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Development of two multiplex PCRs for microsatellite analysis in Alpine chamois (*Rupicapra r. rupicapra*)

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RIASSUNTO – Allestimento di due PCR multiple per l'analisi di popolazioni di Camoscio delle Alpi (*Rupicapra r. rupicapra*) mediante marcatori microsatelliti. *Allo scopo di elaborare un protocollo per l'analisi della struttura genetica di popolazioni di camosci italiani dell'arco alpino, sono stati individuati 16 microsatelliti già utilizzati per gli ungulati selvatici e mappati su cromosomi diversi in capra, pecora e bovino. Sulla base di analisi preliminari condotte su un ristretto numero di campioni dodici marcatori sono risultati sufficientemente polimorfi nel camoscio e coamplificabili. Per automatizzare le analisi e ridurre i tempi sono state messe a punto due PCR multiple con cui sono stati analizzati 42 campioni provenienti da differenti colonie. L'elevato contenuto informativo dei marcatori coamplificati dimostra che le due PCR multiple sono strumenti utili nelle indagini di genetica di popolazione nel camoscio.*

KEY WORDS: chamois, microsatellite, multiplex PCR, DNA.

INTRODUCTION – The study of the genetic diversity gives important information about structure, subdivision in subunits and evolution of populations. Chamois (*Rupicapra rupicapra*, Linneus 1758) are mountain ungulates belonging to the subfamily *Caprinae*. They are presently distributed over most of the medium to high altitude mountains in the Southern Europe. Ten distinct geographical populations have been recognised as subspecies (Masini and Lovari, 1988); one of this subspecies, *R. r. rupicapra*, includes also the chamois living on the Italian Alps.

Microsatellites are the most suitable markers in order to obtain refined pictures of the biodiversity of species (Perez *et al.*, 2002). Indeed, they are ipervariable, uniformly distributed in the genome, and quite easy to analyse. The use of the automatic analyser limits cost and time of tests and improves the reliability of the results reducing the variability in scoring. In order to develop a tool for the genetic analysis of the Italian Alpine chamois, we tested a set of loci commonly used in cattle, sheep and goats. The purposes of this study were (1) to identify a panel of microsatellites highly informative in chamois and (2) to develop and validate multiplex protocols for ABI Prism® 310 Genetic Analyzer.

MATERIAL AND METHODS – From 42 chamois individual samples of blood or tissue were collected and preserved at -20°C. Two different kits were used to isolated DNA from tissue and blood according to the manufacturer protocols: NucleoSpin® Tissue (Macherey-Nagel GmbH, Dueren, Germany) and Invisorb Spin Blood Mini Kit (Invitek GmbH, Berlin, Germany) respectively. Sixteen dinuclotide-repeat microsatellite markers were chosen using information from literature (Table 1): they included 5 caprine, 3 ovine, and 8 bovine loci

mapped on different chromosomes. Some of them have been already used for wild ungulates (Perez *et al.*, 2000; Muntwyler *et al.*, 2002; Sacchi *et al.*, 2002). In order to combine the primers for multiplex PCR analyses, the markers were chosen on the basis of their polymorphism, allele size ranges, and possibility of co-amplifying. Loci with non-overlapping allele lengths were chosen to have the same fluorescent dye (Table 1).

Table 1. Microsatellite loci.

	Map location				Primer	
	Locus	chromosome	source	Reference	μ M	label
1°	*INRA005*	BTA12	cattle	Perez *et al.*, 2000	0.05	HEX
1°	*OarFCB304*	OAR19	sheep	Muntwyler *et al.*, 2002	0.08	6-FAM
1°	*SRCRSP05*	CHI21 OAR21	goat	Perez *et al.*, 2000	0.04	NED
1°	*SRCRSP08*	CHI unknown	goat	Perez *et al.*, 2000	0.20	NED
1°	*INRA040*	BTA2	cattle	Perez *et al.*, 2000	0.08	6-FAM
2°	*BM1258*	BTA23	cattle	Muntwyler *et al.*, 2002	0.20	6-FAM
2°	*BM1329*	OAR6 BTA6	cattle	Muntwyler *et al.*, 2002	0.25	6-FAM
2°	*BMS332*	BTA26	cattle	Stone *et al.*, 1995	0.05	NED
2°	*CSRD247*	OAR14	sheep	Muntwyler *et al.*, 2002	0.20	HEX
2°	*INRA011*	BTA1	cattle	Perez *et al.*, 2000	0.04	6-FAM
2°	*McM527*	OAR5	sheep	Muntwyler *et al.*, 2002	0.15	HEX
2°	*TGLA325*	BTAXY	cattle	George *et al.*, 1992	0.05	HEX
	INRA063	CHI18 OAR14 BTA18	cattle	Perez *et al.*, 2000		6-FAM
	SRCRSP06	CHI19	goat	Perez *et al.*, 2000		HEX
	SRCRSP14	CHI unknown	goat	Perez *et al.*, 2000		NED
	SRCRSP15	CHI unknown	goat	Perez *et al.*, 2000		6-FAM

1° = first multiplex PCR; 2° = second multiplex PCR; μM = primer concentration in multiplex mix reaction

Two multiplex PCRs were developed using the selected primers set that showed to amplify in simplex conditions and yielded polymorphic patterns in the Alpine chamois. The multiplex amplifications were prepared in 10 μl reactions containing 1 μl of genomic DNA, 50 μM of each dNTP, 1.5 mM $MgCl_2$, PCR Buffer 1X (QIAGEN SpA, Milano, Italy), and 0.4U HotStarTaq (QIAGEN SpA, Milano, Italy). Primer concentration ranged from 0.04 to 0.25 μM (Table 1). PCRs were carried out on a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems, Foster City, CA). Protocols included an initial denaturing step of 15 min at 95°C followed by 30 cycles of 30 s at 94°C, 1 min at 58°C and 1 min at 72°C. Final extension was performed at 72°C for 7min. The fragments were separated using the ABI PRISM 310 Genetic Analyzer (Applied Biosystem, Foster City, CA). The data were analysed with GeneScan® Analysis Software V3.1.2. Allele scoring was done with GenoTyper® Software V3.7. Summary statistics were obtained using Cervus V.2 (Marshall *et al.*, 1998) and GENEPOP (Raymond and Rousset, 1995).

RESULTS AND CONCLUSIONS – In a preliminary phase, simplex PCRs were carried out on 10 samples. In simplex conditions two out of the 16 markers could not be amplified (SRCRSP15 and 14) and two loci (INRA063 and SRCRSP06) showed no polymorphism, so they were discarded. The remaining 12 polymorphic markers were combined into two multiplex PCRs. Individual multiplex patterns were in accordance with the corresponding simplex patterns. The markers grouped in the same multiplex and labelled with the same fluorescent dye showed no overlapping. For nearly all loci the peak intensities showed well-balanced PCR products. "N+1" peaks, produced by the HotStarTaq, and "stutter" peaks produced by polymerase slippage during elongation did not interfere with the correct allele scoring.

The two multiplex PCRs were used to analyse the 42 samples (Table 2). A total of 114 alleles were detected

across the 12 loci. The average PIC was 0.749 (sd 0.108). The average observed number of alleles per locus was 9.5 (5÷19). The average observed and expected heterozygosity were 0.681 (sd 0.125) and 0.784 (sd 0.102), respectively. Six loci showed significant heterozygosity deficiency that could arise from non-amplifying alleles or population subdivision. In the case of INRA040 and BM1329 no amplification was obtained in 38% and in 24% of samples, respectively. These results suggest the existence of non-amplifying alleles at high frequencies in agreement with Perez *et al.* (2002). Consequently, both markers should be discarded from further investigations. At the other loci the heterozygosity deficiency is probably due to samples subdivision. In fact, the 42 individuals belonged to geographically isolated colonies.

In conclusion we identified ten microsatellite markers highly informative and easy to combine in multiplex PCRs. The developed protocols will be suitable tools to analyse genetic characteristics of Italian Alpine chamois.

Table 2. Summary statistics.

Locus	Allele Number	N. typed individuals	H(O)	H(E)	P-value	PIC
FCB304	7	42	0.762	0.785	ns	0.743
INRA005	5	42	0.667	0.680	ns	0.635
INRA040	19	26	0.731	0.926	***	0.902
SRCRSP05	7	42	0.714	0.789	ns	0.752
SRCRSP08	10	40	0.675	0.841	***	0.813
BMS332	9	42	0.762	0.745	ns	0.706
BM1258	11	42	0.595	0.863	***	0.836
BM1329	6	32	0.656	0.822	*	0.782
CSD247	14	41	0.707	0.852	**	0.826
INRA011	12	42	0.833	0.842	ns	0.812
McM527	7	42	0.333	0.538	***	0.497
TGLA325	7	42	0.738	0.731	ns	0.681

N. typed individuals = number of individual samples that gave PCR products.
H(O) = observed heterozygosity.
H(E) = Hardy-Weinberg expected heterozygosity.
P-value = probability score test performed using the GENEPOP software.
ns = not significant; * = P<0.05; ** = P<0.01; *** = P<0.001.
PIC = Polymorphism Information Content.

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