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Low diversity in the major histocompatibility complex class II *DRB1* gene of the Spanish ibex, *Capra pyrenaica*

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During the last two centuries, the Spanish ibex (*Capra pyrenaica*) has shown a significant demographic decline as a result of the progressive destruction of its natural habitat, disease epidemics, and uncontrolled hunting. Partial sequencing of the class II MHC *DRB1* gene revealed that the Spanish ibex has remarkably low levels of genetic variation at this locus, with only six different *DRB1* alleles and an observed heterozygosity of 0.429–0.579. The rates of nonsynonymous *vs* synonymous substitutions were significantly different in the peptide-binding region ($d_N/d_S = 5.347$, $P = 0.002$), a feature that indicates that the *DRB1* gene is under positive selection. A phylogenetic analysis of the Spanish ibex and a set of domestic goat *DRB1* alleles revealed that the reported sequences represent four major allelic lineages. The limited allelic repertoire of the *DRB1* gene in the Spanish ibex is likely the direct result of the recent history of population bottlenecks and marked demographic decline of this species. A genetic survey of 13 microsatellite loci was consistent with this idea. The Spanish ibex subspecies *C. p. hispanica* and *C. p. victoriae* consistently showed considerably lower levels of microsatellite heterozygosity ($H_o = 0.184$–0.231) and allelic diversity (mean number of alleles per locus = 2–2.4) than those reported in other wild ruminants. This study demonstrates the significance of both natural selection and the demographic history of populations in determining patterns of genetic variation at MHC loci. In addition, our results emphasize the importance of locally adapted populations for the preservation of genetic diversity.
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Introduction

The major histocompatibility complex (MHC) plays an important role in the adaptive immune response of vertebrates (Trowsdale, 1993). Class II *DRA* and *DRB* genes of the MHC encode heterodimeric glycoproteins formed by α and β chains that bind and present both self and foreign peptides to helper T cells, playing a major role in T-cell recognition. The amino-acid positions of the DR-β chain, which form the peptide-binding region (PBR), have been shown to be highly polymorphic in most vertebrate species. Genetic diversity at this locus is believed to be maintained by balancing selection favouring antigen-presenting cells to bind a wide array of self and non-self-peptides, thus conferring higher resistance to infectious diseases (O'Brien and Evermann, 1988; Hughes *et al*, 1994; Hedrick and Kim, 2000).

Previous studies have shown that the demographic history of populations may affect the genetic diversity present at the MHC (Yuhki and O'Brien, 1990; Hughes and Yeager, 1998). For example, population bottlenecks and genetic drift have been shown to play a major role in shaping MHC diversity of many wild species (Mikko *et al*, 1999). Natural populations of muskox (*Ovibos moschatus*), moose (*Alces alces*), fallow deer (*Dama dama*), beaver (*Castor fiber*), Asiatic lion (*Panthera leo persica*), cotton-top tamarin (*Saguinus oedipus*) and the cheetah (*Acinonyx jubatus*), among others, have shown reduced allelic variation or even monomorphism at the MHC loci, indicating that the presence of high genetic diversity is not a universal feature of this genetic system (O'Brien *et al*, 1987; Yuhki and O'Brien, 1990; Watkins *et al*, 1991; Ellegren *et al*, 1993, Mikko *et al*, 1999). Studying closely related species with different demographic histories (eg, endangered species that have suffered drastic reductions in population size *vs* populations that have not gone through genetic bottlenecks) may provide insights into the relative importance of selective and stochastic processes shaping the genetic diversity present at the MHC.

We have undertaken a population genetic analysis of the MHC *DRB1* gene in the Spanish ibex (*Capra pyrenaica*), a wild goat species distributed throughout the South-Eastern mountains and the Gredos and Batuecas mountain ranges of Spain (Pérez *et al*, 2002). Four different subspecies of Spanish ibex (SI), *C. p. hispanica*

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(CPH), *C. p. victoriae* (CPV), *C. p. pyrenaica* (CPP) and *C. p. lusitanica* (CPL), were originally characterized on the basis of horn morphology, coat colour and other phenotypic features (Cabrera, 1911). At present, only CPH and CPV persist having an allopatric distribution in the Iberian Peninsula (Pérez *et al*, 2002). In contrast, CPL became extinct in the 19th century and the last specimen of CPP died in January 2000 (Pérez *et al*, 2002). Disease epidemics, uncontrolled hunting, overgrazing, and progressive destruction of natural habitats likely played an important role in the marked demographic decline of the SI. Although current estimated population sizes of SI are fairly large in certain areas, for example, 14 000 individuals in the Sierra Nevada population, the occurrence of severe demographic bottlenecks during the 19th and 20th centuries, with effective sizes ranging from 5 to 100 individuals, is well documented (Crampe, 1991; Manceau, 1997; Pérez *et al*, 2002).

The occurrence of recent historical population bottlenecks, increased isolation, and the drastic reduction in the SI distribution range may have had profound effects on the genetic diversity of this species. The study of MHC variation in representatives of three subspecies of the SI provides an appropriate case study for evaluating the potential effects of demographic changes on the genetic variability of the MHC. In this study, we performed PCR-RFLP and DNA sequence analyses to evaluate levels of genetic variation at the MHC class II *DRB1* gene. In addition, the genetic analysis of 13 microsatellite loci provided an independent line of evidence for assessing overall levels of genetic diversity in the SI. Results from this study are discussed in light of the evolutionary processes that may have led to the observed patterns of MHC variation in the SI.

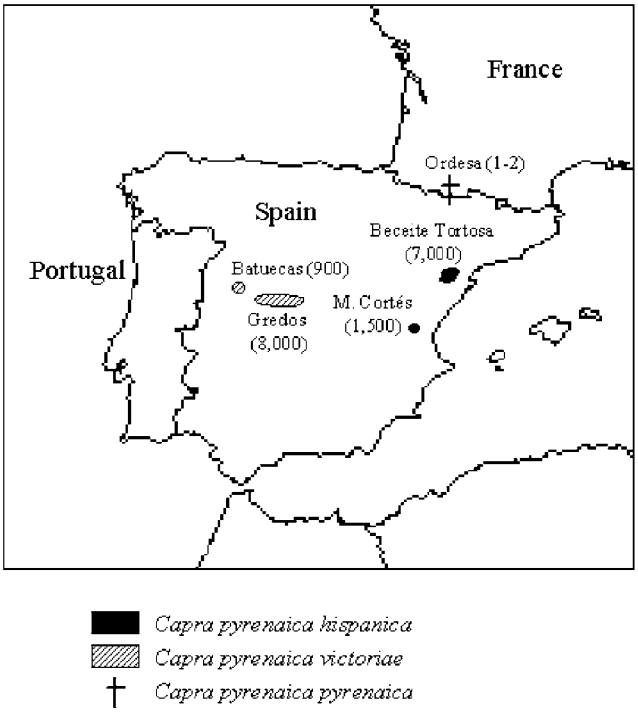

Figure 1 Range of geographic distribution of the five Spanish ibex (*Capra pyrenaica*) populations sampled in this study (Ordesa, Beceite-Tortosa, Muela de Cortés, Gredos and Batuecas). Numbers in parentheses indicate population sizes reported by Pérez *et al* (2002) and corresponding to estimates obtained in 1993–1997. The Ordesa National Park *C. p. pyrenaica* population had 1–2 individuals in 1997 but the last representative of this subspecies died in January 2000.

Materials and methods

Sample sources

Blood and tissue (liver or muscle) samples from 43 SI were used as a source of genomic DNA. Samples from SI were collected from five populations located throughout a wide range of its geographic distribution (Figure 1). These included 23 individuals of subspecies *C. p. victoriae* (CPV), 19 individuals of *C. p. hispanica* (CPH), and the last representative of *C. p. pyrenaica* (CPP).

PCR-RFLP typing and DNA sequencing of the MHC *DRB1* gene

Protocols for genomic DNA isolation from blood samples are described elsewhere (Amills *et al*, 1996a). The purification of genomic DNA from liver samples was performed by digesting 300 mg of tissue in 500 µl lysis buffer (50 mM Tris-HCl, pH 8; 20 mM EDTA, pH 8; 2% SDS; 1 mg/ml Proteinase K) overnight at 37°C. Genomic DNA was then purified through a standard phenol–chloroform extraction followed by an ethanol precipitation (Sambrook *et al*, 1989). DNA samples were re-suspended in 48 µl of sterile water and 2 µl of RNAase (10 mg/ml) for further PCR analyses.

The second exon of the *DRB1* gene was typed by using both PCR-RFLP and direct sequencing of the PCR product. Exon 2 was amplified as previously described (Amills *et al*, 1996a) and PCR products were digested at 37°C for 12 h with 5 U of *Rsa*I restriction endonuclease (Roche Diagnostics SL, Barcelona 08006, Spain). This enzyme has been previously shown to yield at least 15 different RFLP patterns in domestic goats (Amills *et al*, 1996a). Restriction fragments were electrophoresed in 3% high-resolution agarose gels allowing us to identify homozygous and heterozygous genotypes (Amills *et al*, 1996a). All distinct *DRB1.1* to *DRB1.5* PCR-RFLP-characterized alleles present in the SI were cloned and sequenced (GenBank accession numbers AF461692–AF46196). Amplified *DRB1* products were cloned using the pCR 2.1-TOPO vector (Invitrogen BV, 9704 CH Groningen, The Netherlands) following the manufacturer instructions. Each DNA clone reported in this study was sequenced forward and reverse in an ABI Prism 377 DNA sequencer (Perkin Elmer Biosystems, Foster City, CA 94404, USA) using the ABI PRISM™ Cycle Sequencing Kit (Perkin Elmer Biosystems, Foster City, CA 94404, USA). At least two independent clones were sequenced for Capy-*DRB1.1*, *-DRB1.2*, *-DRB1.3* and *-DRB1.5*, whereas a single clone was sequenced for Capy-*DRB1.4*. The sequence of the *Capy-DRB1.6* allele (Genbank accession number AY351788) was inferred from the alignment of amplified exon 2 sequences from different individuals harbouring this particular allele.

Since PCR-RFLP may underestimate the levels of genetic variation, we direct-sequenced exon 2 from each one of the individuals that had been previously typed by this method. Visual inspection of these sequences showed that each one of the RFLP patterns represented a single allele sequence.

Microsatellite typing

A total of 13 microsatellites, previously described in other ruminant species, were selected to evaluate overall levels of genetic diversity in the SI. Microsatellite loci were selected on the basis of their polymorphism, PCR amplification feasibility, and available data for comparison with other goat species (Jiménez *et al*, 1999). These were: *INRA005*, *INRA023*, *INRA032*, *INRA035*, *INRA037*, *INRA063*, *CHIAE54*, *CSSM66*, *HEL9*, *ILST005*, *ETH10*, *ETH152*, *ETH225*. Primer identification, PCR conditions and amplification profiles for each microsatellite locus are described elsewhere (Brezinsky *et al*, 1993; Kaukinen and Varvio, 1993; Steffen *et al*, 1993; Toldo *et al*, 1993; Barendse *et al*, 1994; Vaiman *et al*, 1994; Amills *et al*, 1996b). Fluorescent genotyping was performed in an ABI Prism 310 automated DNA sequencing system (Perkin Elmer Biosystems, Foster City, CA 94404, USA).

Phylogenetic analysis of the *Capy-DRB1* sequences and calculation of the number of synonymous *vs* nonsynonymous substitutions

The DNA sequences of the SI *DRB1* alleles were aligned using the Clustal-X multiple sequence alignment program (Thompson *et al*, 1997). The MEGA 2.1 program (Kumar *et al*, 1993) was used to estimate relative rates of nonsynonymous (d_N) and synonymous substitutions (d_S) according to Nei and Gojobori (1986), using Jukes and Cantor's (1969) correction for multiple hits. To test whether positive selection was operating at this locus we compared the relative abundance of synonymous and nonsynonymous substitutions using a *Z*-test (Nei and Kumar, 2000). The variance of d_N and d_S was estimated using the bootstrap method implemented in MEGA, with 500 replications (Schneider *et al*, 2000). To estimate substitution rates in the domestic goat (*Capra hircus*) we selected all *DRB1* gene sequences currently deposited in GenBank and identified 47 alleles with distinct sequences at the amplified region (GenBank accession numbers: AF376809-813, AB008345-362, U00183-203, X83367, CAZ92717-725).

MEGA was also used to perform phylogenetic analyses of the SI *DRB1* alleles reported in this study and a set of domestic goat *DRB1* sequences (see Figure 2 for GenBank accession numbers). Two independent phylogenetic methods (neighbour-joining and maximum parsimony) were used to confirm the reliability of the observed phylogenetic patterns. The neighbour-joining tree was constructed using Jukes and Cantor (1969) genetic distances. Bootstrap resamplings of 1000 multiple data sets provided estimates of relative confidence for nodes in our phylogenetic trees.

Population data analyses

Both *DRB1* and microsatellite allele and genotype frequencies were estimated by direct counting, and observed (H_o) and expected (H_e) heterozygosities and levels of genetic differentiation among populations (F_{ST}) were estimated using the BIOSYS-2 software (Swofford and Selander, 1999). Population structure was analysed by means of F-statistics using the variance based method described by Weir and Cockerham (1984) and implemented in the FSTAT computer program (Goudet, 2000). In order to test the significance of the different statistics for the null hypothesis of no differentiation at the corresponding level, permutation and resampling tests (jacknifing and bootstrapping) were carried out as implemented in the programs mentioned above.

The F_{ST} values among pairs of populations were calculated using the GENEPOP program (Raymond

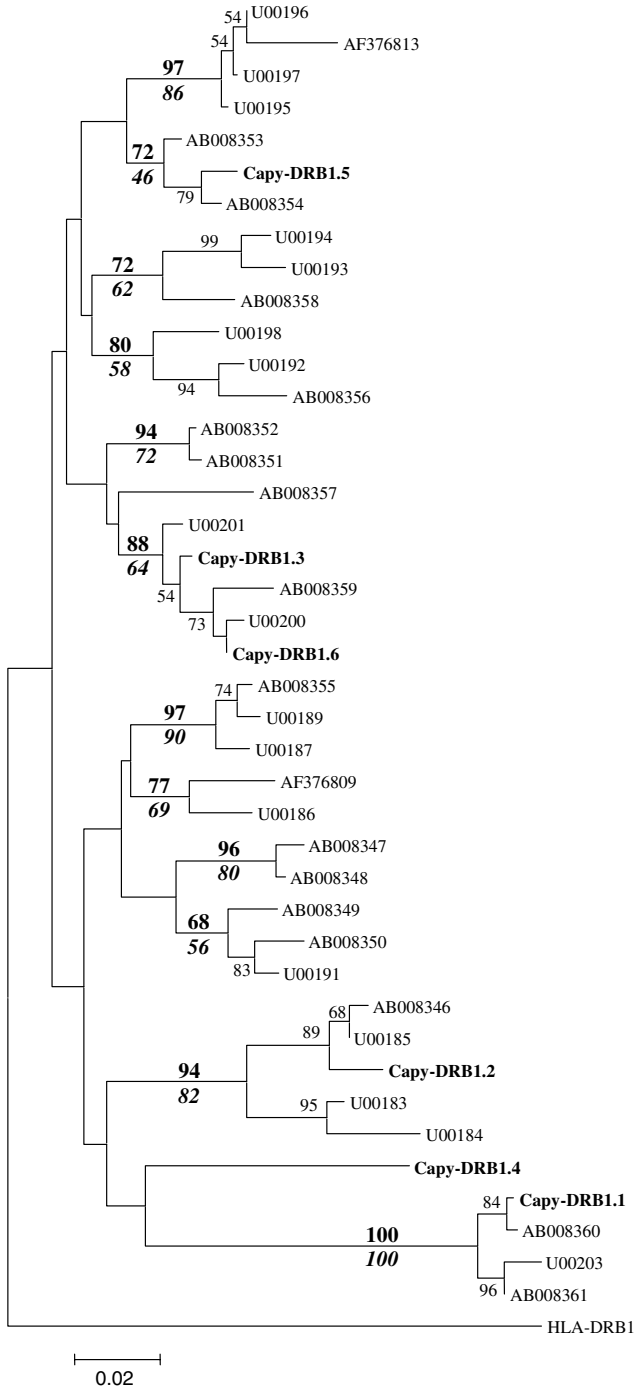

Figure 2 Neighbour-joining phylogenetic tree of the six *Capy-DRB1* sequences from the Spanish ibex *Capra pyrenaica* (in bold) and selected sequences from the domestic goat (*Capra hircus*). Numbers in bold represent bootstrap values for 12 major *DRB1* allelic lineages. Regular font and italics indicates bootstrap values from the neighbour-joining and maximum parsimony analyses, respectively. Domestic goat sequences are indicated by GenBank accession numbers. The human *HLA-DRB1* sequence was used as an outgroup.

and Rousset, 1995). In addition, GENEPOP was used to quantify the effects of migration on genetic structure and population subdivision. Gene flow was estimated between each pair of populations by converting F_{ST} to amount of gene flow (N_em), according to an island model under neutrality and negligible mutation (Slatkin, 1993). N_em indicates the average number of effective migrants per generation to produce the observed F_{ST} under the *n*-island model.

Phylogenetic trees among populations were produced by using the distance measure D_A (Nei *et al*, 1983). Takezaki and Nei (1996) suggested that the D_A distance is particularly suitable for constructing phylogenetic trees when the most critical issue is the topology of the tree rather than evolutionary time. Distance data were analysed with the neighbour-joining method of clustering (Saitou and Nei, 1987). The robustness of the dendrogram was evaluated by bootstrap resampling of loci (1000 replicates). All these calculations were carried out using DISPAN (Ota, 1993).

Results

Genetic characterization of the MHC *DRB1* gene in the Spanish ibex

PCR-RFLP and sequence analyses of the *Capy-DRB1* gene in three subspecies of the SI allowed us to detect six distinct alleles (Genbank accession numbers AF461692-AF46196 and AY351788). One of these *DRB1* alleles (*Capy-DRB1.6*) was found only in CPV whereas two other alleles (*Capy-DRB1.4* and *Capy-DRB1.5*) were solely detected in the only representative individual of subspecies CPP. The observed heterozygosity (H_o) for the *DRB1* gene in the SI subspecies ranged from 0.429 (CPV) to 0.579 (CPH) (Table 1).

We sequenced exon 2 of the *DRB1* gene from five SI displaying the six RFLP-characterized alleles in this species. In total, 55 (23.21%) of the 237 nucleotides sequenced were variable in the *Capy-DRB1* gene. These resulted in 30 (37.97%) variable amino-acid positions from a total of 79 amino acids. A total of 20 variable amino-acid positions were located at the peptide-binding region (PBR) of the DRβ molecule (Brown *et al*, 1993). The rate of nonsynonymous (d_N) and synonymous (d_S) substitutions for the PBR and non-PBR amino-acid positions, and the ratio of d_N/d_S are shown on Table 2. In the SI, the PBR region showed significantly higher rate of nonsynonymous *vs* synonymous substitutions, with a ratio d_N/d_S significantly larger than one ($d_N/d_S = 5.347$; $P = 0.002$). In contrast, the rates of nonsynonymous and synonymous substitutions at no-PBR sites were not significantly different ($d_N/d_S = 0.778$; $P = 0.550$). The analysis of the *DRB1* domestic goat sequences showed similar trends. The 47 DRB1 distinct sequences showed lower levels of amino-acid sequence divergence (18.4%), with a positive and significant d_N/d_S ratio at the PBR region ($d_N/d_S = 3.566$; $P = 0.003$) and no significant differences at sites not involved in antigen recognition (Table 2).

Table 1 Estimates of allelic diversity (N_a, number of alleles per locus), and observed (H_o) and expected (H_e) heterozygosities for the *Capy-DRB1* gene in *C. p. hispanica* (Muela de Cortés and Beceite-Tortosa populations) and *C. p. victoriae* (Gredos and Batuecas populations)

Population	N	N_a	H_o	H_e
C. p. hispanica	19	3	0.579	0.562
Muela de Cortés	3	3	1.000	0.733
Beceite-Tortosa	16	2	0.500	0.516
C. p. victoriae	21	4	0.429	0.659
Gredos	12	4	0.500	0.707
Batuecas	9	4	0.333	0.575
C. p. pyrenaica	1	2	1.000	1.000

N indicates the number of sampled ibexes for each population and subspecies.

Table 2 Rates of nonsynonymous (d_N) and synonymous (d_S) substitutions for positions at the peptide-binding region (PBR), other regions not involved in antigen binding (No-PBR), and the complete sequence (All) of the *DRB1* gene

	d_N	d_S	d_N/d_S	*P*
Spanish ibex				
PBR	0.385 (0.103)	0.072 (0.049)	5.347	0.002
No-PBR	0.056 (0.015)	0.072 (0.024)	0.778	0.550
All	0.134 (0.025)	0.075 (0.022)	1.787	0.063
Domestic goat				
PBR	0.271 (0.066)	0.076 (0.033)	3.566	0.003
No-PBR	0.057 (0.014)	0.060 (0.021)	0.950	0.879
All	0.110 (0.021)	0.065 (0.018)	1.692	0.045

Numbers in parentheses indicate standard deviations. The statistical significance of the test is shown as *P*.

Figure 2 shows a neighbour-joining phylogenetic tree with the six *Capy-DRB1* alleles and 35 *DRB1* sequences representing the major *DRB1* allelic lineages from the domestic goat. The *Capy-DRB1* sequences reported for the SI represent at least four (possibly five) major allelic lineages. These lineages are characterized by the relatively high bootstrap values (using both neighbour-joining and maximum parsimony) clustering together each *Capy-DRB1* sequence with other sequences from domestic goats (Figure 2). Our phylogenetic analysis did not show support for the basal branching order among the characterized allelic lineages, which showed low bootstrap values (<50%) in the neighbour-joining and parsimony analyses. One of the SI *DRB1* alleles (*Capy-DRB1.4*) did not show a consistent grouping with any of the characterized allelic lineages.

Microsatellite analysis

We analysed the polymorphisms of thirteen microsatellite loci in the SI. Average observed heterozygosities were 0.184 and 0.231 for CPV and CPH, respectively (Table 3). When we made the same analysis of genetic diversity excluding monomorphic microsatellites, average observed heterozygosities raised to 0.266 (CPV) and 0.333 (CPH). These values are remarkably low compared to those reported for microsatellites in other domestic goats and in mammal species in general. Moreover, the last representative of the CPP subspecies was homozygous for all 13 microsatellite loci analysed. A significant deficit in the frequency of heterozygotes was observed for five of the nine analysed polymorphic microsatellites, with a mean estimate of $F_{IS} = 0.268 \pm 0.108$ ($P < 0.001$, Table 4).

Table 3 Mean allelic diversity (N_a) and observed (H_o) and expected (H_e) heterozygosities for 13 microsatellite loci in *C. p. hispanica* (Muela de Cortés and Beceite-Tortosa populations) and *C. p. victoriae* (Gredos and Batuecas populations)

Population	N	N_a	H_o	H_e^a
C. p. hispanica	18	2.4±0.4	0.231±0.073	0.336±0.084
		3.0±0.4	**0.333±0.084**	**0.485±0.079**
Muela de Cortés	5	1.7±0.2	0.169±0.059	0.253±0.070
		2.0±0.2	**0.244±0.073**	**0.365±0.075**
Beceite-Tortosa	13	2.1±0.3	0.254±0.086	0.299±0.078
		2.6±0.3	**0.368±0.103**	**0.432±0.078**
C. p. victoriae	23	2.0±0.3	0.184±0.062	0.237±0.060
		2.4±0.2	**0.266±0.076**	**0.342±0.056**
Gredos	14	2.0±0.3	0.192±0.065	0.268±0.063
		2.4±0.2	**0.278±0.078**	**0.387±0.054**
Batuecas	9	1.7±0.2	0.171±0.074	0.183±0.062
		2.0±0.2	**0.247±0.098**	**0.264±0.074**

[a]Unbiased estimate (Nei, 1978).
The number of sampled individuals is indicated as *N*. Since four of the 13 microsatellites were monomorphic, a second analysis that only included the nine polymorphic microsatellites was performed (values indicated in bold).

Table 4 Wright *F*-statistics for nine polymorphic microsatellite loci in two Spanish ibex subspecies (*C. p. hispanica* and *C. p. victoriae*)

Locus	$F_{IS}=f$	$F_{IT}=F$	$F_{ST}=\theta$
ETH 152	0.405***	0.548***	0.240***
ETH 10	−0.133	−0.022	0.098*
HEL 9	1.000***	1.000***	−0.050
ETH 225	1.000***	1.000***	−0.044
CSSM 66	0.181*	0.461***	0.341***
INRA005	0.308*	0.386**	0.112*
CHIRAE 54	0.045	0.368***	0.338***
INRA 023	0.152	0.228**	0.160***
INRA 032	−0.024	0.066	0.088*
Mean estimates +	0.268 (0.108)***	0.409 (0.079)***	0.196 (0.050)***

f, Within-population inbreeding estimate; *F*, total inbreeding estimate; *θ*, measure of population differentiation.
+ Mean estimates obtained by jackknifing over loci. Standard deviations are indicated in parentheses.
*$P<0.05$, **$P<0.01$, ***$P<0.001$, from permutation tests as implemented in the FSTAT program.

Table 5 F_{ST} estimates (below the diagonal) as a measure of genetic distance between *C. p. hispanica* (CPH, Muela de Cortés and Beceite-Tortosa populations) and *C. p. victoriae* (CPV, Gredos and Batuecas populations) and number of effective migrants per generation (N_em) (above the diagonal)

Population	*Muela de Cortés*	*Beceite-Tortosa*	*Gredos*	*Batuecas*
Muela Cortés	—	0.640	0.793	0.692
Beceite-Tortosa	0.281	—	0.809	0.602
Gredos	0.240	0.236	—	31.93
Batuecas	0.265	0.293	0.008	—

The levels of genetic differentiation between CPV and CPH were remarkably high with a mean value of $F_{ST}=0.196\pm0.05$ ($P<0.001$, Table 4). Moreover, when we made the pairwise comparison of four SI populations from Gredos (CPV, $N=14$), Batuecas (CPV, $N=9$), Muela de Cortés (CPH, $N=5$), and Beceite-Tortosa (CPH, $N=13$) the high F_{ST} values were indicative of a high degree of population structure (Table 5). The only exception to this general trend was represented by the pairwise comparison of the Gredos *vs* Batuecas populations, which displayed an F_{ST} value of 0.008 and an N_em estimate of approximately 31.93 (Table 5). This result is consistent with the neighbour-joining phylogenetic analysis of the SI subspecies and populations based on the polymorphisms of the *Capy-DRB1* gene and 13 microsatellites, clustering the Gredos and Batuecas populations (Figure 3).

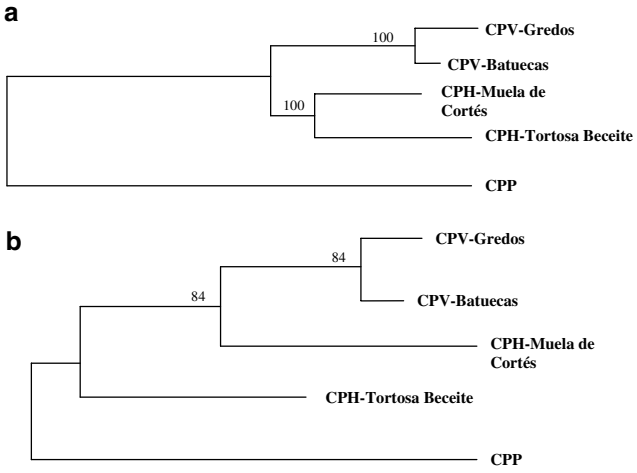

Figure 3 Dendrogram showing the genetic relationships among the *C. p. hispanica* (Muela de Cortés and Beceite-Tortosa populations), *C. p. victoriae* (Gredos and Batuecas populations) and the last representative of the *C. p. pyrenaica* subspecies, according to the polymorphism of (**a**) the *Capy-DRB1* gene and (**b**) a panel of 13 microsatellites. The phylogenetic trees were constructed by using the neighbour joining method and the D_A genetic distances. The consistency of the dendrogram was evaluated by bootstrap resampling of loci (1000 replicates).

Discussion

The genetic analysis of the MHC *DRB1* gene reported in this study demonstrates that the SI, a wild goat species from the Iberian Peninsula, consistently showed low levels of genetic diversity at this locus with only six *Capy-DRB1* alleles. Observed and expected heterozygosities were comparable to those reported in other bottlenecked wild species such as bontebok (*Damaliscus pygargus pygargus*, $N=44$ individuals, $H_o=0.51$, $H_e=0.71$) and the Norwegian and Swedish moose (*Alces alces*, $N=218$ individuals, $H_e=0.76$–0.79) (Mikko *et al*, 1999; Van der Walt *et al*, 2001) (Table 1). With regard to allelic diversity, the number of *Capy-DRB1* alleles identified by PCR-RFLP analysis and direct sequencing was consistently lower in CPV and CPH ($N=40$ individuals, four alleles) than in reindeer (*Rangifer tarandus*, $N=60$ individuals, 11 alleles), North American bison (*Bison bison*, $N=20$ individuals, nine alleles) and bontebok ($N=44$ individuals, six alleles), which are wild ruminant species that have passed through known demographic bottlenecks and consequently have restricted diversity at their MHC loci (Mikko *et al*, 1999; Van der Walt *et al*, 2001).

As MHC class II genes are fundamental for the immune response of organisms against pathogenic and parasite infections, the presence of specific MHC allele variants in the SI may be the result of natural selection favouring host resistance to infectious diseases. DNA sequence analyses of the six *DRB1* alleles in the SI revealed a significantly higher rate of nonsynonymous *vs* synonymous substitutions in the PBR region of the DRβ protein, which is responsible for the binding and presentation of extracellular peptides to T cells (Table 2). A ratio of d_N/d_S greater than one suggests that variation at the antigen-binding sites is under positive selection, probably for the recognition of a wide range of infectious agents (Hughes and Yeager, 1998). Our phylogenetic analyses showed that the *Capy-DRB1* sequences from the SI were widely distributed throughout the *DRB1* gene tree, clustering consistently with sequences from the domestic goat, rather than grouping together with each other. This suggests that the *DRB1* alleles currently present in the SI have originated prior to the divergence of this species and the domestic goat about 1–6 MYR ago (Manceau, 1997).

We believe that the reduced *Capy-DRB1* allelic repertoire we have observed is the direct result of the recent history of population bottlenecks and marked demographic decline that the SI experienced during the last two centuries (Pérez *et al*, 2002). Although current estimates of population sizes for the extant subspecies indicate the existence of several nuclei with thousands of individuals (Beceite-Tortosa, Muela de Cortés, Gredos, Cazorla, Sierra Nevada and others), historical records indicate that most populations have passed through demographic bottlenecks of less than 100 individuals (Manceau, 1997, Pérez *et al*, 2002).

A genetic survey of 13 microsatellite loci provided independent evidence demonstrating that low genetic variation is not only circumscribed to the MHC. Contrary to reported microsatellite data from domestic goat breeds, which displayed 5.6–6.8 alleles per locus and mean expected heterozygosities of 0.57–0.79 (Luikart *et al*, 1999; Saitbekova *et al*, 1999; Li *et al*, 2002), the SI subspecies showed considerably low levels of both heterozygosity and allelic diversity (Table 3), suggesting that the demographic decline of the SI resulted in an overall reduction of genetic diversity at the genome level. Observed ($H_o = 0.266$–0.333) and expected ($H_e = 0.342$–0.485) heterozygosities become higher if monomorphic microsatellites are excluded from the analysis of genetic diversity, but they are still remarkably small. These values are very similar to the ones reported for other endangered species with a similar demographic history of genetic bottlenecks, such as the Alpine ibex (*Capra ibex*, Maudet *et al*, 2002) and the fallow deer (Poetsch *et al*, 2001). Allelic diversity was also very limited, with four microsatellites that were monomorphic in both subspecies. Moreover, four (CPH) and six (CPV) of the remaining polymorphic microsatellites were biallelic (data not shown). The mean F_{ST} value for the 13 microsatellite markers was 0.196, indicating a significant degree of genetic differentiation between CPV and CPH (Table 4). The calculation of the F_{ST} values and the number of effective migrants per generation (N_em) in four CPV and CPH populations made evident the existence of population subdivision, although these data should be interpreted with caution due to the small sample size per population (Table 5). Interestingly, the occurrence of significant gene flow between the Gredos and Batuecas populations was detected (Table 5). This result is consistent with the geographical proximity of these populations (Figure 1) and the phylogenetic analysis of the SI populations based on the polymorphisms of the *Capy-DRB1* gene and 13 microsatellites (Figure 3).

Results from this study emphasize the importance of both natural selection and the demographic history of populations in determining patterns of genetic variation at MHC loci. Sequence analysis of the SI mitochondrial control and cytochrome *b* gene regions by Manceau *et al* (1999) demonstrated that the level of divergence between CPP and the other two extant SI subspecies was almost as high as the divergence between the SI and the Alpine ibex. We hypothesize that the recent extinction of the CPP subspecies might have involved a marked reduction in the allelic pool of the *Capy-DRB1* and other MHC genes. Moreover, it may have led to the extinction of certain MHC alleles that were specific to this subspecies. Since reduced MHC variation has been previously associated with higher susceptibility to infectious diseases in some cases (Potts *et al*, 1994; Paterson *et al*, 1998; Hedrick and Kim, 2000), we may expect consequent effects on fitness, particularly if populations become exposed to disease epidemics. Genetic analyses of *Capy-DRB1* sequences from museum specimens or samples from preserved bones would be necessary to directly assess historical levels of MHC variation in the SI and, thus, evaluate the relative role that demographic changes may have played in determining existing levels of genetic diversity at the MHC.

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