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Reduced major histocompatibility complex class II polymorphism in a hunter-managed isolated Iberian red deer population

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Abstract

The major histocompatibility complex (MHC) contains the most variable functional genes described in vertebrates. Individuals from natural populations deal constantly with a diverse range of pathogens and the polymorphism at MHC loci is what determines the diversity of foreign antigens that the host immune system can recognize. Polymorphism at individual loci may result in variable MHC class II (MHC-II) haplotypes. We characterized for the first time, the allelic diversity at the second DRB locus of the MHC-II in the Iberian red deer *Cervus elaphus hispanicus*. The studied population was sampled from a fenced estate that has been managed for hunting purposes and may provide information of the effect of game management on the genetic diversity of this species. Deer presented high levels of variation at MHC-II DRB-2 with 18 different alleles detected in 94 individuals. However, a significant heterozygous deficiency was found for MHC-II DRB-2 locus (92.5% of individuals only amplified one allele), whereas genotype frequencies at three neutral microsatellite loci were according to Hardy–Weinberg equilibrium, with heterozygosity over 50%. The analysis of control Iberian red deer from different geographic locations identified two expressed DRB-2 loci with a high degree of heterozygosis. The annual diversity index of MHC-II DRB-2 alleles significantly decreased along the 16-year study period, which was confirmed with losses in microsatellite markers. Although we cannot exclude positive non-assortative mating and/or substructured breeding (Wahlund effect) within our reference population, such unexpected apparent homozygosity at MHC DRB-2 loci is suggestive of null alleles occurring at our population. The observed pattern could be the result of a founder effect in this recently established population. Nonetheless, the loss of MHC-II DRB-2 allele diversity could reflect the effects of inbreeding in this fenced population managed for hunting. These findings support the importance of immunogenetic studies to assess management decisions, especially in isolated ungulate populations.

Introduction

The major histocompatibility complex (MHC) is a multigene family of the vertebrate immune system comprising highly polymorphic loci (Klein, 1986). The primary role of the MHC is to recognize foreign proteins, present them to specialist immune cells and initiate an immune response. MHC genes encode cell surface glycoproteins that bind and present antigenic peptides to T cells. The MHC class I (MHC-I) genes are expressed on the surface of all nucleated somatic cells and play an essential role in the immune defence against intracellular pathogens by presenting endogenously derived peptides to CD8+ cytotoxic T cells (Klein & Horejsi, 1997). MHC class II (MHC-II) genes are expressed on antigen-presenting cells of the immune system and present processed exogenous antigens to CD4+ T helper cells.

The MHC contains the most variable functional genes described in vertebrates (Piertney & Oliver, 2006). The extensive polymorphism and unusual persistence of alleles at the MHC loci suggests the action of balancing selection, that is, natural selection works to maintain genetic polymorphism at these loci. Individuals from natural populations deal constantly with a diverse range of pathogens and

the polymorphism at MHC loci is what determines the diversity of foreign antigens that the host immune system can recognize to subsequently trigger a specific immune response. Polymorphism at individual loci may result in variable MHC-II haplotypes. Recent studies of wild vertebrates suggest further that specific MHC-II genotypes confer resistance to a variety of pathogens (Hedrick, Kim & Parker, 2001). In addition to the MHC function in immune response, a large number of studies have reported associations between genetic variation at MHC loci and life-history traits (Finch & Rose, 1995; Lochmiller, 1996; Von Schantz *et al*., 1996) and behavior (Penn & Potts, 1999) of individuals in natural populations. Therefore, the study of MHC polymorphism has become relevant in ecology and conservation.

Wildlife management for hunting has diverse impacts on the ecology and genetics of vertebrates. It may cause a loss of genetic variation and may lead to short-term reduction of fitness components (Keller & Waller, 2002; Altizer, Harvell & Friedle, 2003). Selective harvests in particular, including trophy hunting, can have important implications for sustainable wildlife management if they target heritable traits. This has been shown in bighorn sheep *Ovis canadensis*, where declines in mean breeding values for weight and horn size occurred in response to unrestricted trophy hunting (Coltman *et al*., 2003).

The Iberian red deer *Cervus elaphus hispanicus* is a subspecies of red deer inhabiting the Iberian Peninsula. Many red deer populations from south-central Spain have been managed during the last decades for hunting purposes. Interventions have included fencing, isolation and subsequent disruption of the interconnectivity among populations, along with supplementary feeding and selective hunting. In spite of the current high densities of this game species, such management schemes have probably affected its genetic diversity leading to increased population genetic clustering and to reduced effective population sizes (Slate *et al*., 2000; Martinez *et al*., 2002; Zachos *et al*., 2007). Therefore, humans might be selecting unknowingly for rapid changes in relationships between Iberian red deer and its pathogens (Altizer *et al*., 2003). In this context, it is important to investigate the genetic diversity of genes that can mediate disease resistance as a reduction in the degree of variation in such genes may have a particularly adverse effect on population viability and conservation.

An isolated population of Iberian red deer managed for hunting purposes in south-central Spain was selected to evaluate the potential effect of hunting management schemes on the genetic diversity of a relatively recent founded population. This reference deer population has been studied by our group over the last 16 years and the population dynamics are well known (Landete-Castillejos *et al*., 2004; Vicente, Fierro & Gortazar, 2005). The MHC-II DRB-2 locus was selected for genetic analysis because it composes part of the functional important peptide binding groove, which has been shown to be the most polymorphic part in MHC-II genes (Klein, 1986) and its polymorphisms have been related to variation in parasite burdens in deer (Ditchkoff *et al*., 2005).

Materials and methods

Study site and Iberian red deer population

The study was conducted in a 900 ha hunting estate in the province of Ciudad Real, south-central Spain (38°55′N, 0°36′E; 600–850 m a.s.l.). The habitat is composed of Mediterranean scrublands (mainly evergreen oak *Quercus ilex*) with scattered pastures and small crops (Vicente *et al*., 2004; Acevedo *et al*., 2007). The climate is Mediterranean and most annual rainfall is concentrated between September and May. The dry season is characterized by high temperatures. Deer numbers were estimated yearly through repeated direct counts at the feeding places at the end of July and during the rutting season and resulted in a mean ± SD of 306 ± 27 individuals with a mean density of 0.35 individuals ha^{-1}. The sex ratio was 1 to 1.3 hinds per male in September and the population density moderately increased during the study period (2000–2006) from 0.30 individuals ha^{-1} in 2000 to 0.43 individuals ha^{-1} in 2006.

Deer reproduce naturally in the estate and should be regarded as one single population where no introduction of individuals has been performed since 1991. In 1989, the estate had five females and two calves. Between 1989 and 1991, 126 individuals (85 females and 41 males) were introduced from three different locations. Management schemes for hunting purposes in the estate include fencing in order to restrict movement of wildlife, artificial water holes, supplemental feeding in troughs and selective hunting of a mean of 80 ± 13 deer each year (8.89 per square km), mostly by the same single hunter. Criteria for selective shooting of deer included obvious poor condition in both sexes, lack of a fawn in females and poor trophy characteristics in males, for example, short spikes in yearlings or lack of second tines in stags (Fierro *et al*., 2002). Prime males were shot as trophies only after participating in several rutting seasons.

Sample collection and DNA extraction

Samples were collected in the study site from 94 selectively harvested Iberian red deer (34 females and 60 males) between 2000 and 2006, during the main hunting season from October to February. This sample represented *c*. 30% of the population living in the estate. Lymph node tissue fragments of *c*. 2 cm^3 were prepared and stored at −80 °C for DNA extraction and genetic analyses. Genomic DNA was extracted from mesenteric lymph node samples using Tri Reagent (Sigma, St Louis, MO, USA) by following manufacturer's recommendations.

DNA was extracted from the lower jawbone remains of six deer introduced and hunted before 1992 in the study site. DNA was extracted from 2 cm^2 jaw sections following the procedure described by Lleonart *et al*. (2000) for human remains.

A control red deer population, which included 13 Iberian red deer from different geographic locations, was used to validate the method applied for DRB-2 polymorphism

analysis. DNA was extracted from semen samples frozen in liquid nitrogen using the same procedures described above.

Single-stranded conformation polymorphism (SSCP) analyses

The DRB-2 locus was amplified by polymerase chain reaction (PCR) using primers LA31 and LA32 (Sigurdardottir *et al*., 1991) in a 50 μL volume PCR [1.5 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphate (dNTP), 1X avian myeloblastosis virus (AMV)/*Thermus flavus* (*Tfl*) reaction buffer, 5 U *Tfl* DNA polymerase] using the Access RT-PCR system (Promega, Madison, WI, USA). Reactions were performed in an automated DNA thermal cycler (Techne, Cambridge, UK) PCR machine (model TC-512) for 35 cycles. After an initial denaturation step of 2 min at 95 °C, each cycle consisted of a denaturing step of 1 min at 95 °C, an annealing for 30 s at 50 °C and an extension step of 1 min at 68 °C. Negative control reactions were performed with the same procedures, but adding water instead of DNA to monitor contamination of the PCR. The allelic diversity of DRB-2 was characterized using a modification of the SSCP analysis (Van Den Bussche, Hoofer & Lochmiller, 1999), without isotopic labeling of PCR products and using gel silver staining (Amersham Biosciences, Uppsala, Sweden).

A subsample of the heterozygous and homozygous individuals identified by SSCP and the deer introduced and hunted before 1992 was also analyzed by fluorescent SSCP (F-SCCP). Samples were prepared for F-SSCP following the procedure of Lento *et al*. (2003) using FAM-labeled primers and the ABI 310 manual (Applied Biosystems Inc., Foster City, CA, USA). A 2 μL aliquot of a 1:5 dilution of the *DQB* amplicon in ddH_2O was mixed with 10.5 μL deionized formamide (Gibco, Invitrogen, Carlsbad, CA, USA), 0.5 μL of freshly made 0.3 N NaOH and 1.0 μL GeneScan-500 TAMRA dye standard (ABI Prism, Applied Biosystem Inc., Foster City, CA, USA). The samples were denatured at 96 °C for 2–4 min and then snap-cooled on wet ice slurry for 2–4 min before electrophoresis on an ABI 310 automated capillary sequencer running 3% GeneScan (ABI Prism) polymer in 1 × TBE. Runs were conducted for 18 min and the heating mantle set at a constant temperature of 29 °C. SSCP peak profiles were analyzed using ABI 310 data collection and peak scanner software (v1.0). The relative position of each SSCP peak was calculated with peak scanner by standard interpolation between peaks of the TAMRA standard run in each lane. As in manual SSCP, this relative position does not represent an absolute size of the fragment and was used only to identify unique alleles across runs. The F-SSCP profile was analyzed on both strands by comparing the results obtained with forward- and reverse-labeled primers.

Sequence analyses

Two SSCP conformations were cloned for subsequent sequence analysis. Amplified fragments were resin purified (Wizard, Promega) and cloned into the pGEM-T vector (Promega) for sequencing both strands by double-stranded dye-termination cycle sequencing (Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University). At least five independent clones were sequenced for each cloned allele.

For phylogenetic analysis of the 18 Iberian red deer DRB-2 alleles (*CeelHap*) identified here, we included published DNA sequences of 56 DRB-2 alleles from red deer (*CeelDRB*; Swarbrick *et al*., 1995), 18 DRB-2 alleles from white-tailed deer (*OdviDRB*; Van Den Bussche *et al*., 1999), and 10 DRB-2 alleles from moose (*AlalDRB*; Mikko & Andersson, 1995). Multiple sequence alignment was performed using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA) with an engine based on the Clustal W algorithm (Thompson, Higgins & Gibson, 1994). Nucleotides were coded as unordered, discrete characters with five possible character states: A, C, G, T or N and gaps were coded as missing data. Phylogenetic analyses were implemented using MEGA version 3.0 (Kumar, Tamura & Nei, 2004). Genetic distances among all DRB-2 alleles were calculated based on Kimura two-parameter model. A neighbor-joining phylogenetic tree (Saitou & Nei, 1987) was constructed under the minimum evolution criteria with equal weights for all characters and substitutions, heuristic searches with five random additions of input taxa and tree bisection-reconnection branch-swapping. Stability or accuracy of inferred topology(ies) was assessed via bootstrap analysis (Felsenstein, 1985) of 1000 replications. Relative frequencies of non-synonymous (*dN*) and synonymous substitutions (*dS*) within and among all pairwise comparisons of Iberian red deer DRB-2 alleles were estimated following the method of Nei & Gojobori (1986) and applying the Jukes & Cantor (1969) correction for multiple substitutions.

The detection of 18 alleles among the 94 individuals sampled in this population indicated 170 possible DRB-2 genotypes. A χ^2-test was performed to evaluate whether the observed heterozygosity was in Hardy–Weinberg (H–W) equilibrium, using GenAlEx 6 Software (Peakall & Smouse, 2006). As an estimation of MHC DRB-2 loci diversity, we used the number of different alleles identified in relation to the number of individuals studied. The level of significance was established at $P = 0.05$.

Genotyping with microsatellite loci

Genotyping was performed on 96 deer DNA samples extracted as described above using microsatellite loci CSSM22, ETH225 and CSSM19 (Frantz *et al*., 2006). To avoid linkage, microsatellite loci from different chromosomes were chosen in this study (http://www.marc.usda.gov/genome/cattle/references/twintable.html). None of these loci is linked to MHC. In order to avoid noise from cariable adenylation during the PCR, the 'pigtail' sequence GTTTCTT was added to the 5′-end of each reverse primer (Brownstein, Carpten & Smith, 1996). The multiplex PCR was done with labeled forward (F) oligonucleotide primers (Table 4) in a 50 μL volume (1.0 mM $MgSO_4$, 1X AMV RT/*Tfl* reaction buffer, 0.2 mM each dNTP, 5 U *Tfl* DNA

polymerase, 0.22, 0.09 and 0.12 μM of each oligonucleotide primer for microsatellite CSSM22, ETH225 and CSSM19, respectively) using the Access RT-PCR system (Promega). Reactions were performed in an automated DNA thermal cycler (Techne model TC-512). The PCR consisted of an initial step of 5 min at 95 °C followed by 37 cycles of a denaturing step of 45 s at 95 °C and annealing step of 60 s at 53 °C and an extension step of 45 s at 68 °C. The reaction was terminated after a final extension at 68 °C for 10 min. Control reactions were done using the same procedures, but without DNA added to control contamination of the PCR reaction. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison with a DNA molecular weight marker (1 kb Plus DNA Ladder, Promega). Fragments were separated using an ABI 3730 automated DNA sequencer (Applied Biosystems Inc.) and sized relative to a ROX-labeled size marker with bands of known size (G500LIZ-250). The data were analyzed using program Peak Scanner (Applied Biosystems Inc.).

The expected (H_E) and observed (H_O) heterozygosities were estimated with Genetix 4.05.2 (Brownstein *et al.*, 1996). The exact test for deviation from H–W equilibrium at each locus was performed with Genepop on the Web v1.2 (http://genepop.curtin.edu.au/genepop_op1.html) (Raymond & Rousset, 1995) and significance was tested with 10 000 dememorizations, 1000 batches and 10 000 iterations. The estimated null allele frequency for each locus is compared with the null allele frequencies obtained using methods by Chakraborty *et al.* (1992) and Brookfield (1996). These methods assume that heterozygote deficiency is caused by null alleles and not by other genotyping errors or deviations from panmixia.

GenBank accession numbers

The GenBank accession numbers for Iberian red deer DRB-2 alleles (*CeelHap*) are EU573241–EU573258 and EU573259–EU573296 for *CeelHap*1–18 and *CeelHap*101–137, respectively.

Results

The SSCP and sequence analyses revealed 18 unique DRB-2 sequences among 94 Iberian red deer from a single hunter-managed population in south-central Spain (Fig. 1, Table 1). DRB-2 sequences were confirmed by sequencing the same allele from different individuals and by sequencing several clones from the same PCR. Furthermore, some SSCP conformations were corroborated by F-SSCP analysis of both strands using labeled forward and reverse primers (Fig. 2). However, although F-SSCP was useful to corroborate homozygous and heterozygous genotypes it was not suitable for distinguishing between different allelic variants. The SSCP and F-SSCP analyses of Iberian red deer samples from the study site evidenced the presence of no more than two predominant bands on each individual, a result that was confirmed with the sequence analysis of cloned DRB-2 amplicons.

The analysis of the DRB-2 locus found polymorphic MHC-II loci in Iberian red deer. Seventeen per cent of nucleotide and 25% of amino acid positions were polymorphic among the 18 alleles identified. Non-synonymous substitutions occurred at a significantly lower frequency than synonymous substitutions ($dN = 0.0453 \pm 0.0250$; $dS = 0.0761 \pm 0.0361$; $P < 0.0001$).

Apparent allelic frequencies for the 18 unique Iberian red deer alleles ranged from 0.5 to 18.6% (Table 1, Fig. 3). Except for alleles *CeelHap*9 and *CeelHap*15, which only appeared once as heterozygotes, the rest of the alleles appeared at least once as homozygotes (Table 1, Fig. 4). Overall, 23 different genotypes out of the possible 170 were found within the 94 sampled individuals, ranging in frequency from 1.1 to 18.1% (Table 1, Fig. 4). The proportion

```
            9++ +          +++ +  ++ +  ++        +          + ++          ++  +  ++       +    91
 CeelHap1   EYAKSECHFSNGTQRVQFLQRYFYNGEEYVRFDSDWGEFRAVTELGRPDAKYWNSQKEILEQKRAEVDRYCRHNYGVIESFTV
 CeelHap2   ****************R**H********F********************V*E**************A**T***************
 CeelHap3   **H*R***********G**D********T*********Y***********L**********A**A**T********F******
 CeelHap4   *HL*************Y*D********F**********************************N*********************
 CeelHap5   ****************G**D********F*********Y*********************************************
 CeelHap6   **H************G**D********F*********Y***********L*********RA**A**T***************
 CeelHap7   **Y*R***********G**D***S****T*********Y***********EL*********A******T********F******
 CeelHap8   **H*G***********G**D*****************Y***********F*********RA******T***************
 CeelHap9   *HL************RY*E********F**********************************N*********************
CeelHap10   **H************GY*E**IH****F*********Y**********************R***D**T********F******
CeelHap11   **H************G**E********F*********Y**********************R*******T********F******
CeelHap12   **P************R**D********F*********Y*********************************************
CeelHap13   **L***********GG**E********F**********************************D*********************
CeelHap14   **V************R********R**********************************DN**A**T***************
CeelHap15   ******************H**I**R***A**********************************RA******TV**********
CeelHap16   ****************R**D********F*********Y*********************************************
CeelHap17   ****************R**D*********************************************A**T***************
CeelHap18   ******************D*********************************************R*******T***************
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Figure 1 Amino acid sequence alignment of 18 Iberian red deer *Cervus elaphus hispanicus* DRB-2 alleles. The standard one-letter amino acid code was used. Numbering of amino acid positions was based on white-tailed deer *OdviDRB* sequences (Van Den Bussche *et al.*, 1999). Asterisks denote amino acids identical to *CeelHap*1. Variable amino acid positions are indicated with a cross (+).

of genotypes that were found in this deer population differed statistically from expected H–W proportions ($\chi^2 = 1248.3$, d.f. = 153, $P < 0.001$). This disequilibrium was mainly due to apparent homozygous genotypes being observed at much higher proportion than expected (Fig. 4). Observed apparent heterozygosity was 7.5% (7/94), whereas expected heterozygosity was 89.1%. Four genotypes out of the 23 genotypes found, which appeared as homozygotes, represented 55% of the 94 individuals analyzed: *CeelHap*2 ($n = 10$, 10.6%), *CeelHap*4 ($n = 12$, 12.8%), *CeelHap*5 ($n = 17$, 18.1%) and *CeelHap*6 ($n = 12$, 12.8%). The F-SSCP analysis of individuals introduced and hunted before 1991 revealed that only one of the six samples analyzed was heterozygous for the DRB-2 locus (Fig. 5). This proportion of heterozygotes did not differ from that found in animals sampled after 1991 ($\chi^2 = 0.65$, d.f. = 1, $P = 0.41$). All the heterozygotes were males (seven out of 61, 11.5%), whereas no heterozygote was evidenced among females. The proportion of heterozygotes statistically differed between sexes (Fisher exact test, $P = 0.04$). The annual diversity index of MCH-II DRB-2 alleles significantly decreased along the study period (two-tailed Spearman correlation, RS = −0.62, $P < 0.01$, $n = 17$ from 1989 onwards; Fig. 6). Nonetheless, no temporal patterns were identified for any single allele, especially when attending to the more frequent ones (Table 2).

Table 1 Allelic frequencies ($2n = 188$) and genotype frequencies ($n = 94$ individuals) for the 18 alleles found

Allele	Frequency	Per cent	Genotype	Frequency	Per cent
5	35	18.62	5–6	17	18.09
4	28	14.36	4–4	12	12.77
6	27	14.36	6–6	12	12.77
2	20	10.64	2–2	10	10.64
7	15	7.98	7–7	7	7.45
1	12	6.38	1–1	6	6.38
3	11	5.85	3–3	5	5.32
17	10	5.32	17–17	5	5.32
8	6	3.19	8–8	3	3.19
12	4	2.13	12–12	2	2.13
16	4	2.13	16–16	2	2.13
18	4	2.13	18–18	2	2.13
11	3	1.60	10–10	1	1.06
14	3	1.60	11–11	1	1.06
10	2	1.06	13–13	1	1.06
13	2	1.06	14–14	1	1.06
9	1	0.53	3–6	1	1.06
15	1	0.53	4–5	1	1.06
			4–6	1	1.06
			4–9	1	1.06
			4–11	1	1.06
			6–15	1	1.06
			7–14	1	1.06
Total	188		Total	94	

Although the SSCP and F-SSCP analyses of samples from the reference study population evidenced the presence of no more than two predominant bands on each individual, the study of control Iberian red deer from different geographic locations using the same experimental procedures described herein identified a minimum of two expressed DRB loci in this species as up to four different allelic variants were evidenced (Table 3, Fig. 7). Control deer also evidenced a high degree of heterozygosis as, except for two individuals, all control deer evidenced four different allelic variants.

Phylogenetic analysis of Iberian red deer DRB-2 alleles together with other cervid sequences revealed monophyletic relationships for moose (*AlalDRB*) alleles (Fig. 8). Although some of the Iberian red deer alleles (*CeelHap*) tended to cluster together (*CeelHap*4, 5, 9, 12, 13, 16), *CeelHap* alleles were paraphyletic with respect to white-tailed deer (*OdviDRB*) and red deer (*CeelDRB*) sequences (Fig. 8).

The estimation of null allele frequency per locus ranged from 43.2% (Brookfield, 1996) to 48.5% (Oosterhouth method, Chakraborty *et al.*, 1992).

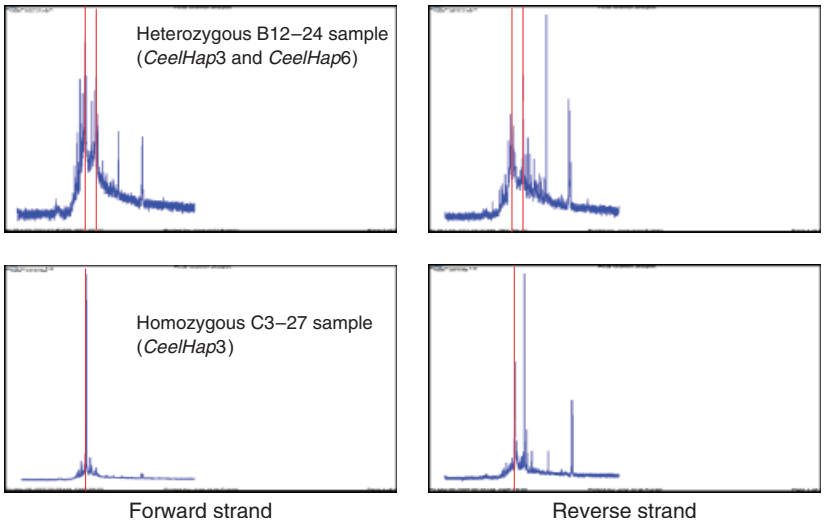

Figure 2 Fluorescent single-stranded conformation polymorphism peak profiles of Iberian red deer *Cervus elaphus hispanicus* DRB-2 alleles. Forward and reverse strands for selected heterozygous and homozygous individuals.

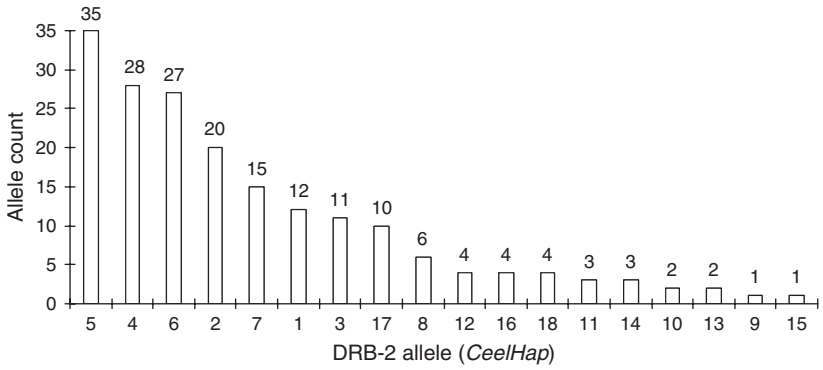

Figure 3 Allelic frequencies of Iberian red deer *Cervus elaphus hispanicus* major histocompatibility complex class II DRB-2 loci. Observed frequency of *CeelHap* alleles ($2n = 188$).

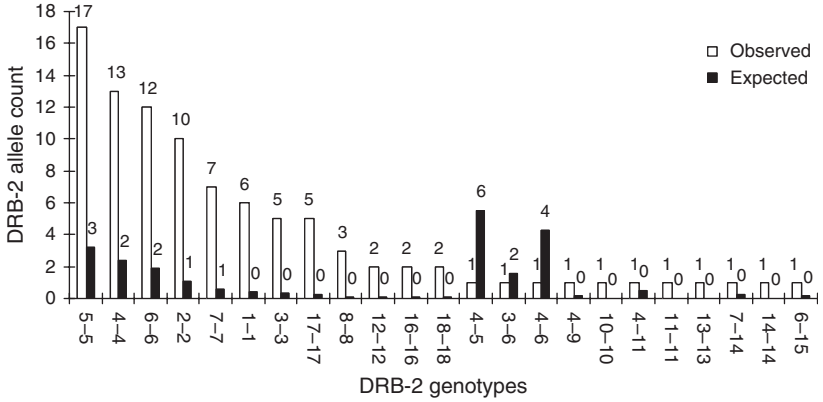

Figure 4 Different genotypes found for the major histocompatibility complex class II DRB-2 locus in Iberian red deer *Cervus elaphus hispanicus* ($n = 94$). Observed versus expected counts of different genotypes for the major histocompatibility complex class II DRB-2 locus in Iberian red deer ($n = 94$). Only those genotypes for which at least one individual was found are shown.

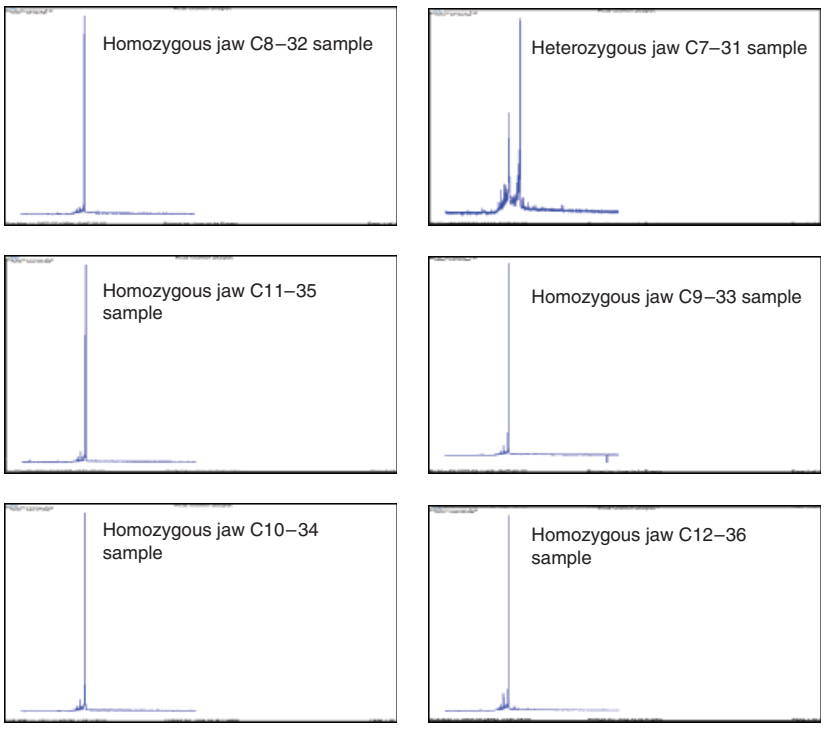

Figure 5 Founding population: fluorescent single-stranded conformation polymorphism peak profiles of Iberian red deer *Cervus elaphus hispanicus* DRB-2 alleles. Forward strand for samples derived from individuals introduced and hunted before 1992.

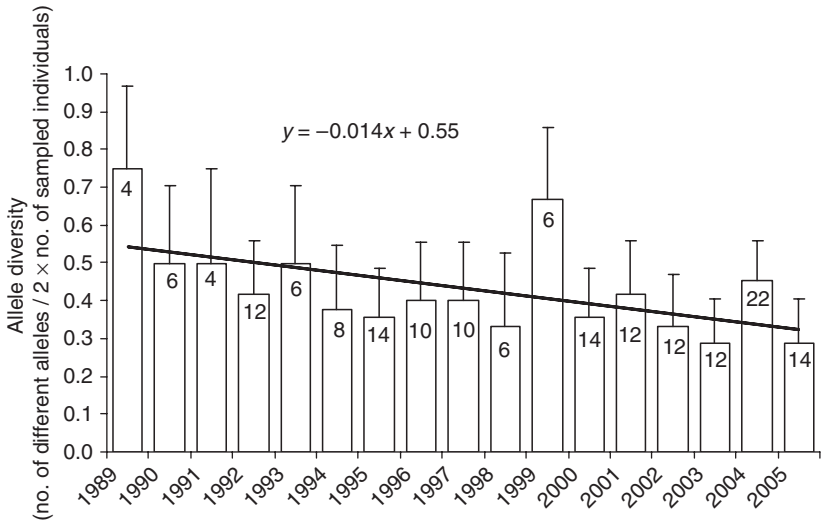

Figure 6 Annual diversity indices of major histocompatibility complex class II DRB-2 alleles. Annual diversity for the 18 unique Iberian red deer *Cervus elaphus hispanicus* alleles found according to individual year of birth across the study period.

Table 2 Allelic frequencies ($2n = 188$) for the alleles found according to individual year of birth across the study period

Haplotypes	1988 $n=7$	1989 $n=2$	1990 $n=3$	1991 $n=2$	1992 $n=6$	1993 $n=3$	1994 $n=4$	1995 $n=7$	1996 $n=5$	1997 $n=5$	1998 $n=3$	1999 $n=3$	2000 $n=7$	2001 $n=6$	2002 $n=6$	2003 $n=7$	2004 $n=11$	2005 $n=7$
1	2	0	2	0	0	0	0	0	0	2	0	0	0	0	0	2	4	0
2	0	0	0	0	0	0	2	0	0	0	0	0	4	2	2	2	4	4
3	2	0	0	0	2	1	0	4	0	0	0	2	0	0	0	0	0	0
4	4	0	2	2	0	0	0	0	3	0	2	1	4	2	2	0	2	4
5	2	2	0	0	2	0	2	4	1	0	4	2	0	4	6	0	2	4
6	0	1	0	0	4	3	0	2	0	4	0	1	2	0	0	6	4	0
7	2	0	0	2	0	0	0	2	0	2	0	0	0	0	2	2	1	2
8	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	0	2	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1	0
12	0	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1	0
15	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0
17	2	0	0	0	2	0	0	0	4	2	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0	0	0	0

Year 1988 includes animals born before population founding.

Genetic diversity at three microsatellite loci was analyzed as genetic reference of neutral markers to separate selective and demographic influences on the MHC-II in this population (Table 4). Expected heterozygosity (H_O) was higher than 0.5 in all three microsatellite loci (Table 4). Microsatellite loci CSSM22, ETH225 and CSSM19 were in H–W equilibrium at the $\alpha = 0.01$ level with a sequential Bonferroni correction ($P < 0.003$). The annual diversity index of CSSM19 microsatellite alleles significantly decreased along the study period (two-tailed Spearman correlation, RS $= -0.49$, $P = 0.04$, $n = 17$ from 1989 onwards), whereas a non-significant decreasing trend was found for CSSM22 (RS $= -0.43$, $P = 0.08$) and ETH225 (RS $= -0.43$, $P = 0.08$) microsatellite alleles.

Discussion

The high levels of allelic diversity found within the studied Iberian red deer population were similar to that found in red deer (Swarbrick *et al.*, 1995) and white-tailed deer (Van Den Bussche *et al.*, 1999). In contrast, the DRB-2 genetic diversity reported in moose and reindeer is greatly reduced (Mikko & Andersson, 1995; Mikko *et al.*, 1999; Wilson *et al.*, 2003), possibly due to limited parasite exposure in boreal ecosystems and/or bottlenecks (Mikko & Andersson, 1995; Ellegren *et al.*, 1996; Mainguy *et al.*, 2007).

The number and distribution of polymorphisms within the Iberian red deer DRB-2 amino acid sequence was similar to that detected in white-tailed deer and red deer alleles

Table 3 Allelic frequencies ($4n=52$) for the 38 alleles found in the control population

Allele	Frequency	Per cent
101	2	3.85
102	1	1.92
103	1	1.92
104	1	1.92
105	2	3.85
106	3	5.77
107	1	1.92
108	1	1.92
109	2	3.85
110	1	1.92
111	3	5.77
112	1	1.92
113	1	1.92
114	1	1.92
115	2	3.85
116	1	1.92
117	1	1.92
118	1	1.92
119	1	1.92
120	1	1.92
121	1	1.92
122	1	1.92
123	1	1.92
124	2	3.85
125	4	7.69
126	1	1.92
127	2	3.85
128	1	1.92
129	1	1.92
130	2	3.85
131	1	1.92
132	1	1.92
133	1	1.92
134	1	1.92
135a	1	1.92
135b	1	1.92
136	1	1.92
137	1	1.92
Total	52	100

(Swarbrick *et al.*, 1995; Van Den Bussche *et al.*, 1999). This fact denotes a strong selection pressure on certain amino acids within the DRB-2 sequence. However, in contrast to reports in other deer species (Swarbrick *et al.*, 1995; Van Den Bussche *et al.*, 1999), DRB-2 alleles were apparently not under positive Darwinian selection in the Iberian red deer population analyzed herein. Positive Darwinian selection involves a selective pressure favoring change so that only a small number of mutational events are retained in a population, the retention of mutations being much closer to the rate of mutation. In our sample, synonymous substitutions occurred more frequently than non-synonymous substitutions, suggesting that positive selection is not acting on the selected protein sequence.

It has been suggested that the high level of MHC allelic diversity is primarily maintained by pathogen-driven positive Darwinian selection and may be the underlying reason for MHC-based mating preferences, a mechanism that evolved to avoid inbreeding (Potts, Manning & Wakeland, 1991; Hedrick, 1992; Paterson, Wilson & Pemberton, 1998; Carrington *et al.*, 1999; Van Den Bussche *et al.*, 1999; Lohm *et al.*, 2002; Wegner, Reusch & Kalbe, 2003; Harf & Sommer, 2005; Schad, Ganzhorn & Sommer, 2005). However, the DRB-2 genotype composition of the Iberian red deer in this study cannot be explained by positive Darwinian selection, leaving other factors acting as selection pressures on these individuals. Possible factors are discussed below.

As in the Iberian red deer population studied herein, white-tailed deer (Ditchkoff *et al.*, 2005), Canadian moose (Wilson *et al.*, 2003) and African buffalo (Wenink *et al.*, 1998) populations have been reported to comprise DRB genotypes in H–W disequilibrium with homozygous individuals at higher frequency than expected. Nonetheless, the strong proportion of (apparent) homozygotes we found has no comparison in the literature when attending to wild or free-ranging ungulates. Selective advantage of heterozygotes over homozygotes would not explain the extreme deficiency of heterozygotes (Penn, Damjanovich & Potts, 2002). Heterozygote deficiencies at loci relative to H–W expectations could be explained by several non-mutually exclusive mechanisms: (1) a Wahlund effect arising from population subdivision; (2) inbreeding due to consanguineous mating

Table 4 Characterization of the microsatellite loci used in this study

Microsatellite	Dye	Primer sequences 5′–3′	Allele size range (bp)	A	H_E	H_O	Hardy–Weinberg equilibrium (P-values)
CSSM22	6-Fam	F: TCTCTCTAATGGAGTTGGTTTTTG R: GTTTCTTATATCCCACTGAGGATAAGAATTC	213–227	4	0.524	0.505	0.72
ETH225	6-Fam	F: ACATGACAGCCAGCTGCTACT R: GTTTCTTGATCACCTTGCCACTATTTCCT	140–173	12	0.851	0.896	0.22
CSSM19	Hex	F: TTGTCAGCAACTTCTTGTATCTTT R: GTTTCTTTGTTTTAAGCCACCCAATTATTTG	140–166	10	0.808	0.792	0.69

Genotyping was performed on 96 deer DNA samples as described by Frantz *et al.* (2006).
The test for Hardy–Weinberg equilibrium was performed at each locus at the $\alpha=0.01$ level with a sequential Bonferroni correction ($P<0.003$).
A, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity.


```
                    9                                                                                                  91
AF082163OdviDRB03   EHHKAECHFSNGTQRVRFLERYIYNQEEYVRFDSNVGEYRAVTELGQPSAKYYNSQKEILEQKRASVDTYCRHNYGVFDNFAV
CeelHap101          *YY*S****F*********H*CFS*G**T*****D**********R*D*ELW*****L***R**A**********VES*T*
CeelHap102          *YY*R****F*********H*CFS*G**T*****D**********R*D*ELW*****L***R**A**********VES*T*
CeelHap103          *YY*R****F*********H*YFS*G**T*****DW*********R*D*ELW********RA**A**********ES*T*
CeelHap104          *YY*R****F******G**D**FS*G**T*****DW*********RPD*ELW*****L***R**A***V*******IES*T*
CeelHap105          LYTTS**********Q**D**F**G**F*****DW*********RPA*EGW*R***F***A**A*********GVES*T*
CeelHap106          LYA*S**********GL*D**F**G**F*****DW**F*******R*A*EGW*R***F***R**A**********IES*T*
CeelHap107          *YA*S**********EY*D**S**G*******DW**F*******R*A*EGW*R***F***R**A***Y*******IES*T*
CeelHap108          *YA*S**********EY*D**F**G*******DW**F*******R*D***W****DFM*****E***V****T*FLRVSL*
CeelHap109          *QL*S********E**Q**H**F**G*******DW**F*******RPD***W****DFM*****E***V****T*FLRVSL*
CeelHap110          *QL*S**********EY*D**F**G**F*****DW**F*******R*D***W****DFM*****E***V********ES*T*
CeelHap111          *YA*S**********EY*D**F**G*******DW**F*******R*D***W****DFM*****E***V********ES*T*
CeelHap112          MYT*K**********GY*D**F**G*******DW**F*******R*D***W****DFM*****E***V********ES*T*
CeelHap113          *YA*S**************D**F**R*******DW**F*******R*********DFM*****E***V*******IES*T*
CeelHap114          **L******F***E*MQ**A**F**G***A****D***F*******R*D***L**********A**R********GES*T*
CeelHap115          **L******F***E*MQ**A**F**G***A****D***F*******R*D***W*********H**E**R******RGR-E*HC
CeelHap116          **L******F***E*MQ**A**F**G***A****D***F*******R*D***L*********N**E**R******RGR-E*HC
CeelHap117          **L*T****F***E*MQ**A**F**G***A****D***F*******R*D***W*********H**E**R********GES*T*
CeelHap118          **L******FH**E*MQ**A**F**G***A****D***F*******R*D***W*********H**E**R********GES*T*
CeelHap119          **L******F***E*MQ**A**F**G***A****D***F*******R*D***W*********H**E**R********IES*T*
CeelHap120          **L******F***E*MQ**A**F**R*******D***F*******R*D***W*********H**E**R********GES*T*
CeelHap121          *YY*R****F******G**D**F**G***A****D***F*******R*D***W*********H**E**R********IES*T*
CeelHap122          *YA*S**********EY*D**F**G*******DW**F*******R*D***W*********H**E**R********GES*T*
CeelHap123          *QL*S**********QY*Q*****G**F*****DW**F*******R*D***L*********H**E**R********GES*T*
CeelHap124          *QL*S**********QY*Q*****G**F*****DW**F*******R*D***W*********H**E**R********GES*T*
CeelHap125          *QL*S**********QY*Q*****G**F*****DW**F*******R*D***L**********A**R********GES*T*
CeelHap126          *QL*S**********QY*Q*****G**F*****DW**F*******R*V*E*L*****L**R***N******Y****GES*T*
CeelHap127          ****C********E**Q**Q*****R*******DW*********R*D**********RS*GA**********VES*T*
CeelHap128          LYA*S***L******GL*D**F**G*******DW**F*******R*D**********RS*GA**********GES*T*
CeelHap129          **A*S**********Q**Q***Y*R*******DW**F*******RL****R********EL**A**RA*******IES*T*
CeelHap130          *YA*S**********Q**Q*****R*******DW**F*******RL****R********EL**A**RA*******IES*T*
CeelHap131          *QL*G**********L*Q*****R***A*Y**DW**F*******R*D*E*W*****YM*EL**A**********IESLT*
CeelHap132          *QL*G**********L*Q*****R***A*Y**DW**F*******R*D*E*W*****YM*EL**A**********IES*T*
CeelHap133          *QL*G**********L*Q*****R***A*Y**DW**F*******R*D*E*W*****YM*KL**A**********IES*T*
CeelHap134          *YA*S**********L*Q*****R***A*Y**DW**F*******R*D*E*W*****YM*EL**A**********IES*T*
CeelHap135a         KLS*TPXLCLQYVSTLARFRSR*SFWLFQYLASGRPSSVT*RYSPQSL*KRTNS*PLWMYLSRYPTRCVPLEKXHSLLXYSR-
CeelHap135b         KLSTTPXLCLQYVSTAPRDRSR*SFWLFQYLASGRPSSVT*RYSPQSL*KRT*S*RLXMYLCRNCTRSVPLEKXHSHLXCPR-
CeelHap136          LYG*S****************HL*SG**L*****DW**F****G**R*V*E*L********R***N**RV*******VES*T*
CeelHap137          *YA*S********E**QY*Q**F**G***A****DW**F*******R*****R********DN**A**********L*S*T*
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Figure 7 Amino acid sequence alignment of 13 Iberian red deer *Cervus elaphus hispanicus* DRB-2 alleles (a control population from different geographic locations). The standard one-letter amino acid code was used. Numbering of amino acid positions was based on white-tailed deer *OdviDRB* sequences (Van Den Bussche *et al.,* 1999). The letter 'x' indicates stop-codon.

(i.e. positive assortative mating); (3) selective hunting effects on deer genetics; (4) the presence of null alleles.

Firstly, heterozygote deficiencies are consistent with a Wahlund effect arising from pooling of discrete subpopulations with different allele frequencies that do not interbreed as a single randomly mating unit, even if the subpopulations are in H–W equilibrium (Excoffier, 2001). This effect has been suggested in populations of white-tailed deer (Van Den Bussche, Ross & Hooper, 2002; Kollars *et al*., 2004) and moose (Wilson *et al*., 2003), but always with a lower degree of homozygosity than this described here. Behavioral barriers to gene flow within the study area could relate to female philopatry and constrained natal dispersal in young males, which can result in the clustering of related individuals, inbreeding and structuring of nuclear genotypes across continuous space (Clutton-Brock, Guinness & Albon, 1982; Kurt, Hartl & Volk, 1993; Nussey *et al*., 2005). Also, spatial aggregation of resources (such as the fixed feeding places in this estate) could induce the aggregation of females (at least during the period of the day they forage) (Carranza, Alvarez & Redondo, 1990; Sanchez-Prieto, Carranza & Pulido, 2004), which may lead to an increase of the level of polygyny and a decrease in effective population size. Nonetheless, the relatively small area used by our study population (<1000 ha), the absence of physical barriers within the area and the intensity of the hypothesized behavioral mechanisms necessary to produce such apparent excess of homozygotes make a strong Wahlund effect improbable as single cause of the reported pattern.

We secondly consider the positive non-assortative mating hypothesis. Any deviation from H–W equilibrium may indicate assortative mating, which may be positive (increases

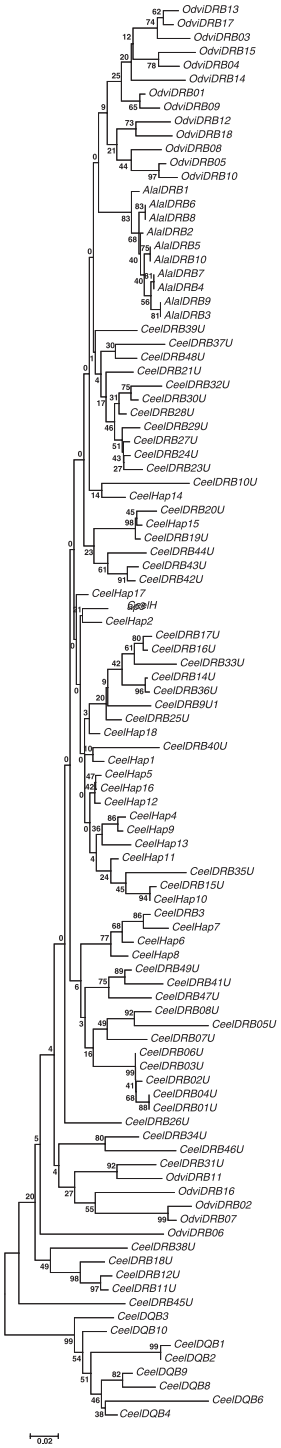

Figure 8 Unrooted neighbor-joining tree of cervid DRB-2 alleles. The DNA sequences of DRB-2 alleles from Iberian red deer (*CeelHap*; *Cervus elaphus hispanicus*), red deer (*CeelDRB*; *Cervus elaphus*), white-tailed deer (*OdviDRB*; *Odocoileus virginianus*) and moose (*AlalDRB*; *Alces alces*) were included in the analysis.

homozygosity, which is our case) or negative (increases heterozygosity). The latter is commonly found in vertebrates and it is accepted that it occurs because inbreeding avoidance and choice of mating partner are interconnected processes. Although some semi-wild ruminant populations seem to mate randomly, despite the presence of pathogen-induced selection on MHC genes (Paterson & Pemberton, 1997; Holand *et al*., 2007), no evidence for positive non-assortative mating has been previously observed. Therefore, although inbreeding avoidance through mating choice seems not to occur in our study population, positive non-assortative mating is not expected to occur from an ecological, behavioral and evolutionary point of view (Paterson & Pemberton, 1997; Holand *et al*., 2007). Our study population is characterized by intense high-quality deer management, and there is a high proportion of mature males (6–10 years) that congregate at feeding places during the rut season, being expected that dominant stags mate with a variable number of hinds, many of them unrelated.

A simpler explanation is based on the hypothetical effect of selective hunting on deer genetics. Keeping in mind that annually almost one-fourth of the animals are removed from the population following trophy-quality criteria (Fierro *et al*., 2002), it seems plausible that selective hunting has an adverse cumulative effect on genetic variability of this deer, as has been seen in other ungulates (Coltman *et al*., 2003). If there is any relationship between heterozygosity at MHC DRB-2 and individual hunter-targeted traits, then the selective culling of stags could lead to increased or reduced heterozygosity depending on the sense of the relationship. For example, if heterozygosity at this locus relates to large antler size, then we would expect an increase in homozygosity, due to the prime stags being removed. If there is any relationship between homozygosity at this locus and poor condition or disease susceptibility, then the selective culling of animals in poor condition would lead to increased heterozygosity. Other explanations could lie in the presence of a disease in the population for which selective pressure is high and a homozygous locus is an advantage. As for the previously hypothesized mechanisms, the intensity of selective hunting would difficultly lead to such apparent excess of homozygotes.

Finally, there is the possibility that many of the apparent homozygotes are, in reality, heterozygotes between a visible and a null allele (Chakraborty *et al*., 1994; Pemberton *et al*., 1995), that is, it is possible that in some samples we did not amplify all loci to a level to be detectable by SSCP or cloning and sequencing analyses. Therefore, expect that the H–W deviation would be an artefact of null alleles. Such null alleles can arise when mutations prevent primers from binding (Callen *et al*., 1993). This idea is supported by several features. Firstly, the analysis of Iberian red deer from different geographic locations identified two expressed DRB loci, corroborating previous results in red deer (Swarbrick *et al*., 1995). However, the analysis of DRB-2 polymorphism in the Iberian red deer population from the study site showed on each individual a maximum of two bands in the SSCP and F-SSCP analyses, a result that was confirmed

with the sequence analysis of cloned DRB-2 amplicons. This is a striking finding when compared with the extreme degree of homozygosis found in our study population in spite of the relatively high allelic variability. We estimated an apparently high null allele frequency per locus [ranging from 43.2%, Brookfield method, to 48.5% (Oosterhouth method, similar for example to that described for T-cell receptor gene segments in human Sardinian population, one of the most elevated so far reported in the literature, Bonfigli *et al.*, 2007). One possibility is that undetermined selective pressure could have played a role in determining the high frequency of this inactivating polymorphism in deer, although alternatively, due to the relatively short period of time since population foundation, these findings are more consistent with a sound founder effect due to original introduction of deer carrying MHC DRB-2 locus null alleles], contrasting with allele frequency data from external control animals.

Secondly, the fact that microsatellite analysis revealed H–W equilibrium is consistent with the idea that real heterozygosity at MCH DRB-2 loci was not detected. Multilocus analyses, such as those also focusing on microsatellites, can distinguish null allele effects because population genetic factors should register more or less concordantly across loci, whereas the effects of null alleles are locus-specific. In this sense, and contrary to our findings, wild populations tend to have excess of heterozygotes for MCH loci in spite of neutral markers being in H–W equilibrium. As a reference, values of H_E for the microsatellite loci analyzed in this study were higher than those reported in a continuous red deer population (Frantz *et al.*, 2006). To deduce the presence and basis of null alleles more directly, molecular approaches are required such as sequencing the nucleotides of the flanking regions in individuals suspected of carrying a null allele (Jones *et al.*, 1998). Also, the presence or absence of null alleles can then be confirmed by pedigree analysis.

Notwithstanding the capacity of potential null alleles across our study individuals to greatly affect apparent heterozygosity, the apparent loss of MHC-II DRB-2 allele diversity through time reflects the effects of inbreeding on this hunted fenced population (the variability of the detected alleles would resemble that of the total). In fact, supporting this possibility, a loss of microsatellite allele diversity was also found. The efficacy of selection in maintaining genetic diversity can vary substantially with the degree of population isolation (Muirhead, 2001). In particular, the management of game species, such as fencing may involve a risk of alteration of their genetic properties due to genetic drift in the subpopulations (Carranza *et al.*, 2003). Intensive game management situations may be analogous to bottlenecks, which increase the risk of loss of rare alleles and probably the risk of outbreaks of severe diseases as a consequence of decreased MHC variation (O'Brien & Evermann, 1988; Carranza *et al.*, 2003).

In the context of overall low homozygosity in the study population, the proportion of heterozygotes was statistically higher in males (seven out of 61, 11.5%), than in females (0%, $n = 34$). In fact, all the detected heterozygotes were males. Different non-exclusive explanations, which cannot be tested in this study, could contribute to this finding. Firstly, heterozygosity may prove more advantageous in males than in females (male deer is more susceptible to parasites, Vicente, Perez-Rodriguez & Gortazar, 2007; and/or females could be more selective when choosing a heterozygous mate, Clutton-Brock *et al.*, 1982), and therefore selecting heterozygous males. Secondly, sex allocation may be influenced by offspring MHC heterozygosity (Olsson, Wapstra & Uller, 2005). Thirdly, hunting, which is undertaken using different criteria in males (selective or trophy) but not in females, could favor the retaining of more heterozygotes among males than in females (Coltman *et al.*, 2003) although our data does not allow explaining how phenotype-based selective harvests would target in genetics. Higher sample size and correlational studies with fitness parameters in our study population are needed to elucidate this aspect. Also, distinguishing the potential confounding effect of null alleles on sex differences is needed.

The Iberian red deer, red deer and white-tailed deer DRB-2 alleles represented paraphyletic groups. As previously discussed by Van Den Bussche *et al.* (1999), this result reflects the high sequence identity that exists among artiodactyl DRB-2 alleles.

Conclusions

With our data, we cannot discard that null alleles were present in our samples, and the reported pattern could be the result of null allele and founder effects (in the form of the introduction of deer carrying MHC DRB-2 locus null alleles) in this recently established population. Putative null (non-amplified) alleles are rarely described or suspected to be present in studies focusing on MHC loci in wild species. Nonetheless, as in microsatellites (Dakin & Avise, 2004), null alleles could be a common feature of MHC genotyping, and can bias estimates of allele and genotype frequencies, thereby hindering population genetic analyses.

The apparent loss of MHC-II DRB-2 allele diversity reflects the effects of inbreeding in hunted fenced populations. These findings raise concern about the ecological consequences of wildlife management on deer genetics, conservation and future life history. Therefore, although red deer is an abundant and widespread species in Spain, its MHC polymorphism (and overall genetic variability), may be threatened (1) by increasing habitat fragmentation and barriers to gene flow (Carranza *et al.*, 2003), (2) by any alteration of mating substructures within managed populations favoring positive assortative mating or (3) by selective hunting. Finally, our findings support the importance of immunogenetic studies to assess management decisions, especially in isolated ungulate populations.

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