Journal of Mammalogy, 2024, **XX**, 1–11
https://doi.org/10.1093/jmammal/gyae008
Advance access publication 12 February 2024
Research Article

Research Article

Diversity of MHC class II *DRB* alleles in the Northern chamois genus *Rupicapra*

Sunčica Stipoljev,[1] Elena Buzan,[2,3,*] Laura Iacolina,[1,2] Toni Safner,[4] Andrea Rezić,[1] Ana Galov,[5] Krešimir Križanović,[6] Hüseyin Ambarli,[7] MariaCruz Arnal,[8] Elmar Babaev,[9] Ferdinand Bego,[10] Attila Farkas,[11] Dragan Gačić,[12] Peter Lazar,[13] Vladimir Maletić,[14] Georgi Markov,[15] Dragana Milošević,[16] Haritakis Papaioannou,[17] Massimo Scandura,[18] and Nikica Šprem[1]

[1]Faculty of Agriculture, Department of Fisheries, Apiculture, Wildlife Management and Special Zoology, University of Zagreb, 10000 Zagreb, Croatia
[2]Faculty of Mathematics, Natural Sciences and Information Technologies, University of Primorska, 6000 Koper, Slovenia
[3]Faculty of Environmental Protection, 3320 Velenje, Slovenia
[4]Faculty of Agriculture, Department of Plant Breeding, Genetics and Biometrics, University of Zagreb, 10000 Zagreb, Croatia
[5]Faculty of Science, Department of Biology, University of Zagreb, 10000 Zagreb, Croatia
[6]Faculty of Electrical Engineering and Computing, Department of Electronic Systems and Information Processing, University of Zagreb, 10000 Zagreb, Croatia
[7]Department of Wildlife Ecology and Management, Düzce University, 81010 Düzce, Turkey
[8]Facultad de Veterinaria, Departamento de Patología Animal, Universidad de Zaragoza, 50002 Zaragoza, Spain
[9]Precaspian Institute of Biological Resources of the Dagestan Federal Research Centre of the Russian Academy of Sciences, 367000 Makhachkala, Russia
[10]Faculty of Natural Sciences, Department of Biology, University of Tirana, 1000 Tirana, Albania
[11]Faculty of Technical and Human Sciences, Sapientia Hungarian University of Transylvania, 540003 Târgu-Mureș, Romania
[12]Faculty of Forestry, University of Belgrade, 11000 Belgrade, Serbia
[13]Department for Breeding and Disease of Game and Fish, University of Veterinary Medicine and Pharmacy, 04000 Košice, Slovakia
[14]Faculty of Forestry, Ss. Cyril and Methodius University, 1000 Skopje, North Macedonia
[15]Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1000 Sofia, Bulgaria
[16]Faculty of Sciences and Mathematics, Department of Biology, University of Montenegro, 80000 Podgorica, Montenegro
[17]Department of Biological Applications and Technology, Biodiversity Conservation Lab, University of Ioannina, 45221 Ioannina, Greece
[18]Department of Veterinary Medicine, University of Sassari, 07100 Sassari, Italy

[*]Corresponding author: Faculty of Mathematics, Natural Sciences and Information Technologies, University of Primorska, 6000 Koper, Slovenia. Email: elena.buzan@upr.si

Associate Editor was Melissa Hawkins

Abstract

Major histocompatibility complex (MHC) genes are commonly used markers for monitoring adaptive genetic and evolutionary potential of species. In this study, we investigated genetic variation of the MHC class II *DRB* locus in the chamois genus *Rupicapra* by using next-generation sequencing. Sequencing of 102 samples led to the identification of 25 alleles, 11 of which are novel. The high ratio of the relative rates of nonsynonymous to synonymous mutations (dN/dS) suggests a signal of positive selection on this locus. We analyzed patterns of genetic variation within and among 2 subspecies of Northern Chamois and compared them to previously published studies using neutral markers to provide a basis for assessing the effects of demographic processes. Our analyses have shown that alleles are likely to be maintained by balancing selection in different populations with similar frequencies and that this mechanism also works in small, isolated populations that are strongly affected by genetic drift.

Key words: allelic polymorphism, major histocompatibility complex, next-generation sequencing, *Rupicapra pyrenaica*, *Rupicapra rupicapra*.

Current climate change is a major threat to biodiversity, especially to mountain-dwelling species that live in high-altitude habitats (Lovari et al. 2020). Species unable to adapt or move will face local or global extinction and this is more likely to happen to species with narrow climatic and habitat requirements. The ability of natural populations to adapt to new environmental conditions is crucial for their survival and mostly determined by the standing genetic variation in each population. It is possible that because of small population sizes threatened taxa have lost the ability to adapt to changing environmental conditions, and this may be 1 reason why such species are at greater risk of extinction (Amos and Balmford 2001). Host genetic diversity may also increase tolerance or resistance to pathogens (Altizer et al. 2003; Spielman et al. 2004), which is especially important when considering the potential impact of ongoing climate change, as it is expected to shift pathogen ranges and potentially expose threatened species to novel pathogens (Štefančíková et al. 2011).

Submitted 26 September 2022; Accepted 30 January 2024

Because neutral genetic variation often provides an incomplete picture of the evolutionary potential of populations (e.g. Bekessy et al. 2003; Hoffmann et al. 2003), it has been proposed adaptive genetic diversity should also be monitored in natural populations. Adaptive variation is defined as "genetic variation that confers a fitness advantage" (Hedrick 2001:633). Genes suitable as indicators of adaptive genetic diversity should be highly variable. Major histocompatibility complex (MHC) genes encode cell surface glycoproteins involved in antigen presentation to T cells and subsequent initiation of the adaptive immune response in vertebrates (Klein 1986). The high levels of polymorphism at MHC class II loci found in most vertebrate species are thought to be maintained by different forms of balancing selection, including heterozygosity advantage (Punt et al. 2019).

MHC genes show extraordinary intraspecific and intraindividual genetic diversity due to both a large number of alleles per locus and the presence of multiple paralogous and presumably functionally equivalent loci (Zagalska-Neubauer et al. 2010). Remarkably, polymorphism at MHC genes is most pronounced at the amino acid sites that encode the peptide-binding region (PBR; Brown et al. 1993).

Pathogen-driven balancing selection has been proposed as one of the most important evolutionary forces for the maintenance of MHC polymorphism, which causes sharing of allelic lineages between different animal species, leading to a pattern of trans-species MHC polymorphism (Piertney and Oliver 2006). Mechanisms resulting from the 'rare-allele advantage' hypothesis are thought to maintain the high diversity of MHC genes at the population level (Ejsmond and Radwan 2015). This hypothesis assumes that rare (e.g. new) MHC alleles that have a higher efficacy in pathogen recognition confer an advantage to the host, spread through the population, and become common (Bernatchez and Landry 2003). Although pathogen-mediated selection is important for preserving MHC functional variation, other mechanisms including disassortative mating preferences, maternal–fetal interactions, recombination, and gene duplication have been suggested as alternative or complementary mechanisms maintaining MHC diversity (Miller and Lambert 2004; Spurgin and Richardson 2010; Juola and Dearborn 2011).

The ability of natural populations to maintain genetic variation in functional genes depends on the selective pressures involved. Balancing selection is thought to counteract the effects of genetic drift and slow the rate of allele fixation (Sommer 2005). In many cases, differentiation patterns between populations can only be detected at functional genes under selection (Awadi et al. 2018). Because MHC allele frequency shifts are considered to have an adaptive value, understanding how such functional variation is generated and maintained within populations is an important component to species conservation and making optimal management decisions (Funk et al. 2012). Strong selection pressures on MHC genes are ultimately responsible for high levels of polymorphism and can lead to discrepancies between patterns of MHC and neutral variation in natural populations (Alcaide 2010).

The chamois is one of the most iconic mammals of Europe and Asia Minor, of which there are currently 2 species recognized: Northern Chamois (*Rupicapra rupicapra*) and Southern Chamois (*Rupicapra pyrenaica*; Corlatti et al. 2011, 2022). The Northern Chamois (with the geographically distinct subspecies *cartusiana*, *rupicapra*, *tatrica*, *carpatica*, *balcanica*, *asiatica*, and *caucasica*) is distributed across a large part of the mountainous regions from Europe to the Caucasus and Turkey. The Southern Chamois (with geographically distinct subspecies *parva*, *pyrenaica*, and *ornata*) has a discontinuous distribution in southwestern Europe including the Pyrenees, the Cantabrian Mountains, and the central Apennines of Italy (Corlatti et al. 2011, 2022). According to the IUCN Red List, both chamois species are classified as Least Concern, with the Northern Chamois having a stable population (Anderwald et al. 2021) of just under 500,000 individuals (Corlatti et al. 2022), while the Southern Chamois has an increasing population trend with a size of around 53,000 individuals (Herrero et al. 2020). Although neither chamois species is threatened, most subspecies are found in restricted areas where they face various threats including poaching, overhunting, habitat loss and degradation, human disturbance, disease, competition with livestock and wild ungulates, hybridization due to translocation of other subspecies, and climate change (Corlatti et al. 2022).

Despite the chamois being a common species, there have been only a few studies analyzing genetic diversity of the MHC *DRB2* gene in this species to date. The first study focused on the Alpine chamois (*R. r. rupicapra*) population in Austria (Schaschl et al. 2004). The subsequent study included the same Austrian population of Alpine chamois and also incorporated the Apennine chamois (*R. p. ornata*) from the Abruzzi Apennines in Italy (Schaschl et al. 2005). Another study investigated the Pyrenean chamois (*R. p. pyrenaica* and *R. p. parva*) populations from 2 locations in Spain (Alvarez-Busto et al. 2007). Furthermore, we recently published a study on Alpine chamois from Croatia and Slovenia (Stipoljev et al. 2020). On the other hand, numerous studies have investigated the influence of population isolation and rocky habitat fragmentation on neutral genetic diversity and population structure (see Corlatti et al. 2022). Rocky habitats are inherently fragmented at different spatial scales, depending on the elevation and geology of the surrounding terrain, and consequently dispersal between habitats may be restricted (Brown 2001). Such habitat fragmentation affects genetic structure by restricting gene flow between isolated populations and keeping effective population sizes low (Ezard and Travis 2006). Limited gene flow between small fragmented populations leads to increased isolation by distance and strong genetic drift (Willi et al. 2007), which was shown by neutral genetic markers (microsatellites). *Rupicapra rupicapra rupicapra* populations from the Italian Alps (Soglia et al. 2010) and Slovenia (Buzan et al. 2013) have higher genetic diversity than populations of *R. r. balcanica* from the Dinaric Mountains (Šprem and Buzan 2016; Rezić et al. 2022b), Bulgaria (Markov et al. 2016), and Greece (Papaioannou et al. 2019), which may indicate stronger genetic drift and bottleneck within these populations.

In this study, we used Ion Torrent sequencing to analyze genetic variability at exon 2 of the MHC class II *DRB* locus of Northern Chamois subspecies. The aims of the study were to: (i) investigate the extent and spatial distribution of genetic variation of the MHC class II *DRB* locus in chamois throughout distribution range and fill the gap in previously published data as suggested by Corlatti et al. (2022); and (ii) better understand how selection shapes the genetic diversity of this locus, thereby informing conservation decisions based on a better understanding of the evolutionary potential of chamois.

Materials and methods

Study area and data collection

We obtained 110 chamois samples—102 from Northern Chamois and 8 from Southern Chamois (Supplementary Data SD1). Samples were collected during regular hunts or after natural death (102 tissue samples) or from museum collections (8 bone

samples). The study was conducted according to the ethical and welfare standards presented in the Official Gazette of the Republic of Croatia (OG 102/2017, Animal Protection Act) and Regulation on the Protection of Animals Used for Scientific Purposes (OG 55/13), with the approval of the Bioethical Committee for the Protection and Welfare of Animals of the University of Zagreb Faculty of Agriculture (UR.BR. 251-71-29-02/19-21-1). Northern Chamois samples include the subspecies *R. r. rupicapra* (Alpine chamois; $n = 57$), *R. r. balcanica* (Balkan chamois; $n = 31$), *R. r. tatrica* (Tatra chamois; $n = 6$), *R. r. carpatica* (Carpatian chamois; $n = 5$), *R. r. asiatica* (Anatolian chamois; $n = 2$), and *R. r. caucasica* (Caucasian chamois; $n = 1$). Since our main focus was on Northern Chamois, we used only 8 samples from the Southern Chamois for genetic comparison. We incorporated an additional 34 chamois MHC *DRB* sequences from GenBank into the analysis, including 21 sequences from Northern Chamois and 13 from Southern Chamois. These sequences had the same length as those generated in our study (Supplementary Data SD2). However, we did not included the shorter alleles published in GenBank due to the loss of polymorphism at the end of the sequence.

MHC genotyping

DNA was isolated from tissue samples using a peqGOLD Tissue DNA Mini Kit (VWR International) according to the manufacturer's instructions, while bone samples were treated following the procedure described in Buzan et al. (2020). MHC sequences were amplified and genotyped according to Stipoljev et al. (2020). Briefly, a 236-bp fragment of the second exon of the MHC class II *DRB* gene was amplified with primers HL030 (ATCCTCTCTGCAGCACATTTCC) and HL032 (TCGCCGCTGCACAGTGAAACTCTC; Schaschl et al. 2004). The forward primers were accompanied by 10- to 12-bp barcodes with a specified "GAT" linker to distinguish individuals/samples and by adaptor sequences required for Ion Torrent sequencing. PCR products from triplicates were pooled and purified using Agencourt AMPure XP beads (Agencourt Bioscience Corporation, Beverly, Massachusetts). Concentrations of the pooled and purified amplicons were estimated with the Qubit 3.0 Fluorometer using the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific). Amplicons were then normalized to 5 ng, pooled, and purified again using Agencourt AMPure XP beads. The size and quality of the pooled amplicons were checked using the Agilent DNA High Sensitivity Kit on the 2100 Bioanalyzer (Agilent, Santa Clara, California). The final library was normalized to 100 pM and sequenced using the Ion Torrent S5 on a 314 chip (Thermo Fisher Scientific), with amplicon sequencing being performed in 3 runs.

AmpliSAS software (Sebastian et al. 2016) was used to derive individual MHC genotypes. Initial quality and length filtering of the raw data was performed using AmpliCLEAN by removing reads with a Phred quality score below 30. AmpliSAS clusters true variants with their potential artifacts based on platform-specific error rates. We used the AmpliSAS default parameters for Ion Torrent sequencing technology: a substitution error rate of 0.5% and an indel error rate of 1%. An exact length (236 bp) was required for the dominant sequence within a cluster. Based on previous work on this species, we expected no more than 2 *DRB* variants per individual, so we left the threshold for "minimum dominant frequency" of clustering at the default values of 25%. The maximum number of reads per amplicon that the AmpliSAS web server can process is 5,000—for this reason samples with a higher number of reads were also genotyped with the locally installed AmpliSAS script, but since the results were the same, we took 5,000 reads as the maximum number. Variants with reads above the threshold and exact length in *DRB* exon 2 were aligned and translated into protein sequences.

MHC *DRB* allelic diversity

Unique sequences that passed the filtering were aligned and confirmed to be chamois MHC *DRB* exon 2 alleles using MEGA X (Kumar et al. 2018) by comparing them with alleles downloaded from GenBank (Supplementary Data SD2). All correctly identified alleles, either newly identified or downloaded from GenBank, were included in calculation of variability and divergence focusing on differences between species—whereas subspecies with 6 or fewer individuals were excluded when measuring differences between Northern Chamois subspecies.

We calculated sequence polymorphism measures, including the number of segregating sites (S), average number of nucleotide differences (k), and nucleotide diversity (π) for PBR and non-PBR sites separately with DnaSP v. 6.12.01 (Rozas et al. 2017). Putative PBR sites were identified based on human HLA molecules (Brown et al. 1993). Average pairwise nucleotide distances (Jukes–Cantor model with a gamma distribution), and Poisson-corrected amino acid distances were calculated in MEGAX for all, PBR, and non-PBR sites.

Supertype identification and clustering

To investigate functional MHC *DRB* diversity within the genus *Rupicapra*, we used 45 unique nucleotide sequences of MHC *DRB* alleles (Supplementary Data SD1 and SD2) and clustered them into supertypes. The first step was to identify amino acids that reflect physicochemical differences between alleles. Therefore, we performed clustering based on amino acid polymorphism at the positively selected amino acid sites (PSSs). For PSSs we retained all codons having the Bayes Empirical Bayes (BEB) posterior probability >95% in model M8. The PSS of each allele was numerically characterized by a set of 4 physicochemical descriptors for each amino acid (hydrophobicity, steric bulk, polarity, electronic effects; Doytchinova and Flower 2005). To describe phylogenetic relationships among *DRB* alleles and supertypes, we created a neighbor-net network in SplitsTree v.4 (Huson and Bryant 2006).

Pattern of genetic structure in Northern Chamois subspecies

We used HP-RARE (Kalinowski 2004) to estimate allelic richness (Ar), expressed as the expected number of alleles for genotyped *R. r. rupicapra* and *R. r. balcanica* samples. The other subspecies were excluded from analysis due to small sample size (<6; Supplementary Data SD1). We calculated pairwise F_{ST} only between *R. r. rupicapra* and *R. r. balcanica* with Arlequin v. 3.5 (Excoffier and Lischer 2010). The neutrality test of Tajima's D (Tajima 1989) was calculated in DnaSP to evaluate demographic changes. To reveal the amount of genetic variation within and among the 2 subspecies we performed discriminant analysis of principal components (DAPC) with "adegenet" package v 2.0.1 (Jombart 2008; Jombart et al. 2010) in R (R Development Core Team 2020).

Signatures of selection on MHC *DRB* exon 2 alleles

We analyzed positive selection separately on the entire MHC *DRB* exon 2 sequences and on the extracted PBR, applying the 1-tailed Z-test with standard errors resulting from 10,000 bootstrap replicates in MEGA X including 45 unique nucleotide sequences of

MHC *DRB* alleles (Supplementary Data SD1 and SD2). We used EasyCodeML v.1.0 (Gao et al. 2019) to identify codons affected by positive selection based on a Bayesian approach. The models implemented in the analysis were M0, M1a, and M7, which do not allow for positive selection and serve as null models for M3, M2a, and M8, respectively. The nested models were compared using the likelihood ratio test, whereas posterior probabilities for site classes in models M2a and M8 were calculated using the BEB approach. The models implemented in this study were M2a and M8. Further, we assessed the influence of positive selection on individual codons using single-likelihood ancestor counting, fixed effects likelihood, mixed effects model of evolution, and fast unconstrained Bayesian approximation methods performed in the Datamonkey 2.0 server (Weaver et al. 2018).

Results

Allele variation and sequence diversity

In the 110 chamois samples genotyped, we detected 25 alleles for MHC *DRB* exon 2—5 alleles were found in Southern Chamois and 21 in Northern Chamois. Alleles Rupy-DRB04 and Ruru-DRB01 were found in both species (Supplementary Data SD1). After initial filtering with a threshold higher than 200 reads per allele of the Ion Torrent raw data, amplicon coverage in samples ranged from 216 to the maximum of 5,000 reads allowed by AmpliSAS, with an average of 2,387 ± 1,880 (SD) reads (Supplementary Data SD3). None of the individuals had more than 2 alleles, confirming previously reported data (Fuselli et al. 2018). Despite variation in sample sizes, we identified unique alleles in many subspecies. Eleven novel *DRB* exon 2 sequences were identified and designated as *Ruru-DRB* from 44 to 54, in accordance with the nomenclature previously established by Klein et al. (1990). The novel alleles have been deposited in GenBank under accession numbers OL421550 to OL421560.

The most common allele was *Ruru-DRB01*, which was identified in 55 individuals with a frequency of 50%. Twenty-three alleles had a frequency <10%, while 7 alleles were identified in only 1 individual each (Supplementary Data SD4 and SD5). Thirty individuals (27.3%) were homozygous, most of which (56.7%) were homozygous for the most common allele *Ruru-DRB01*. Of the 25 alleles found, only 2 (*Rupy-DRB11* and *Rupy-DRB12*) were not present in the Northern Chamois. The putative novel alleles *Ruru-DRB53* and *Ruru-DRB54* were found only in *R. r. carpatica*; *Ruru-DRB51* and *Ruru-DRB52* in *R. r. asiatica*; *Ruru-DRB47*, *Ruru-DRB49*, and *Ruru-DRB50* in *R. r. balcanica*; and *Ruru-DRB48* in *R. r. rupicapra* (Fig. 1).

The nucleotide alignment of 45 *DRB* exon 2 chamois sequences, including both newly generated and GenBank sequences, revealed 38 segregating (variable) nucleotide sites that led to the identification of the abovementioned 25 alleles. The overall nucleotide evolutionary distance calculated using the Jukes–Cantor substitution model with a gamma distribution shape parameter was 5%—whereas the amino acid evolutionary distance calculated using the Poisson substitution model was 11% (Table 1). Average nucleotide diversity was $\pi = 0.04$, and the average number of nucleotide differences among alleles was $k = 9.76$ (8.96 in Northern Chamois and 10.87 in Southern Chamois). Following the model proposed by Brown et al. (1993) for the MHC *DRB* exon 2 protein structure in humans, we attributed 22 of 78 (28%) codons in MHC DRB alleles to the PBR. For Northern Chamois, 14 of 23 (61%) variable codon positions were within the putative PBR. The higher value applies to Southern Chamois, where 14 of 17 (82%) variable codon positions were within the putative PBR.

Functional diversity among MHC *DRB* alleles

The clustering procedure revealed 5 supertypes in chamois based on 14 PSSs (Fig. 1; Supplementary Data SD1)—all present in Northern Chamois but only 4 in Southern Chamois. The number of alleles assigned to each supertype ranged from 7 to 12 (Fig. 1). The mean number of supertypes per individual was 1.50 for Southern Chamois and 1.61 for Northern Chamois (Supplementary Data SD1). The number of supertypes varied not only between species but also between subspecies. Despite the small sample sizes of Southern Chamois and Northern Chamois subspecies *R. r. asiatica* and *R. r. caucasica* and the correspondingly small number of alleles detected, there was no reduction in the number of supertypes, i.e. each allele corresponded to a distinct supertype (Fig. 1; Supplementary Data SD1).

The neighbor-net network did not reveal a clear pattern of allele and supertypes clustering between Northern and Southern Chamois, as alleles and sypertypes of the 2 species were mainly spread across the network (Supplementary Data SD6).

Pattern of genetic structure in Northern Chamois subspecies

Table 2 shows the values of the diversity parameters for Northern Chamois across its 2 subspecies. The number of alleles (*A*) was 12 in *R. r. rupicapra* and 11 in *R. r. balcanica*. Allelic richness (*Ar*) ranged from 10.20 in *R. r. rupicapra* to 11.00 in *R. r. balcanica* and was estimated to be 15.81 in Northern Chamois. The proportion of heterozygous individuals was the highest in the subspecies *R. r. rupicapra*. Tajima's *D* values were positive and significant in Northern Chamois and its *R. r. rupicapra* subspecies, but nonsignificant within *R. r. balcanica* (Table 2). The F_{ST} value between *R. r. rupicapra* and *R. r. balcanica* subspecies was significant ($F_{ST} = 0.12$, $P < 0.05$). The DAPC has clearly separated the subspecies *R. r. rupicapra* and *R. r. balcanica* on the horizontal axis—the additional distinction on the vertical axis is between sampling locations of subspecies *R. r. rupicapra* (Fig. 2).

Signatures of selection on MHC *DRB* exon 2 alleles

Global estimates of ratio of the relative rates of nonsynonymous to synonymous mutations (dN/dS), averaged across all codon sites using the codon-based Z-test of selection, demonstrated positive selection at the *DRB* locus. The nonsynonymous mutation rate (dN = 0.06 in Southern Chamois and 0.05 in Northern Chamois) exceeded the synonymous mutation rate (dS = 0.004 in Northern Chamois and 0.01 in Southern Chamois; Table 3). The *DRB* locus showed signs of strong selection pressure. Methods of calculating dN/dS values on individual codons (models M2a and M8) identified up to 14 codons (17.95%; posterior probabilities >95%) predicted to be affected by positive selection (Table 4). The selection models revealed different levels of selection pressure at the analyzed locus. Mean values of dN/dS calculated using model M2a for individual codons are presented in Supplementary Data SD7.

Discussion

In this study, polymorphism of the MHC class II *DRB* locus was investigated in chamois with Ion Torrent next-generation sequencing. We provide a characterization of the adaptive genetic

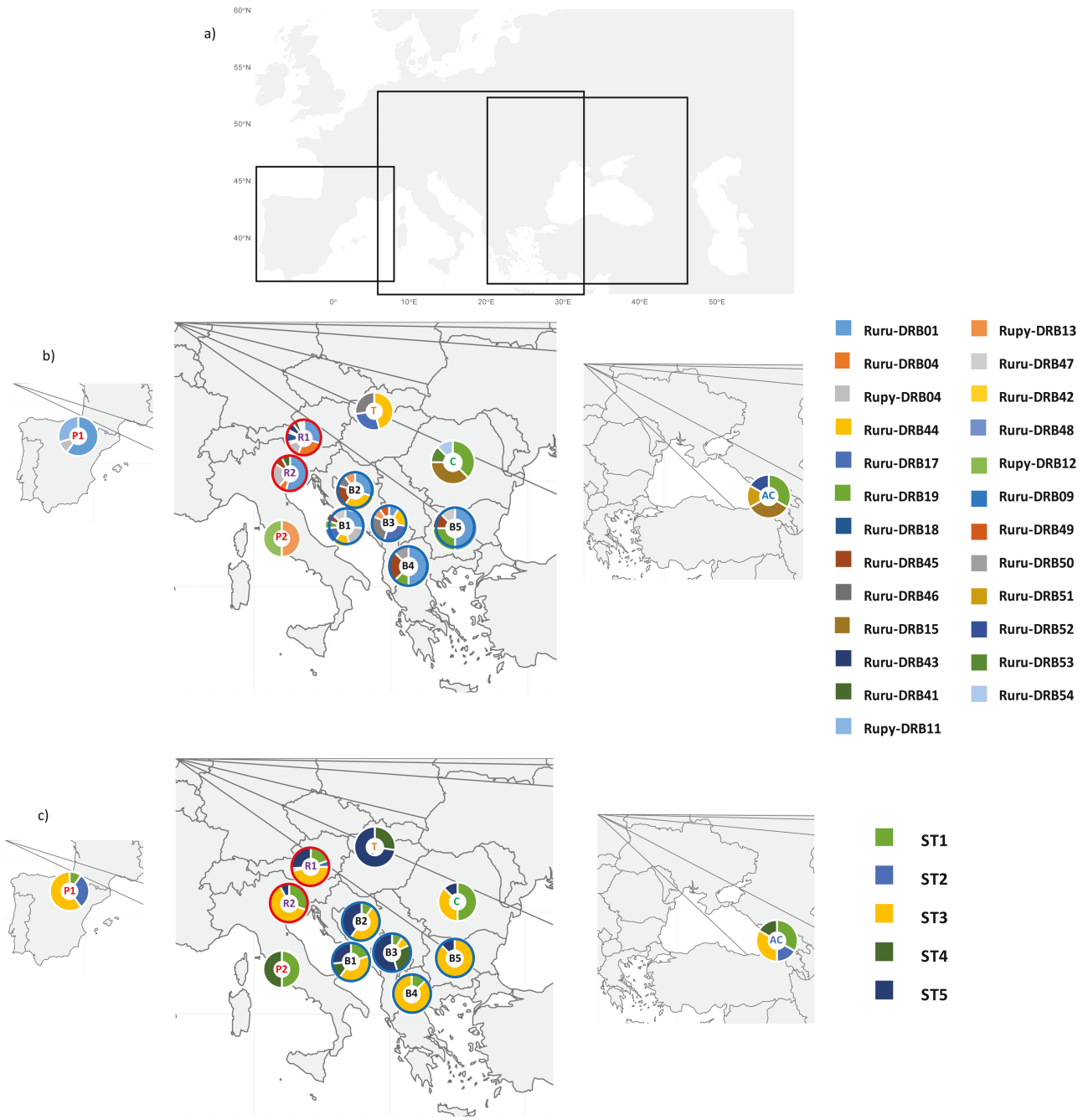

Fig. 1. Distribution of ***DRB*** alleles and supertypes in Northern and Southern chamois. The locations of samples included in study are shown by 3 sections of the map (a). The frequencies of alleles (b) and supertypes (c) in each population are shown by the pie charts with letters (Supplementary Data SD1). Each pie segment represents the frequency of 1 allele (b) or supertype (c). Different font colors and letters represent different taxonomic units (P—*Rupicapra pyrenaica*; R—*R. r. rupicapra*; B—*R. r. balcanica*; T—*R. r. tatrica*; C—*R. r. carpatica*; AC—*R. r. asiatica*; C—*R. r. caucasica*). The color of the pie outline represents the 2 subspecies of *Rupicapra rupicapra* (red—*R. r. rupicapra*; blue— *R. r. balcanica*).

diversity of chamois throughout their distribution range and fill the gap in previously published data for Northern and Southern Chamois (Schaschl et al. 2004, 2005; Alvarez-Busto et al. 2007). Of the 25 exon 2 *DRB* variants identified in 110 chamois, 11 were new and previously undescribed alleles. As in previous studies (Fuselli et al. 2018), we found no evidence of gene duplication.

Demography of chamois populations determined from mitochondrial and nuclear genes (Pérez et al. 2002; Crestanello et al. 2009; Rodríguez et al. 2009, 2010; Šprem and Buzan 2016; Pérez et al. 2017; Papaioannou et al. 2019) revealed a probable reduction in population size due to historical events within the last 5 to 30 Ky, as well as recent habitat fragmentation and isolation (Corlatti et al. 2021; Leugger et al. 2022; Rezić et al. 2022a). Unlike many other species that experienced a demographic contraction within Mediterranean refugia during the Last Glacial Maximum and a subsequent northward expansion when temperatures increased (Petit et al. 2003), results we obtained support the view of Mona et al. (2008) that chamois took a different path. Higher temperatures reduced the available territory for chamois and kept them trapped on mountain peaks, likely reducing population size. A positive and significant Tajima's *D* for the MHC *DRB* locus for Northern Chamois and its subspecies *R. r. rupicapra* suggests that

Table 1. Sequence diversity and average nucleotide and amino acid evolutionary distances of chamois *DRB* exon 2 alleles (found in this study and previously published; Schaschl et al. 2004, 2005; Alvarez-Busto et al. 2007; Stipoljev et al. 2020) calculated for the complete sequences (All), PBR, and non-PBR sites. *k*, average number of nucleotide difference; S, the number of segregating sites; π, nucleotide diversity. Standard error (SE; 10,000 bootstrap replicates) is shown in parentheses.

				Nucleotide distance			Amino acid distance		
	k	S	π	All	PBR	Non-PBR	All	PBR	Non-PBR
Chamois	9.76	38	0.04	0.05 (0.01)	0.14 (0.04)	0.01 (0.01)	0.11 (0.03)	0.43 (0.14)	0.03 (0.02)
Northern Chamois	8.96	33	0.04	0.04 (0.01)	0.13 (0.04)	0.01 (0.01)	0.11 (0.03)	0.40 (0.14)	0.03 (0.01)
Southern chamois	10.87	27	0.05	0.05 (0.01)	0.17 (0.04)	0.01 (0.01)	0.12 (0.03)	0.46 (0.15)	0.03 (0.02)

Table 2. *DRB* exon 2 genetic diversity detected in Northern Chamois and 2 subspecies (Corlatti et al. 2011), with the number of heterozygous and homozygous individuals, the number of supertypes estimated based on 14 positively selected amino acid sites, and Tajima's *D* values: *n*, number of individuals; *A*, number of alleles; *ST*, number of supertypes; *Ar*, allelic richness. Values in bold are significant at $P < 0.05$.

Species/subspecies	*n*	*A*	*ST*	*Ar*	N° heterozygous (%)	N° homozygous (%)	Tajima's *D*
Northern Chamois	102	23	5	15.81	76 (75)	26 (25)	**2.38**
R. r. rupicapra	57	12	5	10.20	44 (77)	13 (23)	**2.38**
R. r. balcanica	31	11	4	11.00	21 (68)	10 (32)	1.25

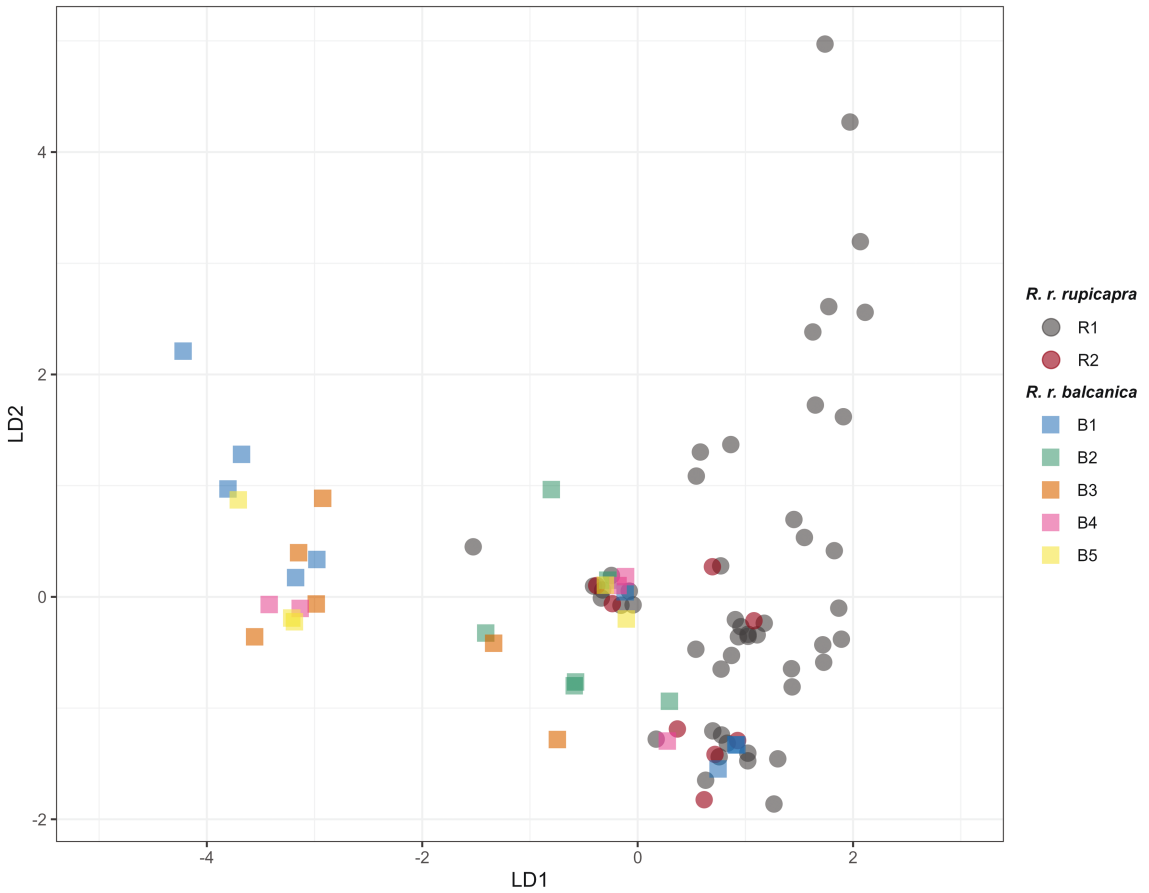

Fig. 2. Scatterplot of genetic differentiation resulting from a DAPC for the genetic structure of Northern Chamois subspecies **R. r. *rupicapra*** and *R. r. balcanica* based on the *DRB* locus (Supplementary Data SD1). Individuals are presented as separate dots with colors denoting chamois subspecies and inclusion of 95% inertia ellipses.

the *DRB* locus did not evolve at random, but under likely non-random processes such as genetic drift, population contraction, and/or balancing selection. Tajima's *D* was not significant for the subspecies *R. r. balcanica*, likely due to its larger distribution range and the presence of multiple populations in our analysis. Discriminant function of principal components including MHC *DRB* alleles also revealed distinction between *R. r. rupicapra* and *R. r. balcanica* (Fig. 2), which was also confirmed by significant F_{ST} pairwise differences. Considering the low migration rate between chamois populations, it is reasonable to assume that some of the

Table 3. Relative rates of synonymous (dS) and nonsynonymous (dN) substitutions, and results of 1-tailed Z-test (Z) for positive selection of chamois *DRB* exon 2 alleles (found in this study and previously published; Schaschl et al. 2004, 2005; Alvarez-Busto et al. 2007; Stipoljev et al. 2020) calculated for the complete sequences (All), PBR, and non-PBR sites. Standard error (SE; 10,000 bootstrap replicates) is shown in parentheses. Values in bold are significant at $P < 0.01$.

	Chamois			Northern Chamois			Southern Chamois		
	All	PBR	Non-PBR	All	PBR	Non-PBR	All	PBR	Non-PBR
dS (SE)	0.01 (0.003)	0.01 (0.01)	0.004 (0.002)	0.004 (0.002)	0.01 (0.01)	0.003 (0.002)	0.01 (0.01)	0.03 (0.02)	0.004 (0.004)
dN (SE)	0.06 (0.01)	0.17 (0.04)	0.02 (0.01)	0.05 (0.01)	0.15 (0.04)	0.02 (0.01)	0.06 (0.02)	0.19 (0.05)	0.02 (0.01)
dN/dS	6.00	17.00	5.00	12.50	15.00	6.67	6.00	6.33	5.00
Z	**3.77**	**3.79**	1.43	**3.70**	**3.69**	1.55	**3.46**	**3.55**	1.10

Table 4. Codon sites under positive selection as predicted by codon evolution models M2a and M8 using the Empirical Bayes approach in EasyCodeML. The codon sites inferred to be under selection with posterior probabilities >99% are listed in bold, and sites with posterior probabilities of >95% are in standard font. Codon numbers correspond to the codons of β1-domain in chamois (Schaschl et al. 2004). * indicates codons corresponding to the PBR.

	Selection model	Codon sites under positive selection
Chamois	M2a	**11*, 13*, 26, 37*, 38*, 47*, 57, 60*, 70*, 71*, 74*, 78*, 86***
	M8	**11*, 13*, 26**, 32*, **37*, 38*, 47*, 57, 60*, 70*, 71*, 74*, 78*, 86***

alleles detected in this study could be private alleles in those subspecies in which they were found, which further suggest diversifying selection, i.e. local MHC adaptation. Hybridization events that can lead to allelic introgression have also been detected in both subspecies (Šprem and Buzan 2016), but the consequences to functional diversity and fitness of individuals are still largely unknown (Iacolina et al. 2019).

Considering this, we suggest that the pattern of MHC genetic variation within and among regions and populations is likely due to both demographic processes, i.e. genetic drift and balancing selection. We support this view based on observed patterns of geographic variation, which demonstrate the distribution of different alleles linked to specific locations. Furthermore, we identified unique alleles in geographically isolated populations, providing further evidence for the influence of geographic factors on MHC genetic diversity (Fig. 1). However, to draw a definitive conclusion, it is necessary to compare the genetic variation of the MHC with neutral variation (Biedrzycka et al. 2020). Uniform selection pressure across different populations could lead to lower differentiation at functional loci due to balancing selection (Oosterhout et al. 2006), but genetic drift in small and isolated populations can overcome balancing selection and decrease genetic diversity (Radwan et al. 2010). On the other hand, we must consider that chamois populations have been subjected to the pressure of unsustainable hunting during the last 2 centuries, leading to local extinction of some subspecies (*R. p. parva*, *R. p. ornata*, *R. r. balcanica*, *R. r. asiatica*; Shackleton 1997). In the late 1970s, populations in the Alps suffered severe bottlenecks caused by sarcoptic mange epidemics with catastrophic outbreaks in which up to 80% of local populations were lost (Rossi et al. 1995; Fuchs et al. 2000; Corlatti et al. 2022), and this scenario may have reoccurred throughout the history of populations. Thus, observed differences in alleles/supertypes within the range of chamois could be due to long-term isolation between populations/subspecies, but may also suggest possible differential pressures from pathogens that may have influenced local MHC adaptation in some mountainous regions (Rossi et al. 1995; Cavallero et al. 2012; Biedrzycka et al. 2020).

Long-term balancing selection throughout the phylogenetic history of chamois is proposed by Mona et al. (2008). This hypothesis is supported by our data, as both maximum likelihood codon-based selection models (M2a, M8) gave a "best fit" to the data compared to models without selection. Positive selection most likely affects only a few codons at a few time points (Yang and Swanson 2002; Nielsen 2005), so interpreting evidence of selection from dN/dS averaged over an entire genetic region could be misleading. As in most other taxa with well-characterized MHC loci, we found codons under positive selection—and most importantly, all codons were located at or near PBR sites involved in pathogen recognition (Piertney and Oliver 2006).

We argue that there is good evidence that selection shaped genetic diversity in chamois MHC *DRB* genes due to: (i) remarkable differences in nucleotide diversity, especially in silent substitutions, ranging from dS = 0.004 in Northern Chamois to dS = 0.01 in Southern Chamois; (ii) rates of synonymous substitutions are lower than in other ruminants (Schaschl et al. 2006), which may indicate a young age of the alleles or influence of species demographic history (Schaschl et al. 2005); and (iii) amino acid positions 11, 71, and 86 were estimated to be under positive selection by at least 3 of our tests (Supplementary Data SD7) which may indicate that MHC genes were involved in pathogen recognition. Amino acid position influences functional differences in pathogen and parasite resistance. It is reasonable to assume that the maintenance and renewal of variation in functionally important parts of the MHC such as in the antigen binding sites by positive selection—either from mutation, recombination, or immigration from other populations—is an important genetic component in the cascade leading to an appropriate immune response when combating new or coevolving virulent pathogens. Excess of nonsynonymous mutations may take many thousands or millions of generations/cohorts to disappear once the selection process is no longer acting (Garrigan and Hedrick 2003).

We identified identical MHC alleles and supertypes in both species, suggesting a trans-specific polymorphism. Complete mitochondrial sequence data revealed several examples of trans-specific polymorphism in tribe Caprini (Hassanin et al. 2009), and the authors noted that these mitochondrial genes may play a potential role in mountain adaptation. Following a speciation event for the Caprini tribe, we can hypothesize that balancing selection favors the retention of some MHC alleles across species divergence events, with the result that these alleles become part of a long-lasting, trans-species polymorphism rather than being maintained by neutral processes (genetic drift and mutation;

Těšický and Vinkler 2015). Because balancing selection on MHC loci is expected to retain individual alleles during the process of species-level diversification, examples of interspecific allele sharing are typically interpreted as evidence of balancing selection on these genes (Schaschl et al. 2006). Another possible explanation for trans-species polymorphism was considered to be relatively recent extensive hybridization between the 2 chamois species during the Late Glacial Maximum (Schaschl et al. 2005; Rodríguez et al. 2010; Pérez et al. 2022). In addition to the effects of selection, variability at MHC genes should reflect the nature and magnitude of processes promoting neutral genetic differentiation. However, we also emphasize that our study is limited due to insufficient statistical power to test the role of rare variants and additional shared alleles, as well as the lack of long time series and small sample sizes. In line with this, our study has shown that further studies on MHC genes, their diversity, and their effects on the resistance and fitness of individuals of different wildlife species are urgently needed.

An important question that remains to be answered is to what extent selection shapes the adaptive genetics of chamois within complex scenarios where other evolutionary processes such as genetic drift and introgression play an important role. This is particularly important at a time when climate change poses a major threat to chamois populations (Lovari et al. 2020). Currently, there is no evidence that measurement of MHC polymorphism is sufficient to predict immunological fitness. Nevertheless, polymorphism in MHC genes under certain pathogenic challenges may determine individual-level survivability and population-level adaptability to the pathogen, but this occurs in combination with the effects of other genes involved in the immune response because MHC genes do not in isolation determine the ability to survive infection (Acevedo-Whitehouse and Cunningham 2006)—although polymorphism at amino acid sites encoding PBR is generally considered an indicator of susceptibility or resistance to infectious disease in wildlife (O'Brien and Evermann 1988), maintenance of variability in other immune genes may most likely account for resistance in species with otherwise uniform or low MHC genetic variation (Acevedo-Whitehouse and Cunningham 2006).

Supplementary data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Basic data on chamois individuals.

Supplementary Data SD2.—*DRB* exon 2 alleles of chamois previously reported.

Supplementary Data SD3.—The number of reads generated by Ion Torrent sequencing.

Supplementary Data SD4.—MHC *DRB* exon 2 alleles frequencies.

Supplementary Data SD5.—Plot of the frequency of MHC *DRB* exon 2 alleles.

Supplementary Data SD6.—Neighbor-net network of chamois DRB alleles.

Supplementary Data SD7.—Distribution of positively selected sites in exon 2 of chamois *DRB* genes.

Acknowledgments

We thank Sandra Potušek for her invaluable assistance with the laboratory work and for conducting Ion Torrent sequencing. We are thankful to the Majella National Park from Italy for providing *R. r. ornata* samples and Hunters Association and Triglav National Park from Slovenia for providing *R. r. rupicapra* samples.

Author contributions

Conceptualization; EB and NŠ. Sample providing; EB, NŠ, KK, HA, MA, EB, FB, AF, DG, PL, VM, GM, DM, HP, and MS. Laboratory and statistical analyses; SS, TS, EB, AR, LI, and AG. Writing—draft preparation, SS and EB; review and editing, SS, EB, NŠ and TS. Funding; NŠ and EB. All authors have read and agreed to the published version of the manuscript.

Funding

This study was supported by (i) the Croatian Science Foundation, project IP 2016-06-5751, "DNA as evidence of distribution and vitality of endangered Balkan Chamois"; (ii) the Slovenian Research Agency (programme group P6-0064); and (iii) the RESBIOS European Union's Horizon 2020 Research and Innovation Program (no. 872146).

Conflict of interest

None declared.

Data availability

The novel alleles have been deposited in GenBank under accession numbers OL421550 to OL421560.

References

Acevedo-Whitehouse K, Cunningham AA. 2006. Is MHC enough for understanding wildlife immunogenetics? Trends in Ecology and Evolution 21(8):433–438. https://doi.org/10.1016/j.tree.2006.05.010

Alcaide M. 2010. On the relative roles of selection and genetic drift in shaping MHC variation. Molecular Ecology 19(18):3842–3844. https://doi.org/10.1111/j.1365-294X.2010.04772.x

Altizer S, Nunn CL, Thrall PH, Gittleman JL, Antonovics J, Cunningham AA, Dobson AP, Ezenwa V, Jones KE, Pedersen AB, *et al*. 2003. Social organization and parasite risk in mammals: integrating theory and empirical studies. Annual Review of Ecology, Evolution, and Systematics 34(1):517–547. https://doi.org/10.1146/annurev.ecolsys.34.030102.151725

Alvarez-Busto J, García-Etxebarria K, Herrero J, Garin I, Jugo BM. 2007. Diversity and evolution of the Mhc-DRB1 gene in the two endemic Iberian subspecies of Pyrenean Chamois, *Rupicapra pyrenaica*. Heredity 99(4):406–413. https://doi.org/10.1038/sj.hdy.6801016

Amos W, Balmford A. 2001. When does conservation genetics matter? Heredity 87(Pt 3):257–265. https://doi.org/10.1046/j.1365-2540.2001.00940.x

Anderwald P, Ambarli H, Avramov S, Ciach, M, Corlatti L, Farkas A, Jovanovic M, Papaioannou H, Peters W, Sarasa M, *et al*. 2021. *Rupicapra rupicapra* (amended version of 2020 assessment). The IUCN Red List of Threatened Species 2021: e.T39255A195863093 [accessed 11 Mar 2022]. www.iucnredlist.org/species/39255/195863093.

Awadi A, Ben Slimen H, Smith S, Knauer F, Makni M, Suchentrunk F. 2018. Positive selection and climatic effects on MHC class II gene diversity in hares (*Lepus capensis*) from a steep ecological

gradient. Scientific Reports 8(1):11514. https://doi.org/10.1038/s41598-018-29657-3

Bekessy SA, Ennos RA, Burgman MA, Newton AC, Ades PK. 2003. Neutral DNA markers fail to detect genetic divergence in an ecologically important trait. Biological Conservation 110(2):267–275. https://doi.org/10.1016/s0006-3207(02)00225-2

Bernatchez L, Landry C. 2003. MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? Journal of Evolutionary Biology 16(3):363–377. https://doi.org/10.1046/j.1420-9101.2003.00531.x

Biedrzycka A, Konopiński M, Hoffman E, Trujillo A, Zalewski A. 2020. Comparing raccoon major histocompatibility complex diversity in native and introduced ranges: evidence for the importance of functional immune diversity for adaptation and survival in novel environments. Evolutionary Applications 13(4):752–767. https://doi.org/10.1111/eva.12898

Brown JH. 2001. Mammals on mountainsides: elevational patterns of diversity. Global Ecology and Biogeography 10(1):101–109. https://doi.org/10.1046/j.1466-822x.2001.00228.x

Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL, Wiley DC. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-*DR1*. Nature 364(6432):33–39. https://doi.org/10.1038/364033a0

Buzan E, Potušek S, Urzi F, Pokorny B, Šprem N. 2020. Genetic characterisation of wild ungulates: successful isolation and analysis of DNA from widely available bones can be cheap, fast and easy. Zookeys 965:141–156. https://doi.org/10.3897/zookeys.965.54862

Buzan EV, Bryja J, Zemanová B, Kryštufek B. 2013. Population genetics of chamois in the contact zone between the Alps and the Dinaric Mountains: uncovering the role of habitat fragmentation and past management. Conservation Genetics 14(2):401–412. https://doi.org/10.1007/s10592-013-0469-8

Cavallero S, Marco I, Lavín S, D'Amelio S, López-Olvera JR. 2012. Polymorphisms at MHC class II DRB1 exon 2 locus in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*). Infection, Genetics and Evolution 12(5):1020–1026. https://doi.org/10.1016/j.meegid.2012.02.017

Corlatti L, Lacolina L, Safner T, Apollonio M, Buzan E, Ferretti F, Hammer SE, Herrero J, Rossi L, Serrano E, *et al*. 2022. Past, present and future of chamois science. Wildlife Biology: e01025. https://doi.org/10.1002/wlb3.01025

Corlatti L., Herrero J., Ferretti F., Anderwald P., García-González R., Hammer S., Nores C., Rossi L., Lovari S. 2021. Northern Chamois, *Rupicapra rupicapra* (Linnaeus, 1758) and Southern Chamois, *Rupicapra pyrenaica* Bonaparte, 1845. In: Corlatti L, Zachos F, editors. Handbook of the mammals of Europe – terrestrial Cetartiodactyla. Switzerland: Springer Nature; p. 1–42. https://doi.org/10.1007/978-3-030-24475-0_30

Corlatti L, Lorenzini R, Lovari S. 2011. The conservation of the chamois *Rupicapra* spp. Mammal Review 41(2):163–174. https://doi.org/10.1111/j.1365-2907.2011.00187.x

Crestanello B, Pecchioli E, Vernesi C, Mona S, Martínková N, Janiga M, Hauffe HC, Bertorelle G. 2009. The genetic impact of translocations and habitat fragmentation in chamois (*Rupicapra*) spp. Journal of Heredity 100(6):691–708. https://doi.org/10.1093/jhered/esp053

Doytchinova IA, Flower DR. 2005. In silico identification of supertypes for class II MHCs. The Journal of Immunology 174(11):7085–7095. https://doi.org/10.4049/jimmunol.174.11.7085

Ejsmond MJ, Radwan J. 2015. Red queen processes drive positive selection on major histocompatibility complex (MHC) genes. PLoS Computational Biology 11(11):e1004627. https://doi.org/10.1371/journal.pcbi.1004627

Excoffier L, Lischer H. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10:564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Ezard THG, Travis JMJ. 2006. The impact of habitat loss and fragmentation on genetic drift and fixation time. Oikos 114(2):367–375. https://doi.org/10.1111/j.2006.0030-1299.14778.x

Fuchs K, Deutz A, Gressmann G. 2000. Detection of space-time clusters and epidemiological examinations of scabies in chamois. Veterinary Parasitology 92(1):63–73. https://doi.org/10.1016/s0304-4017(00)00269-7

Funk WC, McKay JK, Hohenlohe PA, Allendorf FW. 2012. Harnessing genomics for delineating conservation units. Trends in Ecology and Evolution 27(9):489–496. https://doi.org/10.1016/j.tree.2012.05.012

Fuselli S, Baptista RP, Panziera A, Magi A, Guglielmi S, Tonin R, Benazzo A, Bauzer LG, Mazzoni CJ, Bertorelle G. 2018. A new hybrid approach for MHC genotyping: high-throughput NGS and long read MinION nanopore sequencing, with application to the non-model vertebrate Alpine chamois (*Rupicapra rupicapra*). Heredity 121(4):293–303. https://doi.org/10.1038/s41437-018-0070-5

Gao F, Chen C, Arab DA, Du Z, He Y, Ho SYW. 2019. EasyCodeML: a visual tool for analysis of selection using CodeML. Ecology and Evolution 9(7):3891–3898. https://doi.org/10.1002/ece3.5015

Garrigan D, Hedrick PW. 2003. Perspective: detecting adaptive molecular polymorphism: lessons from the MHC. Evolution 57(8):1707–1722. https://doi.org/10.1111/j.0014-3820.2003.tb00580.x

Hassanin A, Ropiquet A, Couloux A, *et al*. 2009. Evolution of the mitochondrial genome in mammals living at high altitude: new insights from a study of the tribe caprini (Bovidae, Antilopinae). *Journal of Molecular Evolution* 68:293–310. https://doi.org/10.1007/s00239-009-9208-7

Hedrick PW. 2001. Conservation genetics: where are we now? Trends in Ecology and Evolution 16(11):629–636. https://doi.org/10.1016/s0169-5347(01)02282-0

Herrero J., Lovari, S., Nores C., Toigo C. 2020. *Rupicapra pyrenaica*. The IUCN Red List of Threatened Species 2020: e.T19771A171131310 [accessed 11 Mar 2022]. www.iucnredlist.org/species/19771/171131310.

Hoffmann AA, Sorensen JG, Loeschcke V. 2003. Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. Journal of Thermal Biology 28(3):175–216. https://doi.org/10.1016/s0306-4565(02)00057-8

Huson DH, Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. Molecular Biology and Evolution 23(2):254–267. https://doi.org/10.1093/molbev/msj030

Iacolina L, Corlatti L, Buzan E, Safner T, Šprem N. 2019. Hybridisation in European ungulates: an overview of the current status, causes, and consequences. Mammal Review 49:45–59. https://doi.org/10.1111/mam.12140

Jombart T. 2008. Adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24(11):1403–1405. https://doi.org/10.1093/bioinformatics/btn129

Jombart T, Devillard S, Balloux F. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genetics 11(94). https://doi.org/10.1186/1471-2156-11-94

Juola FA, Dearborn DC. 2011. Sequence-based evidence for major histocompatibility complex-disassortative mating in a

colonial seabird. Proceedings of the Royal Society of London, B: Biological Sciences 279(1726):153–162. https://doi.org/10.1098/rspb.2011.0562

Kalinowski ST. 2004. Counting alleles with rarefaction: private alleles and hierarchical sampling designs. Conservation Genetics 5(4):539–543. https://doi.org/10.1023/b:coge.0000041021.91777.1a

Klein J. 1986. Natural history of the major histocompatibility complex. New York (NY, USA): John Wiley and Sons.

Klein J, Bontrop RE, Dawkins RL, Erlich HA, Gyllensten UB, Heise ER, Jones PP, Parham P, Wakeland EK, Watkins DI. 1990. Nomenclature for the major histocompatibility complexes of different species: a proposal. Immunogenetics 31(4):217–219. https://doi.org/10.1007/BF00204890

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35(6):1547–1549. https://doi.org/10.1093/molbev/msy096

Leugger F, Broquet T, Karger DN, Rioux D, Buzan E, Corlatti L, Crestanello B, Curt-Grand-Gaudin N, Hauffe HC, Rolečková B, *et al*. 2022. Dispersal and habitat dynamics shape the genetic structure of the Northern Chamois in the Alps. Journal of Biogeography 49(10):1848–1861. https://doi.org/10.1111/jbi.14363

Lovari S, Franceschi S, Chiatante G, Fattorini L, Fattorini N, Ferretti F. 2020. Climatic changes and the fate of mountain herbivores. Climatic Change 162(4):2319–2337. https://doi.org/10.1007/s10584-020-02801-7

Markov G, Zhelev P, Ben Slimen H, Suchentrunk F. 2016. Population genetic data pertinent to the conservation of Bulgarian chamois (*Rupicapra rupicapra balcanica*). Conservation Genetics 17:155–164. https://doi.org/10.1007/s10592-015-0768-3

Miller HC, Lambert DM. 2004. Gene duplication and gene conversion in class II MHC genes of New Zealand robins (Petroicidae). Immunogenetics 56(3):178–191. https://doi.org/10.1007/s00251-004-0666-1

Mona S, Crestanello B, Bankhead-Dronnet S, Pecchioli E, Ingrosso S, D'Amelio S, Rossi L, Meneguz PG, Bertorelle G. 2008. Disentangling the effects of recombination, selection, and demography on the genetic variation at a major histocompatibility complex class II gene in the Alpine chamois. Molecular Ecology 17(18):4053–4067. https://doi.org/10.1111/j.1365-294x.2008.03892.x

Nielsen R. 2005. Molecular signatures of natural selection. Annual Review of Genetics 39:197–218. https://doi.org/10.1146/annurev.genet.39.073003.112420

O'Brien SJ, Evermann JF. 1988. Interactive influence of infectious disease and genetic diversity in natural populations. Trends in Ecology and Evolution 3(10):254–259. https://doi.org/10.1016/0169-5347(88)90058-4

Oosterhout C, Joyce DA, Cummings SM, Blais J, Barson NJ, Ramnarine IW, Mohammed RS, Persad N, Cable J. 2006. Balancing selection, random genetic drift, and genetic variation at the major histocompatibility complex in two wild populations of guppies (*Poecilia reticulata*). Evolution 60(12):2562–2574. https://doi.org/10.1111/j.0014-3820.2006.tb01890.x

Papaioannou H, Fernández M, Pérez T, Domínguez A. 2019. Genetic variability and population structure of chamois in Greece (*Rupicapra rupicapra balcanica*). Conservation Genetics 20(4):939–945. https://doi.org/10.1007/s10592-019-01177-1

Pérez T, Albornoz J, Domínguez A. 2002. Phylogeography of chamois (*Rupicapra* spp.) inferred from microsatellites. Molecular Phylogenetics and Evolution 25(3):524–534. https://doi.org/10.1016/s1055-7903(02)00296-8

Pérez T, Fernández M, Hammer SE, Domínguez A. 2017. Multilocus intron trees reveal extensive male-biased homogenization of ancient populations of chamois (*Rupicapra* spp.) across Europe during Late Pleistocene. PLoS One 12(2):e0170392. https://doi.org/10.1371/journal.pone.0170392

Pérez T, Fernández M, Palacios B, Domínguez A. 2022. Phylogenetic analysis of the complete mitochondrial genomes in the ten *Rupicapra* subspecies and implications for the existence of multiple glacial refugia in Europe. Animals 12(11):1430. https://doi.org/10.3390/ani12111430

Petit RJ, Aguinagalde I, de Beaulieu J-L, Bittkau C, Brewer S, Cheddadi R, Ennos R, Fineschi S, Grivet D, Lascoux M, *et al*. 2003. Glacial refugia: hotspots but not melting pots of genetic diversity. Science 300(5625):1563–1565. https://doi.org/10.1126/science.1083264

Piertney SB, Oliver MK. 2006. The evolutionary ecology of the major histocompatibility complex. Heredity 96(1):7–21. https://doi.org/10.1038/sj.hdy.6800724

Punt J., Stranford S.A., Jones P.P., Owen J.A. 2019. Kuby Immunology. 8th ed. New York (NY, USA): W. H. Freeman and Company.

R Development Core Team. 2020. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing. www.R-project.org/.

Radwan J, Biedrzycka A, Babik W. 2010. Does reduced MHC diversity decrease viability of vertebrate populations? Biological Conservation 143(3):537–544. https://doi.org/10.1016/j.biocon.2009.07.026

Rezić A, Iacolina L, Bužan E, Safner T, Bego F, Gačić D, Maletić V, Markov G, Milošević D, Papaioannou H, *et al*. 2022a. The Balkan chamois, an archipelago or a peninsula? Insights from nuclear and mitochondrial DNA. Conservation Genetics 23(3):527–539. https://doi.org/10.1007/s10592-022-01434-w

Rezić A, Safner T, Iacolina L, Buzan E, Šprem N. 2022b. Traces of past reintroduction in genetic diversity: the case of the Balkan chamois (Mammalia, Artiodactyla). Zookeys 1116:57–70. https://doi.org/10.3897/zookeys.1116.84577

Rodríguez F, Hammer S, Pérez T, Suchentrunk F, Lorenzini R, Michallet J, Martinkova N, Albornoz J, Domínguez A. 2009. Cytochrome b phylogeography of chamois (*Rupicapra* spp.). Population contractions, expansions and hybridizations governed the diversification of the genus. Journal of Heredity 100(1):47–55. https://doi.org/10.1093/jhered/esn074

Rodríguez F, Pérez T, Hammer SE, Albornoz J, Domínguez A. 2010. Integrating phylogeographic patterns of microsatellite and mtDNA divergence to infer the evolutionary history of chamois (genus *Rupicapra*). BMC Evolutionary Biology 10(222). https://doi.org/10.1186/1471-2148-10-222

Rossi L, Meneguz PG, Martin PD, Rodolfi M. 1995. The epizootiology of sarcoptic mange in chamois, *Rupicapra rupicapra*, from the Italian eastern Alps. Parassitologia 37(2-3):233–240. PMID: 8778664.

Rozas J, Ferrer-Mata A, Sanchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sanchez-Gracia A. 2017. DnaSP 6: DNA sequence polymorphism analysis of large data sets. Molecular Biology and Evolution 34(12):3299–3302. https://doi.org/10.1093/molbev/msx248

Schaschl H, Goodman SJ, Suchentrunk F. 2004. Sequence analysis of the MHC class II DRB alleles in Alpine chamois (*Rupicapra r. rupicapra*). Developmental and Comparative Immunology 28(3):265–277. https://doi.org/10.1016/j.dci.2003.08.003

Schaschl H, Suchentrunk F, Hammer S, Goodman SJ. 2005. Recombination and the origin of sequence diversity in the DRB MHC class II locus in chamois (*Rupicapra* spp.). Immunogenetics 57(1–2):108–115. https://doi.org/10.1007/s00251-005-0784-4

Schaschl H, Wandeler P, Suchentrunk F, Obexer-Ruff G, Goodman SJ. 2006. Selection and recombination drive the evolution of MHC class II *DRB* diversity in ungulates. Heredity 97(6):427–437. https://doi.org/10.1038/sj.hdy.6800892

Sebastian A, Herdegen M, Migalska M, Radwan J. 2016. AMPLISAS: a web server for multilocus genotyping using next-generation amplicon sequencing data. Molecular Ecology Resources 16(2):498–510. https://doi.org/10.1111/1755-0998.12453

Shackleton D. 1997. Wild sheep and goats and their relatives. Gland (Switzerland): International Union for Conservation of Nature.

Soglia D, Rossi L, Cauvin E, Citterio C, Ferroglio E, Maione S, Meneguz PG, Spalenza V, Rasero R, Sacchi P. 2010. Population genetic structure of Alpine chamois (*Rupicapra r. rupicapra*) in the Italian Alps. European Journal of Wildlife Research 56(6):845–854. https://doi.org/10.1007/s10344-010-0382-0

Sommer S. 2005. The importance of immune gene variability (MHC) in evolutionary ecology and conservation. Frontiers in Zoology 2(1):1–18. https://doi.org/10.1186/1742-9994-2-16

Spielman D, Brook BW, Frankham R. 2004. Most species are not driven to extinction before genetic factors impact them. Proceedings of the National Academy of Sciences of the United States of America 101(42):15261–15264. https://doi.org/10.1073/pnas.0403809101

Šprem N, Buzan E. 2016. The genetic impact of chamois management in the dinarides. Journal of Wildlife Management 80(5):783–793. https://doi.org/10.1002/jwmg.21081

Spurgin LG, Richardson DS. 2010. How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. Proceedings of the Royal Society of London, B: Biological Sciences 277(1684):979–988. https://doi.org/10.1098/rspb.2009.2084

Štefančíková A, Chovancová B, Hájek B, Dudiňák V, Šnábel V. 2011. Revision of chamois infection by lung nematodes under ecological conditions of national parks of Slovakia with respect to ongoing global climate changes. Helminthologia 48(3):145–154. https://doi.org/10.2478/s11687-011-0022-6

Stipoljev S, Bužan E, Rolečková B, Iacolina L, Šprem N. 2020. MHC genotyping by SSCP and amplicon-based NGS approach in chamois. Animals 10(9):1694. https://doi.org/10.3390/ani10091694

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123(3):585–595. https://doi.org/10.1093/genetics/123.3.585

Těšický M, Vinkler M. 2015. Trans-species polymorphism in immune genes: general pattern or MHC-restricted phenomenon? Journal of Immunology Research 2015:838035. https://doi.org/10.1155/2015/838035

Weaver S, Shank SD, Spielman SJ, Li M, Muse SV, Kosakovsky Pond SL. 2018. Datamonkey 2.0: a modern web application for characterizing selective and other evolutionary processes. Molecular Biology and Evolution 35(3):773–777. https://doi.org/10.1093/molbev/msx335

Willi Y, Van Buskirk J, Schmid B, Fischer M. 2007. Genetic isolation of fragmented populations is exacerbated by drift and selection. Journal of Evolutionary Biology 20(2):534–542. https://doi.org/10.1111/j.1420-9101.2006.01263.x

Yang Z, Swanson WJ. 2002. Codon-substitution models to detect adaptive evolution that account for heterogeneous selective pressures among site classes. Molecular Biology and Evolution 19(1):49–57. https://doi.org/10.1093/oxfordjournals.molbev.a003981

Zagalska-Neubauer M, Babik W, Stuglik M, Gustafsson L, Cichoń M, Radwan J. 2010. 454 sequencing reveals extreme complexity of the class II major histocompatibility complex in the Collared Flycatcher. BMC Evolutionary Biology 10(395). https://doi.org/10.1186/1471-2148-10-395