Sequence analysis of the MHC class II DRB alleles in Alpine chamois (Rupicapra r. rupicapra)

Helmut Schaschl*, Simon J. Goodman b, Franz Suchentrunk a

a Research Institute of Wildlife Ecology, Veterinary Medicine University of Vienna, Savoyenstrasse 1, A-1160 Vienna, Austria
b Institute of Zoology, Zoological Society of London, Regent’s Park, London NW1 4RY, UK

Abstract

This study characterises the expressed MHC class II DRB gene and its genetic variation in exon 2 among 59 Alpine chamois (Rupicapra r. rupicapra) from several locations in the Eastern Alps plus one population in the Western Alps. The expressed DRB gene contains all the expected characteristics including all of the conserved residues found in other mammalian species. The isolated expressed DRB gene from chamois can be considered orthologous to MHC class II DRB genes in other mammalian species. Cattle primers yielded exon 2 sequences of 284 bp length. Sequence analysis revealed 19 DRB exon 2 alleles among 59 chamois. Alleles exhibited a high degree of nucleotide and amino acid polymorphism, with most amino acid variations occurring at positions forming the peptide-binding sites. A significantly higher rate of non-synonymous (dN = 0.053 ± 0.0132) than synonymous (dS = 0.007 ± 0.003) substitutions indicated positive selection for diversity in the DRB locus.

1. Introduction

The major histocompatibility complex (MHC) is a genomic region that encodes cellular proteins that are involved in the immune response. The main functions of MHC class I and class II molecules consist of binding immunogenic peptides inside the cell and presenting them to T-cells. The MHC class II molecules are αβ heterodimers and are mainly expressed on antigen-presenting cells. The α1 and β1 domains form the peptide-binding region (PBR), in which peptides are bound and recognized by CD4+ helper T-cell receptors. The analysis of the three-dimensional structure of the human MHC class II molecules (HLA-DR) has revealed molecular pockets in the PBR that influence the binding of presented peptides and the subsequent T-cell response [1,2].

The usually high polymorphism found in MHC class II genes is generally confined to exon 2, which encodes the PBR in the β1-domain. Among the ruminating ungulates such as cattle (Bos taurus) and sheep (Ovis aries) various MHC class II genes (DRA, DRB, DQA and DQB) have been characterised. The cattle DR-region contains three DRB genes and one DRA gene (reviewed by Lewin et al. [3]). Among the three DRB loci, DRB3 is the predominantly expressed gene and shows extensive polymorphism. The DRB2 locus is expressed at a very low level, DRB1 is
a pseudogene, and the single DRA gene is monomorphic.

More than 100 DRB3 alleles are currently known in cattle (Bola Nomenclature Committee, Web site: http://www.projects.roslin.ac.uk/bola/drtab.html). In sheep the organisation of the MHC class II is similar to that of cattle and several DRB genes exist of which only DRB1 has been found to display extensive polymorphism [4,5]. Sequence variation studies in other ungulate species have found a range of variability, often conflicting with predictions based on known demographic history, making it difficult to draw general conclusion on the role of parasite mediated selection in MHC evolution in these taxa. High polymorphism at the DRB locus was also found in goat (Capra aegagrus, Capra hircus) [6,7], Bighorn-sheep (Ovis canadensis) [8], a population of white-tailed deer (Odocoileus virginianus) [9] and red deer (Cervus elaphus) [10]. On the other hand, Mikko et al. [11] and Ellengren et al. [12] detected only low levels of genetic diversity at this locus in moose (Alces alces) from North America and North Europe. Furthermore, limited allelic diversity at the DRB locus was also reported for roe deer (Capreolus capreolus) and reindeer (Rangifer tarandus) from sampling areas in Norway and Sweden, and musk-ox (Ovibos moschatus) and fallow deer (Cervus dama) were monomorphic at this locus [13]. However, in the African buffalo (Syncerus caffer), high allelic diversity in the DRB3 gene was found in spite of a bottleneck caused by a rinderpest epidemic at the end of the 19th century [14]. Bighorn-sheep revealed a high level of polymorphism, even though populations have declined, primarily due to infectious diseases transmitted to them from livestock [8]. On the other hand, one study that has looked explicitly at MHC genotypes and survivorship in a wild population of Soay sheep revealed that certain MHC class II alleles were associated with low survivorship probability and high level of parasite load [15].

The MHC complex contains many linked genes and so far it is not well understood how these genes interact. The MHC Sequencing Consortium [16] identified 224 genes within the complex in humans and estimated that 40% of these expressed genes are involved in specific immune functions. Social and ecological parameters such as mating system, social structure, and habitat are hypothesised to play an important role in the evolution of the immune system and variation in the pattern of immune system variability across species reflects evolutionary adaptation to such factors. For example, in anthropoid primates it has been found that the concentration of lymphocytes and phagocytes were positively correlated with annual rainfall, potentially due to the higher rates of parasitism in wetter habitat [17]. Mate choice [18,19] social structure [20], and parasite diversity [21] have been shown to be important factors for maintaining MHC variability, either through a direct effect of selection on MHC genes, an indirect effect of selection on other immune response genes in the MHC, or perhaps a combination of the two effects. Collecting MHC sequence information from a range of species is therefore critical to understanding the general mechanisms, which influence the evolution of these genes.

Alpine chamois (Rupicapra r. rupicapra) is an ungulate species of the subfamily Caprinae within the Bovidae (see e.g. Hartl et al. [22] and Randi et al. [23] for biochemical-genetic and phylogenetic conclusions) primarily inhabiting mountainous areas at or above the tree line. The phylogenetic position of several species within the Caprinae has long been discussed and is still being debated. The presumably rapid radiation of the Caprinae results in a reduced resolving power for phylogenetic analyses. Historically, the Rupicaprina (with the four genera Rupicapra (chamois), Capricornis (serow), Naemorhedus (goral), and Oreamnos (mountain goat) have been regarded as the most basal tribe within the Caprinae [22].

The main purpose of this study was to identify expressed MHC class II DRB gene(s) and analyse exon 2 variability within the species. Alpine habitats might provide a smaller range of infectious or parasitic organisms for ungulates than habitats at lower elevation or at lower latitudes, because of more unfavourable climatic conditions, shorter periods for the development of infectious stages or intermediate hosts, and a generally reduced spectrum of biodiversity. Parallel to such a hypothesized lower diversity of infectious or parasitic organisms in unfavourable environments, mountain ungulates might generally display lower diversity in the MHC, because of possible relaxed pathogen-driven
selection. Furthermore, demographic history might have also an influence on the variability at the MHC in chamois due to fragmented habitats throughout the Alps (e.g. due to glaciations and recent human developments) and population crashes caused by sarcoptic mange epidemics [24]. Taken together these factors might have lead to reduced variation in the MHC.

2. Material and methods

2.1. Samples and DNA isolation

Liver samples of 53 chamois from 14 different populations (Achenkirch (Ach), Ennstal (Ens), Gailtaler Alpen (Gai), Kalkalpen (Kal), Karnische Alpen (Kan), Karawanken (Kaw), Kitzbühler Alpen (Kit), Nockberge (Nob), Osterhorngruppe (Ohg), Rax (Rax), district Scheibbs (Sbs), Schladminger Tauern (Sch), Tauern (Tau), Wildalpe (Wal)) in the Eastern Alps were collected during regular hunts in several provinces of Austria. In addition, six chamois were collected from a regional population in the Western Alps (Val de Susa (Vds), Piemont, Italy). Genomic DNA was extracted from frozen liver using standard phenol/chloroform–isoamylalcohol extraction.

2.2. RNA isolation, generation of cDNA

One millilitre fresh blood was obtained from a single chamois from Hellbrunn Zoo (Salzburg, Austria) and stored in EDTA. Total RNA was isolated from leucocytes using the Purscript RNA Isolation-Kit (Gentra). From total RNA, mRNA was isolated with the NucleoTrap® mRNA-Minikit (Macherey-Nagel) according to the manufacturer’s protocol. RT-PCR was performed in the Titan One Tube RT-PCR-System from Roche. The two primers used for first-strand cDNA synthesis and subsequent PCRs were DRBsp: 5’-ATGGCCATAACTGGGTTCCAATA-3’ and DRBcy: 5’-TCACA-GAGGCCCTCGGCGGCTCAAAC-3’ [26]. The primer HL030 is homologous to the intron 1/exon 2 boundary, the primer HL031 is homologous to the intron 2/exon 2 boundary and the nested primer HL032 is complementary to the 3’ end of the exon 2. The 50 µl PCR reaction mixture contained PCR buffer, 2% DMSO, 0.1 mM of each dNTP, 25 pmol of each primer, 1.5 mM MgCl2, 200–300 ng of genomic DNA and 1.0 U Taq Polymerase (DyNAzyme ii). The thermal cycling profile in an initial PCR was hot start for 5 min at 94 °C followed by 10 cycles of 60 s at 94 °C, 60 s at 60 °C and 90 s at 72 °C. Five microlitre products from the first reaction were used as a template in a second PCR. The reaction mixture contained the same components at the same concentrations as in the first PCR with the replacement of primer HL031 with HL032. The thermal cycling profile for the second PCR was: preheating 5 min at 94 °C, followed by 25 cycles of 60 s at 94 °C, 60 s at 60 °C and 90 s at 72 °C with a final extension of 10 min. PCR products were purified on an 1.5% agarose gel and the band corresponding to the amplified product was isolated with the GenElute™ Gel Purification Kit (Sigma). The recovered DNA was re-suspended in 30 µl double distilled water.

2.3. PCR amplification of the exon 2 of the DRB gene

Amplification of DRB exon 2 was achieved by using the two cattle primers HL030: 5’-ATCCTCTCTGCAGCACATTTCC-3’ and HL031: 5’-TTTAAATTCCGCTACCTGAGCT-3’ [26]. To improve the specificity of the amplification, a semi-nested PCR with high stringent conditions was employed with the nested primer HL032: 5’-TCGCCGCTGACAGGTACAATCTC-3’. The primer HL030 is homologous to the intron 1/exon 2 boundary, the primer HL031 is homologous to the intron 2/exon 2 boundary and the nested primer HL032 is complementary to the 3’ end of the exon 2. The 50 µl PCR reaction mixture contained PCR buffer, 2% DMSO, 0.1 mM of each dNTP, 25 pmol of each primer, 1.5 mM MgCl2, 200–300 ng of genomic DNA and 1.0 U Taq Polymerase (DyNAzyme ii). The thermal cycling profile in an initial PCR was hot start for 5 min at 94 °C followed by 10 cycles of 60 s at 94 °C, 60 s at 60 °C and 90 s at 72 °C. Five microlitre products from the first reaction were used as a template in a second PCR. The reaction mixture contained the same components at the same concentrations as in the first PCR with the replacement of primer HL031 with HL032. The thermal cycling profile for the second PCR was: preheating 5 min at 94 °C, followed by 25 cycles of 60 s at 94 °C, 60 s at 65 °C and 60 s at 72 °C with a final extension of 10 min. PCR products were purified on an 1.5% agarose gel and the band corresponding to the amplified product was isolated with the GenElute™ Gel Purification Kit (Sigma). The recovered DNA was re-suspended in 30 µl double distilled water.

2.4. Cloning and sequencing

Purified PCR products from both cDNA and genomic DNA templates were cloned into the TA
vector pCR2.1 (Invitrogen), and then transformed in Escherichia coli competent cells. From each individual between five and seven positive (blue/white selection) clones were picked and grown overnight in 3 ml of LB-Medium. Plasmids were isolated by the QIAprep Spin Miniprep Kit (Qiagen) and subsequently checked for the correct inserts with EcoRI digestion or by running a second PCR with M13 primers. Those clones, which contained the correct inserts, were sequenced bi-directionally with the SequithermExcel II LC Sequencing Kit (Epicentre), using fluorescent labelled M13 universal and M13 reverse primers, on the LI-COR double Laser-System (model 4200). Samples with poor cloning efficiency and sequences produced by PCR recombination were discarded. Sequence data were analysed with BioEdit [27].

2.5. Sequence analysis and statistical methods

All nucleotide and amino acid sequences were aligned with the program ClustalX [28]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 [29]. The relative frequencies of non-synonymous ($d_N$) and synonymous ($d_S$) substitution in the exon 2 were calculated according to Nei and Gjobori [30] and applying Jukes and Cantor’s correction for multiple substitutions [31]. The significance of the difference between these rates was tested with a Z-test of selection at the 5% level, whereby the $P$-values are the probability of rejecting the null hypothesis of neutrality ($d_N = d_S$) [32]. Genetic distances among all MHC class II DRB alleles was calculated based on Kimura’s [33] 2-parameter model (Gamma). Allelic frequencies and gene diversity (expected heterozygosity; $H_E$) were calculated for each population with the FSTAT program (version 2.9.3; [34]). Deviation from the Hardy–Weinberg proportion was tested by using GENPOP [35].

Neighbour-joining trees [36] for the MHC class II DRB exon 2 nucleotide sequences were constructed on nucleotide gamma distances (Kimura 2-parameter) based on all nucleotide sites and based on the third nucleotide sites (tree not shown). Five hundred bootstrap replications were performed to determine the reliability of the branching order. In accordance with the proposed nomenclature for MHC in non-human species [37], we designated the exon 2 alleles Ruru-DRB for Alpine chamois (R. r. rupicapra) with serial numbers attached.

For the calculation of the relative frequencies of non-synonymous and synonymous substitution in exon 2 of the MHC class II DRB gene the following sequences from the GenBank (with accession numbers) were used: goat (C. hircus (Cahi: AB008347-AB008362)); Spanish ibex (Capra pyrenaica (Capy: AF461692-AF461696)); sheep (O. aries (Ovar: U00204-U00237)); bighorn-sheep (O. canadensis (Ovca: AF324840-AF324861)); domestic cattle (B. taurus: Bota-DRB: U00124-U00144)). From these species a representative number of DRB exon 2 alleles were used for the phylogenetic analysis.

3. Results

3.1. cDNA analysis

Sequencing of the chamois MHC class II DRB cDNA clones revealed one sequence with an open reading frame of 792 nucleotides (Ruru-DRB04; GenBank accession number AF336340). A second DRB sequence (Ruru-DRB07) in this individual was amplified using exon 2 primers and genomic DNA as a template: this sequence may not have been amplified from cDNA under the conditions used or it might represent a second, unexpressed DRB locus. By comparison with other MHC class II DRB genes (goat, sheep, cattle) it is assumed that the chamois cDNA encodes a polypeptide of 263 amino acids of which 26 amino acids form the signal peptide, 94 codons the $\beta 1$-domain, 93 codons the $\beta 2$-domain and the remaining 50 codons determine the connecting peptide, transmembrane, and cytoplasmatic regions. A multiple alignment of the deduced amino acid sequences revealed a single gap, of three amino acids (from positions 7–9), in the signal peptide sequence of chamois as compared to all other sequences (Fig. 1). We assumed that this gap might be representing a rare splice variant because it has so far only been identified in this species.

Comparison of the deduced amino acid sequences of chamois with those of the other mammalian species show that the cysteine residues of the $\beta 1$-domain and $\beta 2$-domain as well as the potential NGT glycosylation signal (amino acid position 19–21) are conserved.
Signal peptide -29

Chamois MCVLYS----RMVALIVMLMLPLPLAWA
Goat ..........GQS..........A..........
Sheep ..........RGQ..........A..........
Cattle .V.C......GGS......
Pie .A..C...RGSX.A.T.D.VV...L.L
Cat ......C....MGGSW.T...MLI........
Dog ......C....SH3SW.T...MLI..V.N..F...
Macaque .......KLQGSCX.C..TMT..V..S..L
Gorilla .......RLQGSCX.A.T.T..V..S..L
Mouse ..W..----PQFPCX.V.LL.TV..V..L
Rat ..........W..----ARDCX.V.LL.TV..V..L
Opossum ..SAQPLGQI.W..E.V..T..L..V..TAQV..AD

B1-domain 4

Chamois REIQPFLYEKSSKECHPFFGTQVFLQDYFNYHEEELVRFDSQMGKFRAVEQGRPFDAEYNWNSQKEILEKAKRAAVDTYCHHNGYVESFTVQXR
Human GDRX.R...QV.H............YQD..Y..........V..Y..T.........DL...QR........
Macaque ..QV.P....QV..N..Q..Y..Y......V...Y..T........DF...Q..V...........
Tamarin GDRX.R...QV..Y....V....T........DF...Q..V...........

B2-domain 95

Chamois VPFIYTYVPARYQCPQGGHNLIW...VCSYFVPYQH1IFPWSFRHGGHRRFLQV1SGLLTQSTDWTQTMNIMFLQVPSFYTCQTHMRHRTSPTVYW
Sheep ..............N............................E..Q...........
Human Y.E.............S.................Q..MT..V..V........L........E..L....
Mouse .............Q..V..S............Q..K..V........L........R..E..L....

CP/TM/CY domain 182

Chamois RARSIGAQKSDKSASVGVQPVLGLFLAVGFLFYFRQKDRPRPFLQPTGLLS
Goat ..........G..............E...........
Sheep ..............N............................E..Q...........
Cattle ..............N............................E..Q...........
Cat .Q.E.............L..I.............V.............NSG...........
Dog .............Q..L..I.............V.............NSG...........
Human .......K..E.G.............L.............NSG...........
Macaque .......E..L.............GA.............NSG...........
Gorilla K..E.G.............L.............NSG...........
Tamarin .......K..E.G.............L.............NSG...........
Mouse K.Q.T..N.L.............RA.............QSG...........
Rat .K.Q.T..N.K.............RA.............QSG...........
Opossum S.Q.E.............L.............I.FG.G..IVBM.S.A.NRGS...........

269
among all species. The phylogenetic analysis showed (tree not shown) a striking clustering of the chamois sequence within the Bovidae cluster. Levels of amino acid sequence identities among the ruminants ranged from 88.7 to 93.2%, as compared to the range of 56.3–93.2% for all considered taxa (Table 1). Among the compared ruminants the overall amino acid identity was 93.4% in signal peptide sequences, 87.3% in the β1-domain, 95.4% in the β2-domain and 97.2% in the connecting peptide/transmembrane/cyttoplasmic region. Therefore, apart from the β1-domain that encodes the PBR, all regions of DRB molecules show relatively high levels of sequence identity among the ruminants considered here. The particularly high level of sequence identity in the connecting peptide/transmembrane/cyttoplasmic region indicates that this region is conserved among ruminants as a result of strong structural constraints.

3.2. Polymorphism in exon 2

Using the primers HL030, HL031, and the nested primer HL032, amplification products 284 bp in length (including primers) were obtained for all 59 chamois. From these products a total of 19 unique amino acid sequences were obtained (Fig. 2). All Ruru-DRB sequences have cysteine residues at positions 15 and 79, which are necessary for the correct folding of the DR molecules and none of the sequences showed deletions, insertions, or stop codons, indicating that all sequences found could form functional molecules. In eight individuals, we observed more than two sequences, which indicates that the primers were amplifying a second DRB locus. We excluded all individuals with more than two sequences from the analysis. The nucleotide sequences (236 bp, without the primer sequences) of the DRB exon 2 have been deposited on GenBank (GenBank accession numbers: AY368437–AY368455).

We found that 33 of 236 nucleotide sites (13.9%) and 23 of 78 amino acid sites (29.5%) were polymorphic. Among those residues considered important in peptide binding [1], 14 (66.6%) of 22 amino acid positions were variable (Fig. 2). Most of the variability was found at amino acid positions 28, 37, 38, 49, and 71 with three different residues per site, and at position 11 where five residues were observed. Amino acid replacements among all pairwise comparisons ranged from 1 to 14 with a mean (± standard deviation) of 8.1 ± 1.73. Nucleotide sequence variation among all pairwise comparisons of Ruru-DRB sequences, corrected for multiple substitutions, ranged from 0.9 to 9.1% with a mean of 4.4 ± 1.0%, and amino acid variation ranged from 1.3 to 20.1% with a mean of 11.2 ± 2.6%.

The frequency of non-synonymous substitutions \((d_N)\) was significantly higher than that of synonymous substitutions \((d_S)\) in the putative PBR (Table 2). This trend was also observed for amino acid sites outside of the putative PBR. The high ratio of non-synonymous to synonymous substitutions \((d_N/d_S = 14.2, P = 0.0003)\) indicates strong diversifying selection at the PBR. A comparison of \(d_N/d_S\) in the PBR among different Caprinae included in this study (Table 2) revealed a similarly high \(d_N/d_S\) ratio in bighorn-sheep, while in goat, sheep and Spanish ibex the ratio was significantly lower \((p = 0.02, t\)-test). The distribution of polymorphic amino acid residues among the chamois and other Caprinae-DRB sequences in this study is similar. A neighbour-joining tree in Fig. 3 presents a graphical representation of the phylogenetic relationships among DRB exon 2 alleles of chamois, other Caprinae species, and domestic cattle. This tree revealed a consistent clustering of the chamois DRB alleles into...
a single clade, despite a low level of bootstrap support. The low bootstrap values are the result of a shallow divergence between Caprinae alleles.

Table 3 presents the observed genotypic numbers and the gene diversity ($H_E$) in the studied populations. Gene diversity ranged from 0.417 in population Kaw to 0.958 in populations Ach and Ohg. No significant deviations from Hardy–Weinberg equilibrium were observed. The most frequent (0.297) allele, **Ruru-DRB01**, was found in 11 of the 15 analysed populations and the frequencies of the remaining alleles ranged from 0.008 to 0.119.

4. Discussion

In the chamois individual examined here, cDNA analysis indicated that only one **DRB** gene is

![Alignment of the putative amino acid sequences for MHC class II DRB exon 2 from chamois. Dots indicate identity in the amino acid sequence to the sequence of the Ruru-DRB01 allele and a cross indicate codons involved in the peptide-binding region (PBR) in humans [1].](image-url)
expressed in the peripheral blood. A second locus was either not expressed in the peripheral blood or was not amplified under the conditions used. The protein domains contain many of the expected characteristics including all of the conserved residues found in other mammalian species. Among the Ruminantia the $\beta_2$-domain and the connecting peptide/transmembrane/cytoplasmic region showed high sequence identity. Within the $\beta_2$-domain the putative CD4 binding site is also conserved among the compared Ruminantia. According to the phylogenetic analysis the isolated DRB gene from chamois can be considered orthologous to MHC class II DRB genes in other mammalian species (phylogenetic tree not shown).

In the present study, we have shown that chamois maintain high genetic diversity in exon 2 of the DRB locus. We found 19 alleles in a sample of 59 chamois from diverse regions of the Eastern Alps including six individuals from a local population of the Western Alps. The observed exon 2 alleles showed extensive nucleotide and amino acid divergence in particular at the putative PBR positions. For example, at the PBR amino acid position 11 we found five different amino acids in all alleles. In other analysed ruminant species similarly high amino acid substitutions at this position were found (Bighorn-sheep, goat, sheep, cattle, White-tailed deer; for comparative tables see Jugo and Vicaro [38] and Van Den Bussche et al. [9]. In the analysed chamois populations one predominant (Ruru-DRB01) allele with a frequency of 0.297 has been observed. The second most frequent allele (Ruru-DRB10) appeared already in a more than twofold lower frequency (0.119). The remaining 17 alleles were found with frequencies between 0.008 and 0.085. Gene diversity averaged to 0.76 and varied considerably among populations (from 0.417 in Kaw to 0.958 in Ach and Ohg). However, general conclusions on DRB variability in the examined chamois populations cannot be drawn due to the small sample sizes analysed presently. We suppose, Table 2

Comparison of the rate of nonsynonymous ($d_N$) and synonymous ($d_S$) substitutions for the PB-sites and non-PB-sites and their ratio among different Caprinae species. Standard errors are given in parenthesis. $N$ is the number of codons and $P$-values are the probability of rejecting the null hypothesis of neutrality ($d_N = d_S$).

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Positions</th>
<th>$N$</th>
<th>$d_N$</th>
<th>$d_S$</th>
<th>$d_N/d_S$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chamois</td>
<td>PBR</td>
<td>22</td>
<td>0.142 (0.044)</td>
<td>0.010 (0.007)</td>
<td>14.2</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Non-PBR</td>
<td>56</td>
<td>0.018 (0.009)</td>
<td>0.005 (0.004)</td>
<td>3.6</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>78</td>
<td>0.053 (0.012)</td>
<td>0.007 (0.003)</td>
<td>7.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Bighorn-sheep</td>
<td>PBR</td>
<td>21</td>
<td>0.311 (0.076)</td>
<td>0.033 (0.019)</td>
<td>9.4</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Non-PBR</td>
<td>56</td>
<td>0.045 (0.017)</td>
<td>0.042 (0.019)</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>77</td>
<td>0.106 (0.020)</td>
<td>0.040 (0.015)</td>
<td>2.7</td>
<td>0.007</td>
</tr>
<tr>
<td>Goat</td>
<td>PBR</td>
<td>21</td>
<td>0.280 (0.075)</td>
<td>0.061 (0.033)</td>
<td>4.6</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Non-PBR</td>
<td>56</td>
<td>0.063 (0.014)</td>
<td>0.049 (0.019)</td>
<td>1.3</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>77</td>
<td>0.115 (0.021)</td>
<td>0.052 (0.016)</td>
<td>2.2</td>
<td>0.009</td>
</tr>
<tr>
<td>Sheep</td>
<td>PBR</td>
<td>21</td>
<td>0.242 (0.068)</td>
<td>0.044 (0.030)</td>
<td>5.5</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Non-PBR</td>
<td>56</td>
<td>0.041 (0.011)</td>
<td>0.047 (0.017)</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>77</td>
<td>0.089 (0.018)</td>
<td>0.046 (0.014)</td>
<td>1.9</td>
<td>0.05</td>
</tr>
<tr>
<td>Spanish ibex</td>
<td>PBR</td>
<td>21</td>
<td>0.415 (0.109)</td>
<td>0.091 (0.049)</td>
<td>4.6</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Non-PBR</td>
<td>56</td>
<td>0.065 (0.017)</td>
<td>0.083 (0.033)</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>77</td>
<td>0.144 (0.027)</td>
<td>0.084 (0.027)</td>
<td>1.7</td>
<td>0.09</td>
</tr>
<tr>
<td>All Caprinae</td>
<td>PBR</td>
<td>21</td>
<td>0.290 (0.068)</td>
<td>0.051 (0.022)</td>
<td>5.7</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Non-PBR</td>
<td>56</td>
<td>0.051 (0.024)</td>
<td>0.060 (0.021)</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>77</td>
<td>0.106 (0.019)</td>
<td>0.057 (0.016)</td>
<td>1.9</td>
<td>0.04</td>
</tr>
<tr>
<td>Cattle</td>
<td>PBR</td>
<td>21</td>
<td>0.322 (0.079)</td>
<td>0.121 (0.052)</td>
<td>2.7</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Non-PBR</td>
<td>56</td>
<td>0.037 (0.011)</td>
<td>0.027 (0.015)</td>
<td>1.4</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>77</td>
<td>0.105 (0.020)</td>
<td>0.051 (0.003)</td>
<td>2.1</td>
<td>0.02</td>
</tr>
</tbody>
</table>
that allelic diversity in chamois could be much higher than revealed in the present study, because many parts of the Eastern Alps, and most of the Western Alps have not been analysed. While high levels of DRB polymorphism were found in red deer [10], African buffalo [14], and white-tailed deer [9], clearly less variability was detected in moose from Scandinavia and North America [11–13], and virtually no polymorphism was present in musk-ox and fallow deer [13]. While the authors concluded that the lack of MHC class II DRB diversity in musk ox could be explained as resulting from bottleneck effects, such neutral population genetic mechanisms cannot be discounted for fallow deer. As regards, the low level of diversity at the DRB and DQB loci in moose, the authors suggest both a bottleneck history and a relaxed pathogen-driven selection as possible causes. The low allelic diversity, that is obviously generally typical, has also been hypothesised as resulting from low pathogen-driven selection in this species with a solitary life style contrary to the herd-living cattle, that has generally high diversity [11,12]. The presently studied chamois revealed a distinct partitioning of mitochondrial gene pools into several populations, which was interpreted as originating from immigration into the study region from different Pleistocene refugia [39]. Such a phylogeographic scenario with possible differential adaptation to separate regional Pleistocene environments might have contributed, at least in part, to the revealed high diversity at the MHC class II DRB locus. However, the presently observed alleles found in the Eastern Alps did not parallel the earlier found phylogeographic structuring. In principle, this corresponds to the population genetic pattern in the nuclear genome of the studied chamois as evidenced by a multi-locus allozyme approach, which revealed a rather panmictic network of gene flow and only little average differentiation, supposedly mediated predominantly by dispersing males. The high variability at

Fig. 3. Neighbour-joining tree of different Caprinae species and from cattle based on MHC class II DRB exon 2 nucleotide sites. Alleles from Alpine chamois (Ruru-DRB), sheep (Ovca-DRB), goat (Cahi-DRB), Bighorn-sheep (Ovca-DRB), Spanish ibex (Capy-DRB), musk ox (Ovmo-DRB), and cattle (Bota-DRB) are included. Numbers indicate bootstrap percentage (1000 iterations).
the DRB locus in chamois might also be a consequence of their social behaviour and aggregation into more or less open female groups and male groups maintained throughout long periods of the year. Moreover, it indicates that the initially hypothesised relaxed pathogen-driven selection in harsh Alpine habitats would not play an important role in determining the level of polymorphism at the DRB locus of chamois.

We observed the occurrence of more than two different sequences in a few specimens, indicating that sequences from a duplicated DRB gene might have been amplified. However, we were unable to assign sequences to specific loci and excluded those individuals from the analysis in which more than two sequences were observed. Among ruminants, so far, in only three species (cattle, sheep, and red deer) two functional DRB loci have been reported. In a study by Jugo and Vicario [38] on DRB exon 2 variability in sheep, three or four exon 2 sequences were found in some individuals. The authors concluded that these sequences might have been derived from the second expressed (probably functional) DRB locus in sheep. So far, no haplotype variations in the MHC class II DRB locus in Artiodactyls have been described. In different other taxa groups, however, as e.g. in the green monkey, Cercopithecus aethiops [40], Ma’s night monkey, Aotus nancymaae [41], in cichlids [42], and in three-spined sticklebacks, Gasterosteus aculeatus [43] haplotype variations in class II were reported.

The phylogenetic analysis of the chamois DRB exon 2 alleles and DRB exon 2 alleles from other Caprinae species including domestic cattle (Fig. 3) revealed a scattered distribution of the alleles of the different species throughout the dendrogram, only the chamois alleles forming a single cluster. Though no shared alleles were found among the compared Caprinae species, certain alleles of some species are closer related to alleles of other species than they are to each other (e.g. Cahi-DRBp09 and Ovar-DRB21; Cahi-DRBp15 and Copy-DRB1-1; Ovar-DRB30 and

Table 3
Distribution and allele frequencies of the chamois exon 2 alleles, sample size, gene diversity (H_E), and level of significance for deviations from Hardy–Weinberg equilibrium (P(HWE)) are shown for each population

<table>
<thead>
<tr>
<th>Populations</th>
<th>Ach</th>
<th>Ens</th>
<th>Gai</th>
<th>Kal</th>
<th>Kan</th>
<th>Kaw</th>
<th>Kit</th>
<th>Nob</th>
<th>Ohg</th>
<th>Rax</th>
<th>Sbs</th>
<th>Sch</th>
<th>Tau</th>
<th>Vds</th>
<th>Wal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ruru-DRB01</td>
<td>0.250</td>
<td>0.250</td>
<td>0.500</td>
<td>0.250</td>
<td>0.500</td>
<td>0.750</td>
<td>–</td>
<td>0.667</td>
<td>0.250</td>
<td>–</td>
<td>0.167</td>
<td>0.500</td>
<td>0.433</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB02</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.167</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB03</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.167</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB04</td>
<td>0.250</td>
<td>–</td>
<td>0.500</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.250</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB05</td>
<td>0.125</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.250</td>
<td>–</td>
<td>0.167</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB06</td>
<td>–</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>0.500</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.167</td>
<td>–</td>
<td>0.033</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB07</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.167</td>
<td>–</td>
<td>0.033</td>
<td>0.125</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB08</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB09</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.250</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.500</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB10</td>
<td>–</td>
<td>–</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.125</td>
<td>0.625</td>
<td>–</td>
<td>0.500</td>
<td>0.200</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB11</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.033</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB12</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.500</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB13</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.250</td>
<td>0.500</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.167</td>
<td>–</td>
<td>0.250</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB14</td>
<td>–</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB15</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.083</td>
<td>0.375</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB16</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB17</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.167</td>
<td>0.100</td>
<td>–</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB18</td>
<td>–</td>
<td>0.250</td>
<td>–</td>
<td>0.375</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.125</td>
<td>0.333</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ruru-DRB19</td>
<td>–</td>
<td>0.125</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.417</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sample size</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Gene diversity (H_E)</td>
<td>0.958</td>
<td>0.917</td>
<td>–</td>
<td>0.833</td>
<td>–</td>
<td>0.417</td>
<td>–</td>
<td>0.583</td>
<td>0.958</td>
<td>0.583</td>
<td>0.917</td>
<td>–</td>
<td>0.762</td>
<td>0.600</td>
<td>0.833</td>
</tr>
<tr>
<td>P(HWE)</td>
<td>0.323</td>
<td>1.0</td>
<td>0.671</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.135</td>
<td>1.0</td>
<td>1.0</td>
<td>0.315</td>
<td>0.393</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>
Ovca-DRB′13); this was also evident if we constructed the phylogenetic tree based the third nucleotide position (tree not shown). Similarly, in sheep, certain alleles were closer related to some goat alleles than to other sheep alleles [38]. Among 10 ruminant species (cattle, bison, musk-ox, goat, sheep, moose, roe deer, reindeer, fallow deer, red deer) studied by Mikko et al. [13], no evidence of shared alleles was found. The latter authors pointed out that even between cattle and bison, which are assumed to have diverged from a common ancestor about 1–1.5 million years, no shared alleles were detected. There is still considerable disagreement about the phylogeny of some species within the subfamily of Caprinae. The genus Rupicapra belongs to the most basal tribe (Rupicaprini) within the Caprinae. However, recent phylogenetic studies have contradicted this assumption [23] and indeed it appears that the Caprinae phylogeny remains unresolved [44,45]. To better understand evolutionary patterns of MHC polymorphism in chamois, the presently found alleles should be compared with those of the closely related Pyrenean chamois (Rupicapra pyrenaica), that is considered a sister taxon to the Alpine chamois. Both species likely have separated during the Middle Pleistocene [39,46].

The higher ratio of dN to dS and the high allelic diversity observed in chamois suggests positive selection on the DRB locus for maintaining high allelic variability. The high ratio of dS/dN (Table 2) revealed in chamois is the results of a generally very low rate on synonymous substitutions in the exon 2, and an excess of non-synonymous substitutions in particular in the PBR. In fact, dS is generally lower in chamois than in the other analysed ruminants. It is thus reasonable to assume that the difference in dS/dN may reflect the young age of most of the chamois alleles.

We conclude that particularly those sites, which are most polymorphic, are under selection for variability. In human it was shown that in particular the polymorphic residues at positions 70, 71, and 74 strongly influence the peptide-binding specificity [2]. Comparing the residue motifs at amino acid positions 70, 71, and 74 between chamois and other Caprinae species revealed shared motifs at these PB-sites. A common motif at these positions is RKA; it can be observed in goat, sheep, bighorn-sheep, and in chamois (in six alleles), suggesting either a common ancestry of these specific residues (sympleiomorphic characters) or alternatively convergent evolution for such a motif in relation to an important response to an antigen from pathogens/parasites common to all Caprinae species.

Acknowledgements

We would like to thank the following persons for organising sampling, providing samples, or for logistic support: J Schöchel, A Pacher-Theinburg, F Österbauer, from the Landesveterinärdirektion Salzburg; J Kristan, secretary of the Salzburger Jägerschaft; W Kulterer, secretary of the Kärntner Jägerschaft, and many hunters for shooting and collecting organ samples. We would like to thank B Jordan from the Institute of Zoology, Zoological Society of London, for helpful comments on a previous version of this manuscript. The Government of Lower Austria provided financial support for this study.

References

Ellengren H, Mikko S, Wallin K, Andersson L. Limited
Swarbrick PA, Schwaiger FW, Epplen JT, Buchan GS, Griffin
Jordan WC, Bruford WM. New perspectives on mate choice
The MHC sequencing consortium, Complete sequence and
Mikko S, Roed K, Schmutz S, Andersson L. Monomorphism
Randi E, Fusco G, Lorenzini R, Toso S, Tosi G. Allozyme
evolution within Cervidae. Immunogenetics 1999;49:
329–37.
Swarbrick PA, Schaiger FW, Epplen JT, Buchan GS, Griffin
Jordan WC, Bruford WM. New perspectives on mate choice
The MHC sequencing consortium, Complete sequence and
Mikko S, Roed K, Schmutz S, Andersson L. Monomorphism
Randi E, Fusco G, Lorenzini R, Toso S, Tosi G. Allozyme
evolution within Cervidae. Immunogenetics 1999;49:
329–37.
Swarbrick PA, Schaiger FW, Epplen JT, Buchan GS, Griffin
Jordan WC, Bruford WM. New perspectives on mate choice
The MHC sequencing consortium, Complete sequence and
Mikko S, Roed K, Schmutz S, Andersson L. Monomorphism
Randi E, Fusco G, Lorenzini R, Toso S, Tosi G. Allozyme
evolution within Cervidae. Immunogenetics 1999;49:
329–37.


