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SHORT COMMUNICATION

Microsatellite-based genotyping of MHC class II DRB1 gene in Iberian and Alpine ibex

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Abstract In an analysis of a microsatellite locus (OLADRB1) linked to the MHC DRB1 gene of Iberian and Alpine ibex (*Capra pyrenaica* and *Capra ibex*), we detected strong linkage disequilibrium between both loci. The allele length polymorphism at OLADRB1 was unambiguously linked to a particular DRB1 allele. This allowed us to develop a DRB-STR matching method for both ibex species. Validation of the DRB-STR matching method was performed in 160 Iberian ibex from Spain and 98 Alpine ibex from Switzerland and Italy. This simple and relatively inexpensive protocol may find wide applications in a variety of research areas (e.g., mate choice, pathogen-driven selection) and in the biological conservation and management of the Western European ibex populations.

Keywords *Capra ibex* · *Capra pyrenaica* · Goats · OLADRB1 · MHC DRB1 · Linkage disequilibrium · DRB-STR method

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Introduction

In vertebrates, the major histocompatibility complex (MHC) plays a central role in foreign antigen recognition and immune response to pathogens and parasites (Klein 1986; Trowsdale 1993; Bernatchez and Landry 2003). High levels of allelic diversity have been found in MHC genes (Robinson et al. 2003) making these closely linked genes some of the most polymorphic regions in the vertebrate genome (Klein 1986). The mechanisms for maintaining this extraordinary polymorphism have been hotly debated among immunogeneticists and evolutionary biologists (Hedrick and Thompson 1983; Knapp 2007). Interestingly, populations of domesticated species have also been found to have high levels of MHC variation, higher than expected given the domestication history (Vilà et al. 2005). However, many endangered species exhibit low MHC polymorphisms, caused by their severe population bottlenecks (Hedrick 1999; Mainguy et al. 2007). Low MHC variability can also originate from the social organisation of a species (Ellegren et al. 1996; Sommer et al. 2002), which may result in low transmission rates of parasites and infectious diseases (Slade 1992; Murray et al. 1995).

MHC class II genes encode cell surface glycoproteins that present extracellular foreign peptides to T cell receptors to generate an adequate immune response (Doherty and Zinkernagel 1975). These genes are usually characterized

by high levels of diversity among vertebrates (Garrigan and Hedrick 2003). Because of the high polymorphism of the MHC DRB region, routine typing is generally performed using different techniques, e.g., sequence specific oligonucleotide typing (Schwaiger et al. 1993), single strand conformational polymorphism (Kostia et al. 1998; Jugo and Vicario 2000), PCR followed by restriction fragment length polymorphism (Dutia et al. 1994; Amills et al. 1996), and 454 pyrosequencing (Babik et al. 2009). In addition, DRB cloning and subsequent sequencing often are inevitable (Nagaoka et al. 1999). These methods are powerful and have been successfully employed in several ruminant species. They are, however, laborious and/or expensive and more straightforward typing protocols are, therefore, desirable (de Groot et al. 2008).

A complex microsatellite repeat, designated DRB-STR, located in close proximity to DRB exon 2 is present in many mammalian species, including humans and primates (Andersson et al. 1987; Doxiadis et al. 2007). This microsatellite is located at the beginning of intron 2 and has a compound character (Trtkova et al. 1995; Bergstrom et al. 1999; Kriener et al. 2000). In humans and Rhesus macaques, genotyping of large samples resulted in the definition of DRB-STR patterns that were characteristic for a certain haplotype (Doxiadis et al. 2007). Associations between polymorphisms in an intronic microsatellite and the coding sequences for class II MHC genes were also reported in bovids (e.g., Sigurdardottir et al. 1991; Ellegren et al. 1993) and domestic and wild sheep (Schwaiger et al. 1993; Outteridge et al. 1996; Paterson 1998). To our knowledge, it has not yet been investigated whether similar patterns hold true in wild goats (genus *Capra*). Here we report a unique DRB-STR association in two wild *Capra* species, the Iberian (*Capra pyrenaica*) and Alpine ibex (*Capra ibex*).

Alpine and Iberian ibex share a similar history of overexploitation and severe bottlenecks. Less than 100 individuals of Alpine ibex (*C. ibex*) survived in the Gran Paradiso massif in northern Italy in the 1800s, after centuries of demographic decline mainly due to overexploitation (Couturier 1962a, b; Boitani et al. 2003). Current ibex populations found in the Alps are the result of translocations from this original source population and, to a much lesser extent, natural colonization. Reintroductions started at the beginning of the twentieth century in the Swiss Alps, while in Italy they mostly have been carried out in the last 20–30 years. Nonetheless, Alpine ibex are still absent from a significant portion of their potential distribution range, and its distribution is still scattered (Stüwe and Nievergelt 1991).

Similarly, parasite epidemics, uncontrolled hunting, and progressive destruction of natural habitats played an important role in the demographic decline of the Iberian ibex (*C. pyrenaica*), and the occurrence of severe population bottlenecks during the last two centuries is well documented (Pérez et al. 2002). Four subspecies of Iberian ibex were officially recognized (Cabrera 1911, 1914) but two of them (*Capra pyrenaica pyrenaica* and *Capra pyrenaica lusitanica*) went extinct recently. The persistent subspecies (*Capra pyrenaica hispanica* and *Capra pyrenaica victoriae*) have an allopatric distribution in the Iberian Peninsula (Manceau 1997). The aim of the present study was to develop a simple and relatively inexpensive protocol for Iberian and Alpine ibex MHC DRB1 gene genotyping based on OLADRB1 microsatellite analysis.

Materials and methods

Samples collection and DNA extraction

We collected 160 Iberian ibex samples from several Spanish populations, including both *C. p. hispanica* and *C. p. victoriae*, and 98 Alpine ibex from Switzerland and Italy in the years 2003–2008 (Table 1). Samples consisted of tissue or blood obtained from legally hunted, naturally deceased, or anesthetized animals and from collection with biopsy darts (Biebach and Keller 2009). Tissue samples were stored in 100% ethanol and blood samples in APS buffer at −20°C before genomic DNA extraction with a commercial kit (BioSprint 96 and QIAamp DNA Mini Kit; QIAGEN).

OLADRB1 microsatellite genotyping

PCR (30–36 amplification cycles depending on DNA concentration) was conducted using 2 μL DNA, OLADRB1

Table 1 The number of Iberian and Alpine ibex samples obtained from each country and geographical locality

Species	Country	Geographical locality	Samples number
C. pyrenaica hispanica	Spain	Sierra Nevada Natural Space	60
C. pyrenaica hispanica	Spain	Maestrazgo and Parque Natural de los Puertos de Tortosa y Beceite	72
C. pyrenaica victoriae	Spain	Sierra de Gredos	28
C. ibex	Switzerland	Different geographical localities	87
C. ibex	Italy	Gran Paradiso	11

Table 2 DRB1 gene and associated OLADRB1 microsatellite alleles found in Iberian and Alpine ibex

Capra species	Animal number	MHC DRB1 locus	MHC DRB1 locus frequency (%)	OLADRB1	OLADRB1 frequency (%)
C. p. hispanica	592	Capy-DRB1*1	27.60	169	27.60
		Capy-DRB1*2	5.8	159	5.8
		Capy-DRB1*3	33.57	187	33.57
		Capy-DRB1*5	16.96	172	16.96
		Capy-DRB1*6	16.07	185	16.07
C. p. victoriae	28	Capy-DRB1*1	38	169	38
		Capy-DRB1*2	48	159	48
		Capy-DRB1*3	2	187	2
		Capy-DRB1*6	12	187	12
C. ibex	98	Caib-DRB1*1	86	170, 174, 178	86
		Caib-DRB1*2	14	184	14

primers (Schwaiger et al. 1993; Paterson 1998), 0.25 μM of each primer, and 1× Multiplex PCR Master Mix (QIAGEN) in a final volume of 5 μL. Using 96-well plates, aliquots of 20 μL of formamide with LIZ size standard and 2 μL of PCR product were analyzed on an ABI 3100 Avant automated sequencer. Allele sizes and genotypes were determined using GeneMapper 3.7 (Applied Biosystems) followed by manual proofreading.

PCR amplification and sequencing of MHC DRB1 gene

The second exon of the DRB1 gene was sequenced using seminested PCR as reported by Schaschl et al. (2004). The PCR reaction mixture for PCR I (preamplification) consisted of 2 μL of gDNA, 0.25 μM of each primer (using primer pairs HL030 and HL031 for *C. ibex*, Schaschl et al. (2004), and DRB1.1 and GIo for *C. pyrenaica*, Schwaiger et al. 1993), 0.217 μM dNTP's, 1× buffer (QIAGEN), and 0.1 μL Taq Polymerase (5 U/μL) in a final volume of 10 μL. Samples were subjected to the following thermal profile for amplification in a 2720 Thermal Cycler PTC-0200 DNA Engine Thermal Cycler (Bio-Rad) or a GeneAmp PCR System 9700 (Applied Biosystems): 5 min at 94°C (initial denaturing), followed by 10 cycles of three steps of 1 min at 94°C (denaturation), 1 min at 60°C (annealing), and 90 s at 72°C (extension), before a final elongation of 5 min at 72°C. PCR blanks (reagents only) were included. We used 2 μL of the PCR product of PCR I as a template for PCR II (seminested), using primers HL030 and HL032 for *C. ibex* (Schaschl et al. 2004) and DRB1.1 and DRB1.2 for *C. pyrenaica* (Schwaiger et al. 1993), and the same PCR reaction mixture and thermal profile as in PCR I, with some modifications: the annealing temperature was modified to 65°C, and the number of cycles was increased from 10 to 25. PCR blanks (reagents only) were again included.

Sequencing reactions were carried out using the Big Dye® Terminator v1.1 cycle sequencing kit following the manufacturer's instructions (Applied Biosystems). Fragments were sequenced on an ABI 3100 Avant automated sequencer and DNA sequences were aligned and edited using the software BioEdit v.7.0.9 (Hall 1999). Alleles' inference from heterozygous sequences was carried out with the program PHASE (Stephens et al. 2001) for Iberian ibex, and by eye for Alpine ibex.

Table 3 Estimates of evolutionary divergence between Iberian and Alpine Ibex MHC-DRB1 alleles: the number of base substitutions per site from between sequences are shown

	Caib-DRB1*1	Caib-DRB1*2	Capy-DRB1*1	Capy-DRB1*2	Capy-DRB1*3	Capy-DRB1*5	Capy-DRB1*6
Caib-DRB1*1		0.038	0.038	0.042	0.038	0.043	0.041
Caib-DRB1*2	0.173		0.008	0.048	0.034	0.042	0.036
Capy-DRB1*1	0.173	0.013		0.048	0.036	0.043	0.037
Capy-DRB1*2	0.196	0.228	0.228		0.029	0.036	0.031
Capy-DRB1*3	0.173	0.152	0159	0.119		0.019	0.008
Capy-DRB1*5	0.204	0.196	0.204	0.159	0.067		0.022
Capy-DRB1*6	0.188	0.159	0.166	0.132	0.013	0.083	

Standard error estimates are shown above the diagonal and were obtained by using analytical formulas. Analyses were conducted using the Jukes–Cantor model. The rate variation among sites was modeled with a gamma distribution (shape parameter=0.53)

Molecular analyses

Microsatellite null alleles were estimated using CERVUS v.3.0 (Marshall et al. 1998). The linkage between MHC and microsatellite was evaluated by eye. The most appropriate substitution models for the data were determined by the Akaike Information Criterion (Posada and Buckley 2004) in MEGA 5.04 (Tamura et al. 2011). Estimates of evolutionary divergence between sequences were performed with the same program.

Results and discussion

Sequencing the second exon of MHC DRB1 and genotyping the OLADRB1 microsatellite locus in 160 *C. pyrenaica* and 98 *C. ibex* detected a total of seven DRB1 exon II alleles: five in Iberian ibex: Capy-DRB1*1, Capy-DRB1*2, Capy-DRB1*3, Capy-DRB1*5, and Capy-DRB1*6 (Amills et al. 2004) and two alleles in Alpine ibex: Caib-DRB1*1 (Schaschl et al. 2006) and Caib-DRB1*2 (Takada et al. 1998). Nine OLADRB1 microsatellite alleles were detected: five alleles in *C. pyrenaica* (159, 169, 172, 185, and 187) and four alleles in *C. ibex* (170, 174, 178, and 184). No microsatellite null alleles were detected in this locus ($p<0.05$). These microsatellite alleles were unambiguously linked to the corresponding DRB1 exon II alleles: each DRB1 allele had one or several uniquely associated microsatellite alleles (Table 2). The large sample size used in our study together with the low number of alleles detected allowed us to easily infer gametic association in the case of double heterozygotes, based on the data of the homozygote individuals. Thus, OLADRB1 genotyping provides an accurate and simple MHC DRB1 genotyping matching method for Iberian and Alpine ibex. Interestingly, while we found a 1:1 microsatellite to DRB1 sequence match in most cases, one DRB1 exon II allele in Alpine ibex was associated with three different microsatellite alleles (Table 2). The strong linkage we observed is probably a consequence of the close proximity of the OLADRB1 microsatellite to the DRB1 gene. In sheep, OLADRB1 is located in an intron about 30 bp 3′ to the second exon of the DRB1 gene (Schwaiger et al. 1993). It appears that a similar situation occurs in the *Capra* species studied here.

The best-fitting evolutionary model, inferred from the seven DRB1 gene alleles, was JC (Jukes Cantor model; Jukes and Cantor 1969)+G (shape parameter, $\alpha=0.53$, $-lnL=-731.164$). The evolutionary divergence among all DRB gene alleles, based on the selected model, ranged from 1.3% (between Caib-DRB1*2 and Capy-DRB1*1) to 22.8% (between Capy-DRB1*1 and Capy-DRB1*2; and between Caib-DRB1*2 and Capy-DRB1*2) (Table 3). The two Alpine ibex alleles differed by 17.3%, while the intra-Iberian ibex evolutionary divergence was 13.3%. The inter-ibex species distance (between *C. ibex* and *C. pyrenaica*) was 16.8%. Hence intra-Alpine ibex genetic diversity was bigger than that for Iberian ibex and even bigger than the inter-ibex species genetic distance (Table 3).

In this study we used a large sampling size (258 ibex) including both existing subspecies of Iberian ibex (*C. p. hispanica* and *C. p. victoriae*) throughout its distribution range in Spain, together with representative sampling from Italian and Swiss Alpine ibex, including samples from the Gran Paradiso massif (the source of all existing Alpine ibex populations). The large sample size together with the low number of DRB1 gene alleles supports the applicability of our method to the unstudied ibex populations.

The small size differences among OLADRB1 alleles (in some cases only 1 bp was detected between microsatellite alleles from both ibex species: e.g., 169 allele linked to Capy-DRB1*1 from *C. pyrenaica* and 170 allele linked to Caib-DRB1*1 from *C. ibex*) require the use of positive controls when performing PCR amplifications, to overcome the possible lack of consistency in allele sizes. This is particularly important if different sequencers and running conditions are used in different laboratories (Pasqualotto et al. 2007).

Our new DRB-STR panel in two *Capra* species provides an indirect method for MHC haplotyping without the need for multiple post-PCR manipulations of samples in cloning, sequencing reactions, and/or restriction digests. These manipulations add time and cost to sample processing and increase the possibility of human error and/or contamination. Our DRB-STR panel is particularly helpful when a large number of samples are to be analyzed, as for example in population and/or disease association studies.

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