCTCGCCACTAGTCCTTC	Polymorphic	216–250	6
CSSM43§	U03824	AAAACTCTGGGAACTTGAAAACTA GTTACAAATTTAAGAGACAGAGTT	Polymorphic	256–312	8
CSSM66§	AF232764	ACACAAATCCTTTCTGCCAGCTGA AATTTAATGCACTGAGGAGCTTGG	Polymorphic	170–190	6
ETH 10§	Z22739	GTTCAGGACTGGCCCTGCTAACA CCTCCAGCCCACTTTCTCTTCTC	Nonspecific amplification		
ETH152§	Z14040	TACTCGTAGGGCAGGCTGCCTG GAGACCTCAGGGTTGGTGATCAG	Monomorphic	195	1
ETH225§	AF232767	GATCACCTTGCCACTATTTCCT ACATGACAGCCAGCTGCTACT	No amplification		
RBP3§	NW001494477	GTATGATCACCTTCTATGCTTCC CCCTAAATACTACCATCTAGAAG	Polymorphic	184–188	3
SR-CSRP01‡	L22192	TGCAAGAAGTTTTTCCAGAGC TCATTCTCAGGAAACTCTGAAAC	Monomorphic	114	1
SR-CSRP05‡	L22197	GGACTCTACCAACTGAGCTACAAG TGAAATGAAGCTAAAGCAATGC	No amplification		
ILST011‡‡	L23485	GCTTGCTACATGGAAAGTGC CTAAAATGCAGAGCCCTACC	No amplification		
MAF70‡‡	M77199	CACGGAGTCACAAAGAGTCAGACC GCAGGACTCTACGGGGCCTTTGC	Nonspecific amplification		
TGLA122§	NC007319	CCCTCCTCCAGGTAAATCAGC AATCACATGGCAAATAAGTACATAC	Monomorphic	144	1

Table 1 *Continued*

Locus (Ref)	GenBank Accession no.	Primers (5′–3′)	Amplification	Size range	N_a
TGLA94*	AF288204	CGAATCTCTAGGGATTGAGACTGTG CATCAAAACAGTGAAGGATGATTGCCAG	Polymorphic	134–152	5
MB25‡‡	AB204988	GGACACGTTCTGCAGATACAACTAC GAACTCTCCTTAAGCATACTTGCTC	Polymorphic	195/203	4
JMP29‡‡	U30893	GTATACACGTGGACACCGCTTTGTAC GAAGTGGCAAGATTCAGAGGGGAAG	No amplification		
SR-CSRP24‡‡	Unpublished	AGCAAGAAGTGTCCACTGACAG TCTAGGTCCATCTGTGTTATTGC	Nonspecific amplification		
SR-CSRP26‡‡	Unpublished	ACAGAGGTGAAGAATAAGGAGAGTG GATAGTTTCAGAAGACCCAGTTGAG	Monomorphic	116	1
NVHRT16††	AF068206	ATTCTAAGCCCAAATAATCTT TCTAAGGGGTCTGTGTCTT	Polymorphic	155–159	2
$NVHRT21_{(g)}$	AF068207	GCAGCGGAGAGGAACAAAAG GGGGAGGAGCAGGGAAATC	Polymorphic	149–165	7
NVHRT48††	AF068214	CGTGAATCTTAACCAGGTCT GGTCAGCTTCATTTAGAAAC	Polymorphic	086–114	4
NVHRT66††	AF068216	GCAGAGTCCGTGGGATTG TTGGAGGCAGCTTGGCTTAT	No amplification		
NVHRT73††	AF068218	CTTGCCCATTTAGTGTTTTCT TGCGTGTCATTGAATAGGAG	Polymorphic	204–226	6
RT1**	U90737	TGCCTTCTTTCATCCAACAA CATCTTCCCATCCTCTTTAC	Polymorphic	203–217	5
RT6**	U90739	TTCCTCTTACTCATTCTTGG CGGATTTTGAGACTGTTAC	No amplification		
$RT7_{(f)}$	U90740	CCTGTTCTACTCTTCTTCTC ACTTTTCACGGGCACTGGTT	No amplification		
RT13**	U90743	GCCCAGTGTTAGGAAAGAAG CATCCCAGAACAGGAGTGAG	Polymorphic	292–312	7
RT25**	U90747	TGCCAAGGAACCAAGATGTC CATTCCAGTATTATTGCCTGA	Nonspecific amplification		
RT27**	U90748	CCAAAGACCCAACAGATG TTGTAACACAGCAAAAGCATT	Monomorphic	133	1
OarFCB193†	L01533	GCTTGGAAATAACCCTCCTGCATCCC TTCATCTCAGACTGGGATTCAGAAAGGC	Polymorphic	100–132	7
OarFCB304†	L01535	CGCTGCTGTCAACTGGGTCAGGG CCCTAGGAGCTTTCAATAAAGAATCGG	Polymorphic	124–140	5

N_a is number of alleles per locus. Ref is the source of microsatellites: *Georges & Massey (1992); †Buchanan & Crawford (1993); ‡Arevalo *et al.* (1994); §Barendse *et al.* (1994); ¶Bishop *et al.* (1994); **Wilson *et al.* (1997); ††Roed & Midthjell (1998); ‡‡Vial *et al.* (2003).

microsatellite markers was carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) using the following conditions: an initial denaturation step at 95 °C for 10 min followed by 35 cycles of 30 s at 94 °C, 1 min at 54 °C and 1 min 30 s at 72 °C and a last cycle of extension of 10 min. The PCR conditions were kept constant for each locus, to make the subsequent multiplex easier. The products of amplification were visualized and scored via gel electrophoresis and ethidium bromide staining.

Of the 45 microsatellite primers tested, 18 failed to amplify or gave nonspecific amplification products while 27 yielded a PCR product of the expected size, but only 21 of them were polymorphic (Table 1). A total of 15 markers were selected to multiplex because of their high levels of polymorphism visualized in agarose gels and the absence of dimer formation among them as detected by the oligo 6.4 software (Molecular Biology Insights).

To multiplex, we carried out a high number of tests, grouping several markers of different allelic size in a single reaction and adjusted the concentration of primers according to the signal. All other PCR conditions were kept as outlined above. Four different combinations of primers were optimized in a total of 11 microsatellite markers. F-primers of these four reactions were 5′-end

Table 2 Description of the 11 microsatellites typed on 30 Iberian red deer in Andalucia, Spain

	Locus	Primer (μm)	N_A	Size range (bp)	H_O	H_E	P_{HW}
PCR1	TGLA94	0.2	7	134–156	0.8333	0.7833	0.6664
	RT1	0.25	7	203–229	0.8667	0.8122	0.7840
	RT13	0.35	11	292–318	0.8276	0.8787	0.0393
PCR2	OarFCB193	0.25	12	092–140	0.8214	0.8807	0.1021
	MB25	0.15	5	195–209	0.4333	0.6850	0.0004
	CSSM43	0.6	14	254–334	0.9310	0.8639	0.8748
PCR3	NVHRT48	0.1	7	084–118	06000	0.6167	0. 1759
	BM302	0.25	5	140–156	0.6333	0.6861	0.4194
	NVHRT73	0.25	8	206–228	0.9000	0.7900	0.9538
PCR4	OarFCB304	0.15	8	118–142	0.7667	0.7617	0.3785
	BM203	0.4	8	208–266	0.7667	0.7678	0.6630

N_A, number of alleles detected per locus; P_{HW}, probability of Hardy–Weinberg equilibrium test; H_O and H_E, observed and expected heterozygosities, respectively.

labelled with PET, NED, FAM or VIC and amplified fragments were resolved on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems).

We analysed the genotypes of 30 individuals of a natural red deer population from Andalusia using the developed multiplex PCRs. Hardy–Weinberg (P_{HW}) and linkage tests of disequilibria were performed with the genepop version 3.4 software (Raymond & Rousset 1995) using Markov chain with 10 000 dememorizations, 1000 batches and 10 000 iterations per batch. Of the 11 microsatellites, only MB25 differed significantly from Hardy–Weinberg expectations. Tests for linkage disequilibrium between pairs of loci revealed no significant results. genetix software version 4.05 (Belkhir *et al*. 2000) was used to calculate the number of alleles per locus and the heterozygosity. The selected microsatellite markers showed high levels of polymorphism with five (MB25) to 14 (CSSM43) alelles per locus (Table 2). Average allelic diversity across loci was 8. Average expected (H_E) and observed (H_O) heterozygosities over loci were 0.7751 and 0.7618, respectively.

These PCR conditions have been thoroughly tested in several hundred samples and thus, are suitable for future studies in this species.

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