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Variability of the DRB locus of MHC genes class II in red deer (*Cervus elaphus*) from a mountain region of Croatia

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ABSTRACT

Red deer (*Cervus elaphus*) are large indigenous mammals in Croatia. Even though Major Histocompatibility Complex (MHC) genes play a pivotal role in initiating immune response, there are no reports on their variability in red deer from Croatia. In this study, the variability of the DRB locus of MHC genes class II was analysed in 23 red deer from a mountain region of Croatia (Gorski Kotar). NGS sequencing revealed 23 unique DRB exon 2 sequences in 23 individuals, of which only six had been previously identified. The number of allelic variants per individual ranged from 1 to 6 (mean 3.09), with one individual having only one allele and one individual having all six allelic variants recognised. The most frequent allele, Ceel-DRB*HR01, was identified in 12 individuals (16.9%). The number of variable nucleotide sites was 84 (33.7%), and the number of variable amino acid positions in translated sequences was 44 (53%). The mean nucleotide evolutionary distance, based on the Jukes-Cantor model, with the gamma distribution shape parameter, was 16.5%, while the pairwise nucleotide distance ranged from 0.04% to 14.24%. The amino acid evolutionary distance, based on the Jones-Taylor-Thornton model, was 34.7%. Compared to the literature, it is clear that the mountain population of red deer from Croatia possesses considerable variability on the DRB locus, which is further confirmed by the notable nucleotide evolutionary distance.

Key words: red deer; MHC genes; DRB locus; variability; mountain region

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Introduction

Major Histocompatibility Complex (MHC) genes are among the most variable genes in vertebrates, having an essential role in their immune response (CONNER and HARTL, 2004.). They encode the trans-membrane glycoproteins responsible for presenting antigens to the T lymphocytes. These glycoproteins are divided into two main groups: i) MHC class I antigens, located on all nucleated cells, with the role of presenting viral and tumour antigens to cytotoxic T-cells, and ii) MHC class II antigens, present on certain cells of the immune system, such as macrophages, B-cells and dendritic cells, with the role of presenting antigens to T helper cells. The MHC class II genetic region is divided into three main loci: DP, DQ and DR, each one consisting of α- and β-chain genes, named as DPA and DPB, DQA and DQB, DRA and DRB (MALE et al., 2006; DAY and SCHULTZ, 2014). Genes that encode the β-chain are more polymorphic compared to those of the α-chain, and may contain hundreds of alleles in a population. Studies about MHC genes class II in mammals have been mainly directed towards the exon 2 region, as it encodes part of the molecule responsible for antigen binding. On this region, the highest diversity among alleles was found, making this locus the holder of the functional receptor diversity and an indicator of genetic variability (MURRAY et al., 1999; SOMMER, 2005).

Red deer (*Cervus elaphus*) are a large indigenous game species in Croatia. This Laurasiatherian mammal belongs to the order Cetartiodactyla, and the *Cervidae* family (HU et al., 2012). Red deer inhabit the majority of Croatia, with two main habitats, lowland areas along the Danube, Drava and Sava Rivers, and the mountain regions of mainly Gorski Kotar, Velika and Mala Kapela, Velebit and Lička Plješivica. Previous studies on the variability of the DRB locus in red deer revealed 34 alleles in samples from New Zealand/Wisconsin USA (SWARBRICK et al., 1995) and 46 alleles in samples from Poland (BUCZEK et al., 2016). At the same time, PÉREZ-ESPONA et al. (2019) found 25 different alleles in a Scottish wild red deer population, while FERNÁNDEZ-DE-MERA et al. (2009) found only 18 different alleles in an Iberian red deer population.

The aim of this paper was to obtain basic data on MHC variability in red deer from mountain regions of Croatia. The specific aims were to: i) investigate the variability of the MHC class II genes in red deer from the Gorski Kotar area, including the number of DRB loci and the number of functional allelic variants present, and ii) compare the obtained results to data reported in previous studies.

Materials and methods

Location and sampling. Liver samples from 23 red deer were collected during the regular execution of the game management plan in the open hunting ground No.: VIII/110 - "Crna Gora" (ANONYM., 2017). This is a mountain habitat located in Gorski Kotar, in the northern part of the Primorje-Gorski Kotar County, near the border with Slovenia. During evisceration, a sample of the liver was taken and stored at -20 °C in a labelled Eppendorf tube with 96% alcohol, until further analysis. Isolation of DNA was performed using a commercial Wizard Genomic DNA Purification Kit (Promega, USA), according to the manufacturer's instructions. Isolated DNA was stored at 4 °C.

Next generation sequencing. We amplified a 249-bp fragment of the 2nd exon of the red deer MHC II DRB gene. For this purpose, we designed forward extended primers for the Ion Torrent PGM system as follows: adaptor sequence (30bp), 10-bp barcodes with a specified "GAT" linker to distinguish individuals, and the MHC-specific forward primer, LA31: 5'< GATCCTCTCTCTGCAGCACATTTCCT > 3' and reverse extended primer; adaptor sequence and MHC-specific reverse primer (LA32 - TTCGCGTCACCTCGCCGCTG). MHC-specific primers LA31 and LA32 were initially designed for cattle (SIGURDARDOTTIR et al., 1991).

PCR amplification was performed in triplicates in 25 µL reaction mixtures, which contained 50 ng of genomic DNA, 0.5 mM of each primer, 0.5 µL dNTPs, 2.5 mM $MgCl_2$, 0.5 µL HotStarTaq (Qiagen AllTag), 5.0 µL Q solution and 6.2 µL H2O. The PCR program consisted of 2-min initial denaturation at 95 °C, followed by 35 cycles of 10-s denaturation at 95 °C, 30-s annealing at 60 °C and

30-s extension at 72 °C. The final elongation step was run at 72 °C for 10 min.

Amplicons from triplicates were then pooled and purified with Agencourt® AmPure® magnetic particles (Agencourt Bioscience Corporation, A Beckman Coulter Company, Beverly, MA). The concentration of pooled and cleaned amplicons was measured with Qubit 3.0 fluorometry, using Qubit dsDNA HS (High Sensitivity) Assay Kit reagents (Invitrogen, Carlsbad CA). The samples were then normalized on the 5ng and merged to one common library. The library was purified again with Agencourt® AmPure® magnetic particles. The size and quality of the amplicons were determined with the Agilent DNA High Sensitivity Kit on a 2100 Bioanalyzer (Agilent), following the manufacturer's recommendations. Before loading the library onto 314 chips, the library was normalized to 100 pM, following the manufacturer's recommendations.

All sequences were analysed with an Ion Torrent Software Suite (Vs. 5.10.0) using the plug-in variant caller (Vs. 3.2.43647) that employs the TMAP Smith-Waterman alignment optimization (LI and HOMER, 2010). The output of the variant caller was presented in tabular format, as a list of differences from the rCRS, without a graphical display of the aligned reads.

Processing of raw Ion Torrent data was conducted using AmpliSAT, a suite of next-generation amplicon sequencing analysis tools, developed by SEBASTIAN et al. (2016) and available as a web server at: http://evobiolab.biol.amu.edu.pl/amplisat/index.php. For the preliminary exploration of the data, AmpliSAT's AmpliCHECK tool was used with default Ion Torrent parameters: 0.5% substitution error rate, 1% indel error rate and minimum per amplicon frequency of 1%. AmpliCHECK revealed the clear predominance of 270-bp sequences. Four variants of similar lengths (169, 171 and 173 bp) were found in five individuals, with three variants recorded in a single individual. Alignment of these sequences to the other variants showed homopolymer indels (base insertion or deletion). Thus, we considered these variants as sequencing artefacts. Also, short-length (148 - 150 bp) sequences were considered as artefacts. The AmpliSAS tool was used for the final de-multiplexing, clustering, and filtering of the amplicon sequences. For clustering, default Ion Torrent expected error rates were set with 25% minimum dominant frequency. Based on these parameters, AmpliSAS clustered putative parental variants, with artefacts derived from these variants occurring within the same amplicon, thereby increasing the read depths of the true variants. In the filtering step, we removed chimeras and low frequency variants that had less than 3% frequency.

Identified sequences were aligned, processed and analysed in BioEdit (HALL, 1999). The number of nucleotide and amino acid variable sites, as well as nucleotide and amino acid evolutionary distances were calculated in MEGA X (KUMAR et al., 2018).

Results

Ion Torrent DRB amplicons assigned from 224 to 2258 reads per amplicon (depth amplicon) (Table 1). After clustering and filtering (removal of chimeras and low frequency variants that had less than 3% frequency), the number of reads per variant occurring within the same amplicon (variant depth) was from 16 (allele Ceel-DRB*HR08, individual J24_GK) to 1223 (allele Ceel-DRB*HR02, individual J10_GK) (Table 1). In total, 71 sequences were identified in 23 individuals collected in the Gorski Kotar region of Croatia, which constituted 23 unique red deer DRB exon 2 alleles. The number of different alleles per individual ranged from 1 to 6 (mean number 3.09), with one individual having only one allele, and one individual having all 6 allelic variants (Fig. 1, Table 1). The most frequent allele, Ceel-DRB*HR01, was identified in 12 individuals (16.9%) while 7 alleles were identified in only a single individual each (frequency 1.41%) (Fig. 2, Table 1).

A region covering most of the second exon of the DRB locus was analysed and included nucleotide positions 10 - 258. None of the detected sequences contained indels or stop codons. All the observed DRB sequences encoded products with unique amino acid sequences. The number of variable nucleotide sites in the detected alleles was 84 (33.7%), and the number of variable amino acid positions in the translated sequences was 44 (53%).

Table 1. Results of genotyping variants with AmpliSAS (numeric values show the variant depths after clustering and filtering), allelic frequencies and matching sequences (previously found alleles) information. Legend: Depth amplicon - number of reads per amplicon; Depth alleles - total number of reads after clustering and filtering per individual; Count alleles - number of alleles per individual

Individual ID	J14	J16	J18	J24	J6	J25	J26	J1	J3	J4	J7	J10	J13	J19	J2	J5	J8	J11	J15	J17	J20	J23	J9	Frequency (%)	Matching sequences
Ceel-DRB*HR01	216	121	268	56		85	128							373				523	444	547	486	539		12 (16.9)	
Ceel-DRB*HR02		20		39		43	67		927			1223			733									7 (9.86)	
Ceel-DRB*HR03			62											83				102	99	131	176	150		7 (9.86)	
Ceel-DRB*HR04				30	278	34	68				373		266			314								7 (9.86)	Ceni-DRB*20[1] and CeelHap113[2]
Ceel-DRB*HR05	50	69	179	24			26																	5 (7.04)	
Ceel-DRB*HR06											50		192										494	3 (4.23)	
Ceel-DRB*HR07	41	21												62										3 (4.23)	
Ceel-DRB*HR08	29		57	16																				3 (4.23)	
Ceel-DRB*HR09					43						112					65								3 (4.23)	
Ceel-DRB*HR10									515						433									2 (2.82)	CeelHap105[2]
Ceel-DRB*HR11									433	100														2 (2.82)	
Ceel-DRB*HR12						23							267											2 (2.82)	CeelHap115[2]
Ceel-DRB*HR13								98									75							2 (2.82)	
Ceel-DRB*HR14					223					247														2 (2.82)	Ceni-DRB*17[1]
Ceel-DRB*HR15					42					122														2 (2.82)	Ceni-DRB*6[1]
Ceel-DRB*HR16								227									122							2 (2.82)	
Ceel-DRB*HR17												247												1 (1.41)	
Ceel-DRB*HR18												228												1 (1.41)	Ceni-DRB*4[1]
Ceel-DRB*HR19	80																							1 (1.41)	
Ceel-DRB*HR20			43																					1 (1.41)	
Ceel-DRB*HR21								40																1 (1.41)	
Ceel-DRB*HR22	37																							1 (1.41)	
Ceel-DRB*HR23		21																						1 (1.41)	
DEPTH_AMPL	767	602	1055	498	662	567	850	562	2258	524	580	2072	952	1079	1461	435	224	1014	1077	1124	1188	1263	567		
DEPTH_ALLE	453	252	609	165	586	185	289	365	1875	469	535	1698	725	518	1166	379	197	625	543	678	662	689	494		
COUNT_ALLE	6	5	5	5	4	4	4	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2	1		

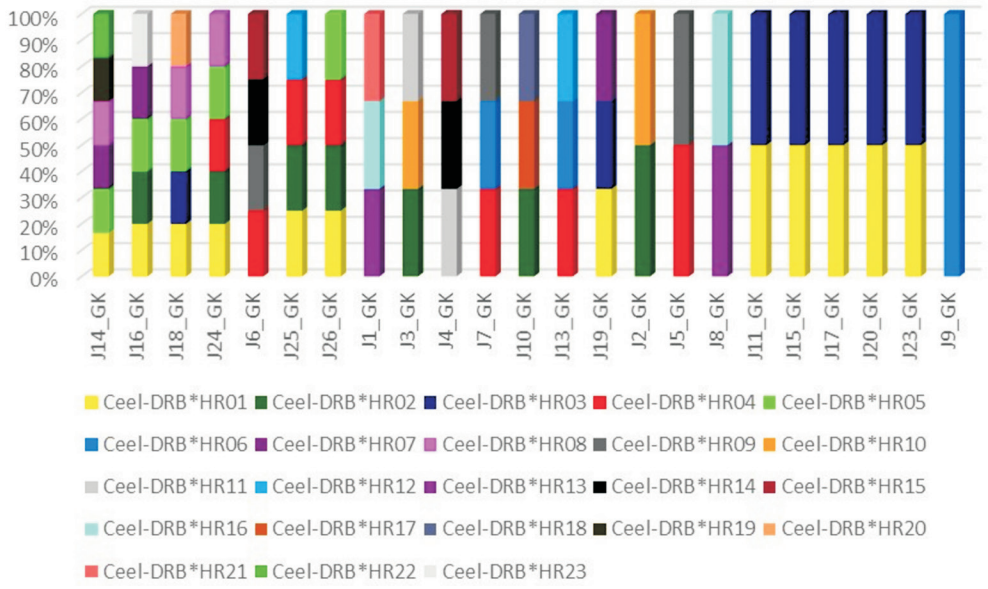

Fig. 1. Distribution of DRB alleles in 23 red deer individuals from Gorski Kotar, Croatia

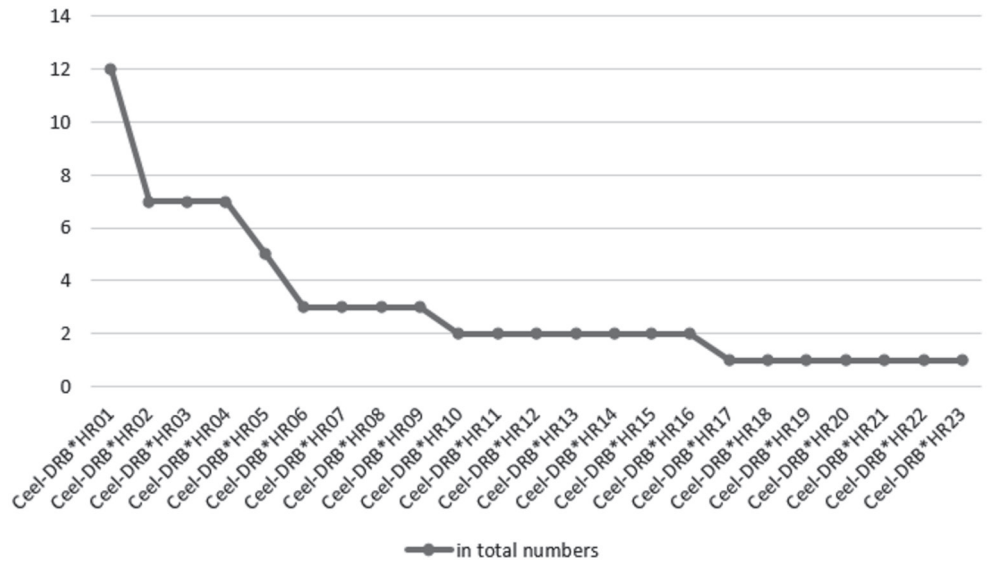

Fig. 2. Frequencies of 23 unique DRB alleles found in sample of 23 red deer from Gorski Kotar, Croatia (out of 71 alleles identified in total)

Twenty-three of the variable amino acid positions matched antigen-binding amino acid positions identified in the human DRB ortholog (BROWN et al., 1993). Further, 11 variable amino-acid positions were found to be neighbouring antigen-binding positions identified in the human DRB ortholog (BROWN et al., 1993), implying a similar receptor domain structure between species. The mean nucleotide evolutionary distance, calculated using the Jukes-Cantor substitution model, with a gamma distribution shape parameter, was 16.5%, while the pairwise nucleotide distance ranged from 0.04% to 14.24%. The amino acid evolutionary distance calculated using the Jones-Taylor-Thornton substitution model was 34.7%. Despite the extreme variability of the detected alleles, we found conserved residues in the amino acid alignment of our sequences. Namely, all translated alleles contained cysteines at positions 10 and 74, which are involved in disulphide bridge formation,

which is important for the folding and function of a protein. Three of our sequences (Ceel-DRB*HR07, Ceel-DRB*HR20 and Ceel-DRB*HR22) contained an RDNS amino acid motif in place of the RFDS motif, which is usually conserved in the β1 domain (MAZEROLLES et al., 1988).

The alleles detected in this study were named Ceel-DRB*HR01 - Ceel-DRB*HR23 and deposited in GenBank with accession numbers MN601742 - MN601764.

Out of the 23 identified different alleles, six had been found previously, while the remaining 17 were novel. Alleles Ceel-DRB*HR10 and *HR12 were previously found in an Iberian red deer population (alleles CeelHap105 and CeelHap115, respectively, FERNÁNDEZ-DE-MERA et al., 2009) and alleles Ceel-DRB*HR14, *HR15 and *HR18 were previously found in a Scottish population (alleles Ceni-DRB*17, *6 and *4, respectively, PÉREZ-ESPONA et al., 2019), while allele Ceel-DRB*HR04 was previously found in both the Iberian and Scottish red deer populations (alleles Ceel-Hap113 and Ceni-DRB*20, FERNÁNDEZ-DE-MERA et al., 2009; PÉREZ-ESPONA et al., 2019, respectively) (Table 1).

Discussion

Despite the large distribution of red deer and the importance of MHC loci in population fitness assessment, only a few studies on deer MHC have been published so far (SWARBRICK et al., 1995; FERNÁNDEZ-DE-MERA et al., 2009; BUCZEK et al., 2016; PÉREZ-ESPONA et al., 2019). This study represents the first analysis of MHC gene variability in red deer from Croatia. In this research we found 23 DRB exon 2 alleles in 23 individuals. A similar number of alleles (25) was found in red deer from the Scottish highlands, however they analysed 48 animals (PÉREZ-ESPONA et al., 2019). Likewise, relatively lower allelic variability was reported in studies by BUCZEK et al. (2016) with 46 alleles in 155 individuals, FERNÁNDEZ-DE-MERA et al. (2009) detecting 18 alleles in 94 individuals and SWARBRICK et al. (1995) who found 34 alleles in 50 individuals. Other parameters of genetic variability, namely the percentage of variable nucleotide and amino acid sites, determined in our study as 33.7% and 53%, respectively, were in the range of the values obtained from Polish (BUCZEK et al., 2016) and New Zealand/Wisconsin USA (SWARBRICK et al., 1995) samples. Considering all these data, we conclude that the mountain population of red deer from Croatia possesses considerable variability on the DRB locus, which is additionally confirmed by the notable nucleotide evolutionary distance (16.5%).

Regarding the number of DRB loci, we found up to six alleles per individual, implying that three DRB loci might be present in red deer. Former studies indicated the presence of multiple DRB copies, but the number of copies varied between different studies and ranged from one to four (SWARBRICK et al., 1995; FERNÁNDEZ-DE-MERA et al., 2009; BUCZEK et al., 2016; PÉREZ-ESPONA et al., 2019). Only one locus was found in the Spanish population (FERNÁNDEZ-DE-MERA et al., 2009), and one possible explanation was the fact that the analysed population was managed for hunting purposes, which included fencing, isolation, population disruptions and selective culling. Indeed, isolated populations might suffer from reduced genetic variability, which makes it important to monitor their genetic status. In the research on the Spanish population, the single strand conformational polymorphism method (SSCP) was used to distinguish different allelic variants. SSCP is an electrophoresis-based method, where sequence variants are separated according to conformation differences, which in turn are determined by nucleotide compositions. It is, however, possible that different sequences show an identical pattern on gel, and hence cannot be distinguished. Before the advent of the NGS methodology, SSCP was the most common method used in genotyping multiplied MHC loci. Nonetheless, as it identifies each nucleotide site, the NGS approach is more accurate and sensitive in detecting different sequences present on multiplied loci.

The allelic variants obtained were compared with those previously reported in the literature. Six allelic variants were previously identified in Scottish wild deer (PÉREZ-ESPONA et al., 2019)

and an Iberian deer population (FERNÁNDEZ-DE-MERA et al., 2009) (Table 1). Among them, we found the allele Ceel-DRB*HR04 to be present in both the aforementioned populations. One of the main features of MHC genes is their involvement in molecular adaptation (HUGHES and YEAGER, 1998; BERNATCHEZ and LANDRY, 2003). Different pathogen pressures result in variations in MHC genotypes between populations. Adaptation of the MHC genes according to habitat variations along the time and geographic scale is consistent with the diversifying selection that shapes MHC variability (SOMMER, 2005). We can hypothesize that the allele Ceel-DRB*HR04, which is present in the Scottish Highlands, the Iberian peninsula and Croatia, represents a widespread variant that contributes to immunity against certain, common pathogens.

To assess the functionality of the nucleotide sequences found in this study, we checked for stop codons, reading frame interruptions and conserved regions. Interestingly, the usually conserved amino acid RFDS sequence motif was replaced by an RDNS motif in three of our sequences, which might influence protein structure and function. Namely, hydrophobic amino acid phenylalanine was replaced by aspartic acid, which is charged. As the RFDS motif is important for the interaction of antigen presenting cells with the CD4 cell surface receptor present on T lymphocytes (MAZEROLLES et al., 1988), the amino acid variations that we found might suggest variations in interaction between antigen presenting cells and T lymphocytes. On the other hand, these three alleles might represent non-functional sequences, *i.e.* pseudogenes.

The NGS technology applied and the results obtained in this study provide the basis for future genetic research on this ecologically and economically important species, and will improve game and wildlife management.

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SAŽETAK

Jelen obični (*Cervus elaphus*) jest zavičajna vrsta divljači u Republici Hrvatskoj. Unatoč činjenici da glavni sustav tkivne podudarnosti ima vodeću ulogu u pokretanju imunosnog odgovora, nema dostupnih podataka o njegovoj varijabilnosti u jelena običnog na području Hrvatske. U ovom je radu istražena raznolikost lokusa DRB gena MHC klase II na uzorku od 23 jelena iz gorskoga staništa (Gorski kotar). Sekvenciranje nove generacije (NGS) potvrdilo je postojanje 23 jedinstvene sekvencije DRB egzona II, od kojih je prethodno identificirano svega 6. Broj alela kolebao je od 1 do 6 (prosjek 3,09) po životinji, dok je jedna jedinka imala svega 1 alel, a jedna svih šest alela. Najčešći alel, Ceel-DRB*HR01, utvrđen je u 21 jedinci (16,9 %). Broj varijabilnih nukleotidnih mjesta bio je 84 (33,7 %), a broj varijabilnih pozicija aminokiselina 44 (53 %). Prosječna evolucijska udaljenost nukleotida, temeljena na Jukes-Cantorovu modelu, s gama-distibucijskim parametrom, bila je 16,5 %, dok je nukleotidna udaljenost sekvencija (temeljena na baznim parovima) iznosila od 0,04 % do 14,24 %. Aminokiselinska evolucijska udaljenost, temeljena na Jones-Taylor-Thorntonovu modelu, iznosila je 34,7 %. Usporedbom s literaturom vidljivo je da gorska populacija jelena običnoga u Hrvatskoj posjeduje zadovoljavajuću raznolikost na lokusu DRB, što je nadalje potvrđeno primjetnom nukleotidnom evolucijskom udaljenošću.

Ključne riječi: jelen obični; geni MHC; lokus DRB; raznolikost; gorsko stanište