Lack of population-specific patterns of major histocompatibility complex (MHC) diversity in roe deer from lowland and mountain habitats in Croatia

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Abstract:
Roe deer (*Capreolus capreolus*) are widespread across Europe and exhibit adaptability to various habitats. In the last couple of decades, population numbers have significantly increased, except for certain areas which have experienced population declines due multifactorial reasons, including the impact of fascioloidosis. In Croatia, roe deer are primarily found in lowland region, while their population in mountain areas is smaller and more scattered due to habitat limitations and presence of large predators. The variability of major histocompatibility complex (MHC) genes offers insight into the population's ability to combat new pathogens and to cope with changing environments. Here, we examined the variability and selection patterns of MHC class II DRB locus in roe deer from two distinct habitats in Croatia. Ten alleles were identified in 133 individuals accompanied by high amino acid evolutionary distance (41.1%). The lack of significant structuring on the DRB was observed between the two habitats, with ambiguous results from contemporary and historical selection analysis. Furthermore, our results highlight the need to investigate other immune loci, which could provide insight into the relationship between pathogen-mediated selection and adaptation potential in roe deer.

Keywords: MHC class II, Capreolus capreolus, next-generation sequencing, immunity genes, balancing selection, adaptive diversity.

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MHC diversity in roe deer from Croatia

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ABSTRACT

Roe deer (*Capreolus capreolus*) are widespread across Europe and exhibit adaptability to various habitats. In the last couple of decades, population numbers have significantly increased, except for certain areas which have experienced population declines due multifactorial reasons, including the impact of fascioloidosis. In Croatia, roe deer are primarily found in lowland region, while their population in mountain areas is smaller and more scattered due to habitat limitations and presence of large predators. The variability of major histocompatibility complex (MHC) genes offers insight into the population's ability to combat new pathogens and to cope with changing environments. Here, we examined the variability and selection patterns of MHC class II DRB locus in roe deer from two distinct habitats in Croatia. Ten alleles were identified in 133 individuals accompanied by high amino acid evolutionary distance (41.1%). The lack of significant population structuring on the DRB was observed between the two habitats, with ambiguous results from contemporary and historical selection analysis. Furthermore, our results highlight the need to investigate other immune loci, which could provide insight into the relationship between pathogen-mediated selection and adaptation potential in roe deer.

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INTRODUCTION

The major histocompatibility complex (MHC) encodes membrane proteins that bind peptide antigens derived from pathogens and present them to T cells which enables adaptive immune response. Extraordinarily high polymorphism at the MHC, particularly in the peptide-binding region (PBR), is presumably driven by pathogen-mediated selection (Spurgin and Richardson, 2010). The diversity of amino acids in the PBR affects capability of binding specific antigens and advancement in defence against pathogens (Stern and Wiley, 1994). Due to the functional importance of MHC in pathogen recognition, populations encompassing substantial MHC diversity might be better equipped for environmental challenges and could possess higher adaptation potential, and thus be less vulnerable to declines and extinction (Sommer, 2005). For this reason, MHC has been routinely studied in vertebrate populations with the aim to investigate how natural selection affects local adaptation at the molecular level (Bernatchez and Landry, 2003). Apart from survival, MHC genes have also been linked to other fitness-related traits, including mate choice (Jordan and Bruford, 1998), body condition (Lenz et al., 2009) and secondary sexual characteristics such as ornaments (Whittingham et al., 2015).

It is postulated that a special type of selection, termed balancing selection, promotes variability on the MHC with three mechanisms: heterozygote advantage (Doherty and Zinkernagel, 1975), negative frequency-dependent selection or rare allele advantage (Slade and McCallum, 1992) and temporally and spatially fluctuating selection (Hill, 1991). The heterozygote advantage hypothesis presumes that heterozygous individuals have the ability to recognise a broader spectrum of pathogens and therefore have selective advantage over homozygotes. In case of negative frequency-dependant selection hypothesis, rare alleles are considered advantageous since pathogens are more likely to adapt to the more common host MHC genotype and avoid host immunological defence, making individuals with rare alleles less susceptible to the disease (Lively and Dybdahl, 2000). The mechanism of temporally and spatially fluctuating selection is similar to the rare allele advantage mechanism with the major difference being that in the former the selective pressure of pathogens on their hosts is determined by biotic and abiotic environment, chance dispersal and extinction events, while in the latter by their co-evolution

(Spurgin and Richardson, 2010). This way balancing selection maintains large number of alleles in a population, promoting long-term survival of alleles as they are less likely to diminish by random processes (Hedrick, 1994).

In this study, we examined genetic diversity at the MHC in two roe deer (*Capreolus capreolus*) populations from Croatia. Roe deer is an important game species and currently, the most abundant cervid in Europe. It inhabits various types of landscapes, including pastures, forests, and mixed agricultural areas (Lovari et al., 2016). Its distribution range covers most of the European continent and spreads further to the east to the Caucasus Mountains and the Middle East (Andersen, 2000). Lorenzini et al. (2014) showed that by using mtDNA, roe deer can be separated into four distinct groups: Eastern European, Southern Iberian, Central-Southern Italian and Central European. While the Central European group can be found throughout Europe, the distribution of other groups corresponded to the specific geographic areas. Similar phylogeographical distinction was later confirmed by Plis et al. (2022), describing mainly admixed central European population. The majority of the Croatian population, belonging to the Central European group, is located in the lowland region (Kusak and Krapinec, 2010), which covers parts of the Pannonian Plain and the hilly peri-Pannonian area. In the mountain region of Gorski Kotar and Lika, the density of the roe deer population is lower as a consequence of habitat conditions and the presence of large carnivores, with the exception of locally high densities around feeding sites (Kusak et al., 2012). Horizontal pathogen transmission is expected to decrease in fragmented and scattered populations (Anderson and May, 1979). Absence of livestock in close proximity to deer populations, and arid karst conditions assumingly further promote lower infection rates in this area. In contrast, lowland parts of Croatia might have been under stronger pathogen selection pressure due to higher roe deer population density (Kusak et al., 2012) which could promote higher pathogen transmission rates in the area (Wilson et al., 2002; Wilson and Reeson, 1998). One example is the allochthonous fluke *Facioloides magna*, now widespread in the majority of lowland Croatia, and a growing threat to deer populations. Infected roe deer experience excessive immunological reaction accompanied by heavy tissue damage made by migrating juvenile fluke and usually do not survive the infection (Konjević et al., 2021). To date,

F. magna has not spread to the mountain regions as territory conditions seem unfavourable for the fluke and its intermediate hosts, freshwater snails from the family Lymnaeidae.

To date, only a few studies have focused on MHC diversity in roe deer (Bužan et al., 2022; Mikko et al., 1999; Quéméré et al., 2015), regardless of the species' omnipresence in Europe. Mikko et al. (1999) inspected patterns of MHC variation in roe deer from Norway and Sweden, Quéméré et al. (2015) compared diversity between three roe deer populations in France, while Bužan et al. (2022) assessed and compared MHC diversity of Slovenian populations. All studies found limited levels of diversity in comparison with European red deer (*Cervus elaphus*) populations (Buczek et al., 2016; Bujanić et al., 2020; Fernández-de-Mera et al., 2009; Pérez-Espona et al., 2019) and some cervids inhabiting other continents (Cook et al., 2022; Li et al., 2013; Van Den Bussche et al., 2002). Studies on the French and Slovenian roe deer populations detected signatures of positive selection. Additionally, the patterns of variation observed at neutral loci did not align with those at MHC loci, implying that balancing selection exerted a stronger influence than historical demographic processes.

This study presents the first examination of MHC diversity in roe deer from Croatia. Our primary aim was to assess genetic diversity within the MHC-DRB exon 2 and compare diversity and selection patterns with data from other European roe deer populations, including the neighbouring Slovenian population. Additionally, we investigated whether selection influenced diversity differently across mountain and lowland habitats, potentially resulting in population structuring evident in the MHC. Lastly, we explored the utility of the DRB locus in roe deer as a marker for assessing population adaptation potential in future studies, particularly in response to the recent threat of fascioloidosis.

MATERIALS AND METHODS

For this study we used 133 samples from animals culled during regular hunting management operations in Croatia, including 14 samples published previously (Svetličić et al. 2022). Muscle and liver samples were collected from Bjelovar-Bilogora County (N=54), Zagreb County (N=39),

Lika-Senj County (N=20) and Primorje-Gorski Kotar County (N=20). Samples were categorised into two distinct populations: lowland (Bjelovar-Bilogora and Zagreb County, N=93) and mountain population (Lika-Senj and Primorje-Gorski Kotar County, N=40) (Fig. 1).

DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, Maidson, WI, USA), following the recommended protocol for animal tissue. Extracted DNA was sent to Novogene sequencing facility (UK) for library preparation and Illumina MiSeq PE250 sequencing. The amplification target was a 249 bp segment of exon, which encompasses the functionally important PBR of the MHC class II. Amplifications were performed using specific LA31 and LA32 primers (Sigurdardóttir et al., 1991), previously successfully used in other roe deer MHC sequencing projects (Bužan et al., 2022; Mikko et al., 1999; Quéméré et al., 2015; Svetličić et al., 2022), tagged with unique sample-specific barcodes to allow for sample multiplexing, followed by addition of Illumina sequencing adapters. Upon receiving raw reads from the sequencing facility, we performed merging of pair-end reads, quality and length filtering, as well as final allele calling using AmpliSAT integrated web tool (Sebastian et al., 2016), as described in Svetličić et al. (2022). The suitability of the utilised sequencing and allele calling method for roe deer DRB was previously confirmed through comparison with other methods, ensuring accurate allele attribution without ambiguities and null alleles (Svetličić et al., 2022). The raw sequences obtained from next-generation sequencing are available at the NCBI Sequence Read Archive (SRA) under reference number PRJNA1198488.

Number of nucleotide variable sites, nucleotide diversity and mean number of pairwise differences were determined using DnaSP (Librado and Rozas, 2009). Pairwise and mean nucleotide and amino acid evolutionary distances according to the best-fitting substitution model, were calculated in MEGA11 software (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2021).

Allelic richness, measure of expected number of alleles assuming the smallest sample size, was estimated using the rarefaction method implemented in FSTAT version 2.9.3 (Goudet, 2002). The effective number of alleles, as well as "evenness" - the ratio of the effective number of alleles to the recorded number of alleles - was calculated in R using the ALRATIO script (Pojskić, 2019). Deviation from Hardy–Weinberg equilibrium (HWE) was tested by applying the exact test, as

implemented in the software Arlequin version 3.11 (Excoffier et al., 2005). Arlequin was also used for the Ewens-Watterson test (Watterson, 1978), as modified by Slatkin (1996) as well as AMOVA analysis among and within the studied populations. Program STRUCTURE v2.3.4.59 (Pritchard et al., 2000) was used to identify possible genetic structure in the analysed samples. This program identifies the number of genetic clusters (K) within a population and assigns individuals to these clusters using a Bayesian clustering approach. Analyses were conducted for K values ranging from 1 to 5, with five iterations for each K. Each iteration included a burn-in phase of 100,000 generations, followed by a Markov Chain Monte Carlo (MCMC) run of 1,000,000 generations. The analyses were performed using the admixture model and assuming correlated allele frequencies. We uploaded the results to StructureSelector web server (Li and Liu, 2018), which plots the log probability of the data (LnP(K) to determine the optimal K value.

To supplement Ewens-Waterson test and further investigate possible role of natural selection we conducted other neutrality tests, including Tajima's D, Li's F* and Fu and Li's D*, implemented in DnaSP. A selection test based on the rate of nonsynonymous (dN) and synonymous substitutions (dS) was conducted using MEGA 11 (Tamura et al., 2021) for entire sequences and antigen binding sites (ABS). Moreover, we tested for the presence of selection on individual codons using several programs. EasyCodeML (Gao et al., 2019) was used to detect positive selection, applying the maximum likelihood approach. We compared two models: M7, which proposes neutral evolution (null hypothesis), with M8, which represents an alternative model that implies positive selection ($\omega>1$). Both models assume beta distribution of ω. Additionally, we applied methods available at Datamonkey web server (Martin et al., 2010), including FEL (Fast, Unconstrained Bayesian AppRoximation) (Murrell et al., 2013), FUBAR (Fast, Unconstrained Bayesian AppRoximation) (Murrell et al., 2013) and MEME (Mixed Effects Model of Evolution) (Murrell et al., 2012).

RESULTS

We identified a total of 10 MHC-DRB alleles in 133 roe deer individuals, all of which were previously documented in the literature (Table S1). Consistent with published data, we found that there were no more than two alleles per individual, further confirming the presence of only one DRB locus in roe deer. Alleles were either 249 bp or 246 bp long, depending on whether they contained a deletion of codon 65. Alleles in which deletion was detected were Caca-DRB*0301, Caca-DRB*0302, Caca-DRB*0303 and Caca-DRB*0402. The number of polymorphic sites was 22 (8.84%), without considering the aforementioned codon deletion. Ten identified alleles could be translated to 9 unique amino acid sequences since alleles Caca-DRB*0302 and Caca-DRB*0402 differed in only one nucleotide and coded for the same amino acid sequence. None of the detected sequences included any stop codons, implying their functionality.

Nucleotide diversity (π) was estimated at 4.2% and the average number of nucleotide differences among alleles (k) was 10.29. The mean nucleotide distance was 10.8%, with pairwise values ranging from 0.4% to 38.2%. Mean amino acid distance was higher in comparison and was estimated at 41.1%, suggesting sufficient levels of functional divergence (Table 1). The largest amino acid pairwise distance was observed comparing allele Caca-DRB*102 to alleles Caca-DRB*0302 and Caca-DRB*0402 (12 substitution steps) since the last two alleles are identical on the amino acid level.

Allele with the highest overall frequency was the Caca-DRB*0301 (39.8%), followed by Caca-DRB*0302 (19.5%). The remaining eight alleles had a frequency of <10%. The rarest allele was Caca-DRB*0403, found only in two individuals (0.8%) (Table 2). Thirty-two individuals (24.1%) were homozygous, 21 (65.6%) of which were homozygous for the most common allele, Caca-DRB*0301. The p-values of tests for Hardy-Weinberg Equilibrium (HWE) deviation were not significant, indicating that neither the population data nor the overall dataset deviated from Hardy-Weinberg expectations (Table 3).

Eight alleles were shared between the lowland and the mountain population, the allele Caca-DRB*0403 was detected only in two individuals from the lowland population, and allele Caca-DRB*0405 was detected only in five individuals from the mountain population. In both populations, allele frequencies of the most common alleles (Caca-DRB*0301 and *0302) followed

the trend for the overall data (Table 2). Expected heterozygosity was estimated at 0.815 in the mountain population, which was a bit higher than in the lowland population (0.763, Table 3). Allelic richness, based on the minimum sample size of 40 individuals, was slightly higher in the mountain population as well (9.0 vs 8.6, Table 3). Results of the AMOVA calculations attributed 96.83% of variance to the within population variation and only 0.70% to the between populations variation. The value of F_{ST} was notably very low, only 0.007, and statistically insignificant (Table 4). STRUCTURE analysis further confirmed the absence of visible structuring of Croatian roe deer population at the DRB locus, as the most probable number of identified clusters was K = 1 and higher values of K resulted in lower probabilities (Figure S1).

Ewens-Watterson-Slatkin test revealed significantly higher values of expected homozygosity than the observed (Fexp>Fobs), more than would be expected under the mutation-drift equilibrium, implying evenness in allele frequencies and the presence of balancing selection (Table 5). Evenness was further tested through ratio of recorded (A_n) and effective number of alleles (A_e) (Table 3). Values close to zero indicate uneven distribution of allelic frequencies while values closer to one imply evenness due to the role of balancing selection. Ratio of A_n to A_e had values close to 0.5 in the overall data and for the lowland population, and was a bit higher in the mountain population (0.618, Table 3), without statistical significance.

Results of dN/dS tests of selection conducted in MEGA 11 on entire sequences and specifically on ABS showed values greater than 1, indicating potential selection, but these values were not statistically significant (Tables S2 and S3). Fu and Li's D* as well as Fu and Li's F* neutrality tests showed statistical significance for specific populations and overall data, while Tajima's D was only significant for the overall data (Table 5). After the analysis of positive selection performed in EasyCodeML, the null model M7 was rejected in favour of the alternative M8 for three codon sites (13, 57, 86). Additionally, codon site 86 was found to be under the influence of positive selection by the methods FEL, FUBAR and MEME (Table S4).

DISCUSSION

After analysing the DRB locus in 133 roe deer from Croatia, we observed low to moderate levels of diversity. Ten identified alleles corresponded to nine different amino acid sequences, which is notably lower than the number of alleles identified in majority of DRB studies on other cervids or related species e.g. red deer (46 alleles in 155 individuals, Buczek et al., 2016), white-tailed deer (18 alleles in 126 individuals, Van Den Bussche et al., 2002), caribou (21 alleles in 114 individuals, Kennedy et al., 2011), Ussuri sika deer (15 alleles in 43 individuals, Li et al., 2013), forest musk deer (seven alleles in 52 individuals, Cai et al., 2015), Chinese muntjac (20 alleles in 12 individuals (Jian et al., 2010). Furthermore, percentage of variable nucleotide sites was also quite low (8.84%) in comparison with some other studies on DRB alleles e.g. 27.6% in mule deer (Cook et al., 2022), 31.3% in Scottish red deer (Pérez-Espona et al., 2019), 34.9% in Ussuri sika deer (Li et al., 2013). Four out of the ten identified alleles exhibit a deletion at nucleotide position 65, contributing to sequence diversity. This deletion has been previously documented in the DRB loci of cattle (Mikko and Anderson, 1995), European bison (Radwan et al., 2006), and forest musk deer (Cai et al., 2015). Codon 65 encodes a residue in the α-helical chain, suggesting its potential impact on peptide binding (Mikko et al., 1997).

Since MHC genes code for the molecules that present antigens to the T-lymphocytes, reduced diversity in MHC could increase the vulnerability of populations to infections. However, selection may favour a specific number of alleles for their effectiveness, even if that number appears deficient (Radwan et al., 2010). A small number of retained alleles in a population can be compensated for by high functional divergence between those alleles, particularly following sudden demographic changes, as indicated by the divergent alleles hypothesis (Wakeland et al., 1990). Because of the socioeconomic changes in rural areas, forest plantations and reintroductions, roe deer population numbers in the last decades have dramatically increased (Apollonio et al., 2017), so presently, the estimated number of mature individuals in central Europe is around 15 million (Lovari et al., 2016). This population expansion could have influenced MHC diversity, due to forces of genetic drift and migration, which has already been argued in Bužan et al. (2022). Although the number of recorded alleles in roe deer is relatively low, the

amino acid distance value (41.1%) is notably high, indicating a significant level of functional diversity among the recorded alleles and recognition of a larger array of antigens compared to alleles that are more similar. Moreover, in line with the "heterozygote advantage hypothesis" (Doherty and Zinkernagel, 1975), increased levels of heterozygosity contribute to a wider spectrum of antigen recognition. Heterozygosity levels determined in this study were consistent with Hardy-Weinberg equilibrium (HWE), albeit ranging from moderate to high values (Table 3).

Examination of the presence of selection yielded ambiguous results. Concerning the tests capable of detecting recent selection patterns, the frequency-based Ewens-Watterson-Slatkin test indicated a potential for selection, while the ratio of observed to effective number of alleles, known as allelic evenness, generally fell within intermediate values. Only the mountain population exhibited somewhat higher than intermediate value of allelic evenness. Therefore, the results of the Ewens-Watterson-Slatkin test should be interpreted cautiously, particularly since they are not supported by genotype-based evidence of selection, such as an excess of heterozygosity (Garrigan and Hedrick, 2003). Standard neutrality tests Fu and Li's F* and D* were positive and significant, indicative of balancing selection or demographic changes. Selection over a larger time scale was further analysed with Tajima's D and dN/dS tests on the whole sequence. While Tajima's D was significant only for the overall dataset, dN/dS tests were not significant for entire sequences or ABS inferred from the human ortholog. Selection typically targets specific codons rather than the entire sequence (Hughes and Nei, 1988). However, if the rate of nonsynonymous mutations significantly surpasses that of synonymous mutations, signs of positive selection should be detectable across the entire sequence (Yang and Bielawski, 2000). In this study, however, such evidence was not observed. The historical signal of selection on the roe deer DRB locus generally seems quite weak, as we could only identify three codons with a distinct signature of selection, when we used site specific tests in CodeML , two of which corresponded to the human ortholog (Brown et al., 1993).

We compared the results of our study to other research on European roe deer populations, namely findings by Mikko et al. (1999) on the Scandinavian (Sweden, Norway) roe deer population, Quéméré et al. (2015) on the French population and Bužan et al. (2022) on the Slovenian population. Interestingly, identical number of alleles (10) was found in studies on the

Croatian, French and Slovenian population, regardless of the sample size (133 in this research, 156 in Bužan et al., 2022; 476 in Quéméré et al., 2015). Nine out of 10 alleles detected in this study were shared with Slovenian population, seven with French and two with the Scandinavian population (Mikko et al., 1999). The pronounced contrast in MHC diversity between Central European populations and Scandinavian populations is likely attributed to an extreme population bottleneck during the severe regional cooling period in the Middle Ages. This is evidenced by the remarkably low MHC diversity reported in the study by Mikko et al. (1999), which identified only four alleles in 62 animals, along with low levels of heterozygosity ranging from 0.24 to 0.58.

Conversely, Croatian and Slovenian populations show a high similarity in the MHC diversity pattern, demonstrated by an almost complete match in detected alleles. Additionally, the most common alleles in both populations are Caca-DRB*0301 and *0302. While there is potential for the continuous distribution of roe deer between neighbouring Slovenia and Croatia without any major geographical barriers to prevent gene flow between the populations, the roe deer are predominantly territorial animals that rarely migrate long distances. Therefore, the observed similarity at the MHC level might be a relic from the glacial refugia. What stands out as a noteworthy difference between these populations is substantially higher proportion of homozygotes in Slovenian roe deer population, i.e. 46% (Bužan et al., 2022) vs. 24.1% in this research, and consequently deviation from HWE in overall dataset as well as in each of the three clusters in Slovenia, and conformation to HWE of Croatian populations. However, deviation from HWE with notably higher homozygosity than expected, as in the case of Slovenian population, is not unprecedented, and in fact was previously reported in some cervids (Cook et al., 2022; Van Den Bussche et al., 2002; Wilson et al., 2003).

Differences in selection pressures across the geographical landscape can lead to population structuring at the MHC, as demonstrated in various vertebrate populations (Babik et al., 2005; Bryja et al., 2007; Cook et al., 2022; Ekblom et al., 2007; Evans et al., 2010; Herdegen et al., 2014). In contrast, balancing selection can lower level of genetic structure on the MHC since it maintains allelic distributions across populations (Sutton et al., 2011). However, apart from selective forces, neutral forces are also acting on the MHC region, which can in some cases counteract selective forces resulting in ambiguous genetic patterns. Similarly, in two populations from Croatia, larger

lowland population and smaller scattered mountain population, we also failed to detect evidence of population genetic structuring based on the DRB locus. Results of AMOVA and STRUCTURE analysis further confirmed absence of distinct structure on the MHC level. Expected and observed heterozygosity, as well as allelic richness were slightly higher in the mountain than in the lowland population. Population-specific alleles were Caca-DRB*0405 detected exclusively in Croatian mountain region (Svetličić et al., 2022), and the least frequent allele Caca-DRB*0403, previously reported in Slovenian population.

The DRB locus is widely recognized as the most variable class II MHC locus in humans (Barker et al., 2023) making it a staple marker in the majority of wildlife MHC studies (e.g. Murray and White 1998; Babik et al., 2005; Froeschke and Sommer 2005; Schaschl et al., 2006; Lenz et al., 2013; Arbanasić et al., 2019; Dong et al., 2023). In roe deer, the limited number of recorded alleles and the weak evidence for long-term positive selection suggest somewhat reduced degree of variability at the DRB locus. Demographic events, particularly past bottlenecks, and recent population expansions, have likely influenced this, as mentioned above. Previous research on deer MHC has primarily focused on DRB, with other MHC loci being considered only rarely (Liu et al., 2013; Swarbrick and Crawford, 1997; Wan et al., 2011; Wu et al., 2012). Exploring other MHC loci might be beneficial for disentangling neutral and selective effects acting on roe deer immunity genes. Furthermore, other genes involved in immune response act in combination with the MHC, especially in case of species with lower MHC variation (Acevedo-Whitehouse and Cunningham, 2006). Quéméré et al. (2015; 2021) investigated the role of innate immunity in maintenance of immunogenetic variability in roe deer. They found levels of variability at toll like receptors at least comparable to those detected at the MHC, suggesting that they have synergistic effects on overall immune competence. Further pathogen-specific research with broader candidate gene targets, including other MHC loci as well as innate immunity genes, could potentially elucidate roe deer immunogenetic contributions to disease resistance and population viability.

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590 **TABLES**

591 **Table 1.** Sequence polymorphism results for 10 MHC-DRB alleles detected in roe deer individuals
592 from lowland and mountain populations, and overall.

593

Population	A_n	N	π	k	Nucleotide distance *	Amino acid distance **
594 Lowland	9	93	0.043	10.611	0.133	0.433
595 Mountain	9	40	0.043	10.611	0.115	0.440
596 Overall	10	133	0.042	10.289	0.108 (0.07)	0.411 (0.73)

597 A_n- number of recorded alleles, N – number of individuals, π – nucleotide diversity, k-mean number of
598 pairwise differences, * T92 + G substitution model, ** JTT + G substitution model, SD is given in
599 parenthesis.

600

Table 2. Frequencies and relative frequencies of 10 roe deer MHC-DRB alleles from Croatia

	Lowland (N=93)		Mountain (N=40)		Overall (N=133)	
Allele	**frequency**	**relative frequency**	**frequency**	**relative frequency**	**frequency**	**relative frequency**
Caca-DRB*0102 [1,2]	12	6.5%	6	7.5%	18	6.8%
Caca-DRB*0201 [1,2,3]	14	7.5%	2	2.5%	16	6.0%
Caca-DRB*0301 [1,2,3]	79	42.5%	27	33.8%	106	39.8%
Caca-DRB*0302 [1,2]	34	18.3%	18	22.5%	52	19.5%
Caca-DRB*0303 [1,2]	18	9.7%	3	3.8%	21	7.9%
Caca-DRB*0304 [1,2]	11	5.9%	6	7.5%	17	6.4%
Caca-DRB*0401 [1,2]	11	5.9%	5	6.3%	16	6.0%
Caca-DRB*0402 [1]	5	2.7%	8	10.0%	13	4.9%
Caca-DRB*0403 [1]	2	1.1%	0	0.0%	2	0.8%
Caca-DRB*0405	0	0.0%	5	6.3%	5	1.9%

Alleles with deletion of one codon are underlined. [1-] previously detected in Bužan et al. (2022), [2-] Quéméré et al. (2015), [3-] Mikko et al. (1999)

Table 3. Hardy-Weinberg equilibrium test results, allelic richness and ratio of recorded and effective number of alleles (A_e/A_n) for lowland and mountain roe deer populations and the overall data set.

	H_o	H_e	P_{HWE}	Allelic richness	A_e/A_n
Lowland	0.731	0.763	0.156	8.61	0.463
Mountain	0.825	0.815	0.668	9.00	0.618
Overall	0.761	0.778	0.167	10.00	0.455

625 **Table 4**. Results of AMOVA analysis and fixation index (F_{ST}) values between mountain and
626 lowland population.

Source of variation	Variance components	Percentage of variation	F_{ST} (p-value)
Among populations	0.00273	0.70	
Among individuals within populations	0.00968	2.47	
Within individuals	0.37970	96.83	
Total	0.39212	100	0.007 (0.24)

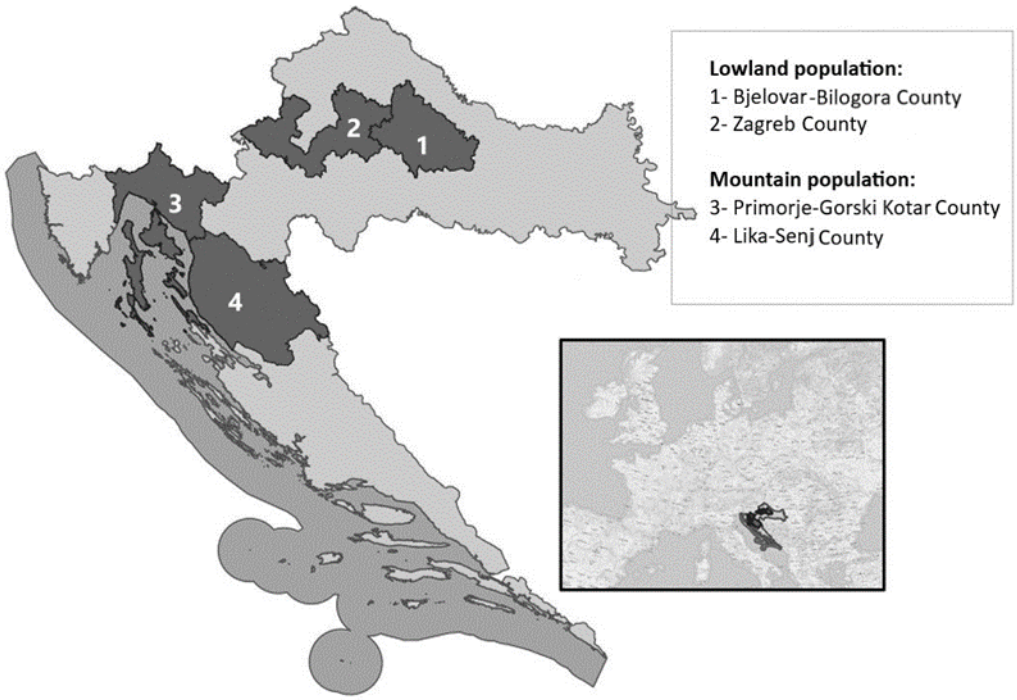
632 ---

Table 5. Results of neutrality tests conducted on two roe deer populations and the overall data

	Ewens-Watterson-Slatkin test					
	F obs.	**F exp.**	**p (Slatkin's exact test)**	**Fu and Li's D***	**Fu and Li's F***	**Tajima's D**
Lowland	0.241	0.342	**0.028**	**1.835**	**2.250**	1.925
Mountain	0.196	0.285	**0.031**	**1.792**	**2.088**	1.678
Mean (Overall)	0.218	0.314	**0.030**	**1.834**	**2.430**	**2.275**

Significant values are given in bold.

644 **FIGURES**



645 **Figure 1.** Map of Croatia showing the locations of lowland and mountain populations sampled
646 across four Croatian counties, each marked by number. A legend indicates the corresponding
647 county numbers.

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