SEQUENCE DIVERSITY OF MHC CLASS II DRB GENES IN THE MOOSE *ALCES ALCES* FROM ITS CONTINUOUS RANGE IN POLAND

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Abstract

Many vertebrate species show a very high degree of polymorphism of the genes of the major histocompatibility complex (MHC), which is associated with its key role in their immune response. However, studies on MHC class II DRB gene diversity in moose populations in Europe and North America have shown a low level of polymorphism, with clear differences between populations. In our study we examined the diversity in a fragment of an MHC class II DRB gene with 226 base pairs in 196 moose from Poland from seven populations. We identified eight (from 5 to 8 in particular populations) MHC II DRB alleles, of which *DRB1*11* was unique to the moose population in the Biebrza Valley. This population has experienced a series of numerous significant reductions in historical times and has been largely isolated from other moose populations. Although genetic differentiation among populations for the MHC II DRB gene was low, PCA analysis clearly showed the distinct character of the Biebrza population. This may confirm the relict character of this moose population.

Key words: *Alces alces*, DRB gene, MHC, moose, relict

Introduction

Major histocompatibility complex (MHC) genes play a key role in vertebrate immune response (Janeway et al., 2004). They are characterized by significant allele diversity, high durability, and high heterozygosity (Klein et al., 1993). The high diversity of MHC loci potentially contributes to increased resistance to environmental pathogens and thus to the survival of the species. Many studies conducted on wild mammals show a correlation between low MHC gene diversity and decreased adaptation (Yasukochi et al., 2012). Reduced MHC diversity has been observed in populations or species at risk of extinction or those that had previously experienced a bottleneck effect (Babik et al., 2005, 2009; Mainguy et al., 2007; Radwan et al., 2007). However, although many studies do show correlations between MHC diversity and resistance to infectious diseases, it does not seem to be a universal principle. MHC diversity in the California sea lion (*Zalophus californianus*) – a non-threatened species with a healthy level of

genetic diversity – is much smaller than in other mammalian species (Bowen et al., 2004). On the other hand, a very low level or even lack of MHC diversity, including the DRB locus, in the snow goat (*Oreamnos americanus*), most probably does not increase its susceptibility to diseases (Mainguy et al., 2007). Reduced diversity in the MHC loci may be the result of population reductions followed by postglacial expansion in temperate and boreal areas (Babik et al., 2009). MHC genes are potentially influenced by natural selection, with the pressure of parasites being the likely main factor influencing their diversity (Hedrick et al., 2000; Lachish et al., 2007). Another significant evolutionary force playing a role in MHC evolution is genetic drift (Garrigan and Hedrick, 2003). When population size is small, MHC diversity is shaped mainly by demographic processes and genetic drift, as indicated by a number of studies comparing the diversity of MHC and neutral loci (Radwan et al., 2010).

The moose (*Alces alces*, Linnaeus, 1758) is the second largest living representative of the terrestrial mammals of Europe, smaller only than the European bison (*Bison bonasus*). It was an important element of Polish fauna from the end of the ice age to the early Middle Ages (Gumiński, 2003). However, at the beginning of the 19th century the moose population in Poland collapsed to such an extent that only the forests near Rajgród in the Biebrza Valley were the westernmost range of this species in Poland (Brincken, 1826). That population survived World War II as a group of only several to a dozen individuals in the Biebrza Valley, which then gave rise to a herd consisting of the descendants of the native population that has lived in the area since the original settlement (Dzięciołowski and Pielowski, 1993; Raczyński, 2006). The total moose population in Poland peaked in 1981 at nearly 6,200 between various populations around the country (Dzięciołowski and Pielowski, 1993; Gębczyńska and Raczyński, 2001). An important role in that recovery was played by the immigration of moose through the northern and eastern borders of Poland and the dynamic development of the reintroduced moose in the Kampinos National Park in the 1950s. However, excessive hunting in the 1980s and 1990s soon led to a significant reduction in the population size and range. That is why in 2001, the Polish Minister of the Environment announced a hunting ban on moose, which helped rebuild the population of this *Cervidae* species.

In this study, we examined variations at the MHC II DRB locus in the moose, a Cervidae species that has undergone an extreme bottleneck in Poland after World War II.

Material and methods

Sample collection and DNA extraction. A total of 229 moose samples were collected in 2010 – 2011 from seven moose populations in Poland (Fig. 1).

The 70 samples of muscle and skin were taken from animals killed in road accidents, found dead or poached. Additionally, 159 stool samples were collected in winter. Samples were stored at –20°C pending DNA extraction, using a DNeasy Blood and Tissue Kit or, for stool samples, the QIAamp DNA Stool

Mini Kit (Qiagen, Hiden, Germany), in accordance with the manufacturer's instruction.

Molecular and statistical analyses. Each tissue and stool sample was examined according to 11 microsatellite loci (Świsłocka et al., 2015), to identify multilocus genotypes of moose individuals. In total, 229 samples from 196 individuals were examined and included in further analyses. The sample size from the analyzed populations ranged from 7 to 68 individuals (Table 1).

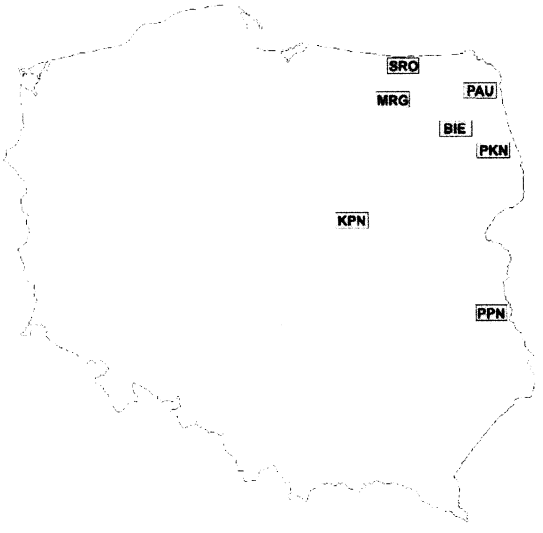

Figure 1. Study area and moose sampling in Poland. BIE – Biebrza Valley, PAU – Augustów Forest, PKN – Knyszyn Forest, MRG – Mrągowo State Forest, SRO – Srokowo State Forest, KPN – Kampinos National Park, PPN – Polesie National Park. Green color – moose range in Poland.

Table 1. MHC II DRB loci diversity indices for seven moose populations studied in Poland. N – sample size; Nh – number of alleles; h – alleles diversity; π – nucleotide diversity (%); S – number of segregating sites; PD – mean number of pairwise differences; SE – standard error. BIE – Biebrza Valley, PAU – Augustów Forest, PKN – Knyszyn Forest, MRG – Mrągowo State Forest, SRO – Srokowo State Forest, KPN – Kampinos National Park, PPN – Polesie National Park.

Population	N	Nh	h ± S.E.	π ± S.E. (%)	S	PD ± S.E.
BIE	68	8	0.759 ± 0.018	1.616 ± 0.912	8	3.653 ± 1.861
PAU	20	5	0.787 ± 0.026	1.637 ± 0.939	8	3.700 ± 1.910
PKN	15	5	0.775 ± 0.041	1.574 ± 0.916	7	3.556 ± 1.859
MRG	7	5	0.725 ± 0.104	1.391 ± 0.860	8	3.143 ± 1.732
SRO	19	6	0.751 ± 0.041	1.416 ± 0.831	8	3.200 ± 1.691
KPN	35	6	0.745 ± 0.030	1.475 ± 0.850	8	3.334 ± 1.732

Population	N	Nh	h ± S.E.	π ± S.E. (%)	S	PD ± S.E.
PPN	32	6	0.728 ± 0.033	1.426 ± 0.827	8	3.224 ± 1.686
TOTAL	196	8	0.7268 ± 0.009	1.549 ± 0.875	8	3.501 ± 1.788

The largest sample (68 individuals) was from the relict moose population in the Biebrza Valley, the largest natural wetland area in Central Europe. PCR amplification of the MHC II DRB locus was performed in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems) at a final volume of 5 μL containing ~25 ng genomic DNA, 1.7 μL Qiagen multiplex PCR Master Mix (1×), 0.3 μL mix of the primers HLO30 and HLO32 (Mikko and Andersson, 1995) and 1 μL RNase-free water. The following thermocycling parameters were used: initial denaturation step at 95 °C for 15 min, followed by 35 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, followed by final elongation for 3 min at 72 °C. The multiplex PCRs were performed in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems). Amplified PCR products of the MHC II DRB locus were purified with shrimp alkaline phosphatase (SAP) and Exonuclease I (Thermo Scientific) in an enzymatic reaction following the manufacturer's protocol, and then processed for cycle sequencing PCR with a BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA). Unincorporated dideoxynucleotides were eliminated from the sequencing reaction with an ExTerminator Kit (A&A Biotechnology, Gdynia, Poland). The sequencing products were run on an automated capillary sequencer ABI 3130 (Applied Biosystems). The resulting 226-bp-long sequences of the MHC II DRB locus were aligned in BioEdit v.7.0.4 (Hall, 1999) and reviewed manually. UNPHASE option in DnaSP v.5 (Librado and Rozas, 2009) was used to identify different haplotypes (alleles). The levels of gene diversity of the MHC II DRB locus were calculated within seven moose populations as the number (Nh) and frequency of alleles, alleles diversity (h), nucleotide diversity (π, in percent), the number of segregating sites (S) and the mean number of pairwise differences (PD), using the software Arlequin v3.5.1.2 (Excoffier and Lischer, 2010) and DnaSP v.5 (Librado and Rozas, 2009). Genetic differentiation between moose populations was described as pairwise Φ_{ST} and F_{ST} values for the seven populations using Arlequin v3.5.1.2 (Excoffier and Lischer, 2010) and statistically tested in Arlequin. Principal component analysis (PCA) was performed on MHC II DRB F_{ST} data using GenAlEx v.6 (Peakall and Smouse, 2006).

Results

In a sample of 196 moose from Poland, out of the eight identified alleles of the MHC II DRB, seven had previously been described by Mikko and Andersson (1995) and by Udina et al. (2002) in moose from Europe, Far East and North America: *DRB1*1* (GenBank X82398), *DRB1*2* (GenBank X83278), *DRB1*3* (GenBank X83279), *DRB1*4* (GenBank X83280), *DRB1*5* (GenBank X83281), *DRB1*8* (GenBank X83284) and *DRB1*9* (GenBank X83285). One allele *DRB1*11*, had

not been described before. The number of alleles in the studied moose populations was generally low and similar, and ranged from 5 (pop. PAU, PKN and MRG) to 8 (pop. BIE; Table 1), despite the fact that sample size varied from 7 to 68 individuals. Alleles *DRB1*4* and *DRB1*11* were found only in the Biebrza Valley moose population. The most common alleles were *DRB1*1*, *DRB1*3* and *DRB1*9*, which were identified in all studied populations, with a frequency ranging from 14% to 50%. Frequencies of particular alleles were generally similar among the studied populations (Table 2).

Table 2. Frequency of eight MHC II DRB alleles found in the moose populations studied in Poland.

Allel Population	DRB1*1	DRB1*2	DRB1*3	DRB1*4	DRB1*5	DRB1*8	DRB1*9	DRB1*11
BIE	0.32	0.04	0.15	0.01	0.02	0.13	0.32	0.01
PAU	0.30	0.17	0.17	0.00	0.00	0.06	0.30	0.00
PKN	0.35	0.07	0.21	0.00	0.13	0.00	0.24	0.00
MRG	0.14	0.07	0.50	0.00	0.00	0.07	0.22	0.00
SRO	0.18	0.03	0.40	0.00	0.08	0.05	0.26	0.00
KPN	0.39	0.06	0.28	0.00	0.07	0.04	0.16	0.00
PPN	0.41	0.07	0.30	0.00	0.02	0.06	0.14	0.00

The haplotype (alleles) diversity (h) in particular populations were remarkably similar and ranged from 0.725 ± 0.104 (pop. MRG) to 0.787 ± 0.026 (pop. PAU), while the nucleotide diversity (π) ranged from 1.391 (%) ± 0.860 (pop. MRG) to 1.637 ± 0.939 (pop. PAU; Table 1).

Genetic differentiation among populations was low (average $F_{ST} = 0.024$) and insignificant (Table 3). Only 10% (Φ_{ST}, range from 0.04 to 0.07) and 24% (F_{ST}, range from 0.03 to 0.07) of the pairwise comparisons significantly differed from zero ($p < 0.001$; Table 3). In the case of Φ_{ST}, significant values, according to the Wright scale (1978) mean small and moderate genetic differentiation, were obtained by comparing the population SRO with KPN and PPN populations (0.04 and 0.07, respectively). The values significant for F_{ST} were obtained mainly for the comparisons of the BIE population with MRG, SRO, KPN, PPN (0.07, 0.04 and 0.03 respectively) and for SRO *vs* PPN population pair (0.03).

Table 3. Genetic differentiation of MHC II DRB loci between moose population pairs in Poland, as measured by Φ_{ST} (below the diagonal) and F_{ST} (above the diagonal).

Population	BIE	PAU	PKN	MRG	SRO	KPN	PPN
BIE	-	0.00	0.01	**0.07**	**0.04**	**0.03**	**0.03**
PAU	0.00	-	0.00	0.04	0.03	0.02	0.02
PKN	0.00	0.00	-	0.05	0.02	0.00	0.00

Population	BIE	PAU	PKN	MRG	SRO	KPN	PPN
MRG	0.02	0.05	0.03	-	0.00	0.03	0.04
SRO	0.03	0.05	0.03	0.00	-	0.02	**0.03**
KPN	0.00	0.01	0.00	0.03	**0.04**	-	0.00
PPN	0.01	0.02	0.02	0.05	**0.07**	0.00	-

Significant values ($p < 0.05$) are indicated in bold.

PCA analysis based on the F_{ST} matrix showed that the main components accounted for 94.39% of the system's variation (PC1: 69.10%, PC2: 25.29%). This analysis confirmed that the relict population of the moose in the Biebrza Valley is separate from other moose populations from Poland (Fig. 2). The BIE and PAU populations differed significantly from SRO and MRG populations in terms of PC1 values, and additionally from the populations of KPN, PPN and PKN with respect to PC2 scores.

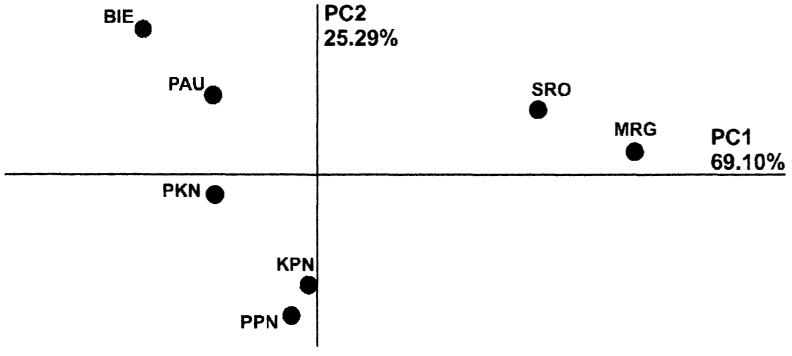

Figure 2. Principal component analysis (PCA) performed on pairwise F_{ST} values of the studied seven moose populations in Poland. The first and second axes of the PCA explained 69.10 and 25.29% of the total variability, respectively.

Discussion

In this paper we determined the diversity of the MHC II DRB, which is the most variable gene of MHC class II in humans, domestic cattle, and other ruminants (Marsh and Bodmer, 1990; Andersson et al., 1991). Previous research on MHC class I and II in moose (Mikko and Andersson, 1995; Ellegren et al., 1996; Udina et al., 2002; Wilson et al., 2003) has shown that both Eurasian and North American moose populations are characterized by low levels of polymorphism, with clear differences between populations. In our sample of 196 moose individuals, we identified eight MHC II DRB alleles, seven of which had been described in the moose throughout the range of the species (Mikko and Andersson, 1995;

Udina et al., 2002; Wilson et al., 2003). The *DRB1*11* allele found in the Biebrza Valley moose population is new to this species. At the same time we did not identify the *DRB1*6* allele present in the Swedish relict moose population and *DRB1*7*, and *DRB1*10* reported in Canadian and Far East moose populations (Mikko and Andersson, 1995; Mikko et al., 1999; Udina et al., 2002; Wilson et al., 2003). The most frequent allele in moose in Poland was *DRB1*1*, which is also one of the most frequent allele in moose in the Eurasian range of this species, carried by every four out of five individuals (Mikko and Andersson, 1995; Udina et al., 2002). The second most frequent *DRB1*9* allele in Poland is rare in Sweden and in the Far East, while the third *DRB1*3* allele is very rare in the Eurasian part of the range, but occurs in 20% of moose individuals from Canada (Wilson et al., 2003). Interestingly, the *DRB1*5* allele found in moose in Poland, is the dominant allele in North America, where it occurs in over half of all individuals (Mikko and Andersson, 1995; Mikko et al., 1999; Wilson et al., 2003).

The highest number of MHC II DRB alleles was identified in the Biebrza moose population (Table 2). Out of eight alleles, two – *DRB1*4* and *DRB1*11* – were not detected anywhere in Poland outside the Biebrza Valley. *DRB1*11* was unique for the Biebrza population. In the light of previous analyses of the MHC II DRB gene, the relict population in Sweden has the unique *DRB1*6* allele (Mikko and Andersson, 1995), while the relict population in the Biebrza Valley has the *DRB1*11* allele. Interestingly, the *DRB1*4* allele, which occurred only in the Biebrza Valley moose populations, has so far been identified in the relict moose population in Sweden and in the Russian part of the moose range (Mikko and Andersson, 1995; Udina et al, 2002). In the relict Biebrza population a very low frequency was observed for as many as four alleles of the MHC II DRB gene: *DRB1*2*, *DRB1*4*, *DRB1*5* and *DRB1*11* showed, compared to three (*DRB1*2*, *DRB1*5* and *DRB1*8*) in the remaining populations of moose in Poland (Table 2). The distinctness and uniqueness of the Biebrza population in terms of the MHC II DRB sequence was further confirmed by PCA analysis based on population pairwise F_{ST} values and especially the fact that the Biebrza moose population was characterized by the largest number of significantly different from zero comparisons of F_{ST} values with other populations (Fig. 2).

Ungulate mammals are generally characterized by a high diversity of MHC loci, with the highest levels of polymorphism found in chamois (*Rupicapra rupicapra)*, bighorn sheep (*Ovis canadensis*), white-tailed deer (*Odocoileus virginianus*) and red deer (*Cervus elaphus*) (De et al., 2011). Low levels of MHC diversity in some ungulates may be largely due to Pleistocene glaciations (Mikko et al., 1999; Loehr et al., 2006), when gene pool depletion was caused by inbreeding combined with genetic drift (Mainguy et al., 2007). During Pleistocene glacial-interglacial cycles, moose – belonging to the group of species living in temperate and boreal regions – experienced a reduction in population size and a related reduction in genetic diversity. Babik et al. (2009) argue that the reduced genetic diversity was due to the founder effect in populations that underwent a bottleneck before the postglacial expansion.

According to Mikko and Andersson (1995), moose has lost most of its MHC diversity over its evolutionary history, which could have been caused by random

favouring of one or more MHC haplotypes, or a very drastic bottleneck effect that preceded the separation of individual phylogenetic groups. Given that European and North American moose share the same limited diversity of MHC, Mikko and Andersson (1995) suggest that the ability of the moose population to survive at this low diversity of MHC must have been kept in equilibrium for at least 100,000 years, allowing moose to colonize the circumpolar areas of the northern hemisphere.

The current MHC diversity which has evolved since the bottleneck is characterized by a small number of alleles differing mainly in non-synonymous substitutions (Mikko and Andersson, 1995) – the effect of positive selection. In addition, Mikko and Andersson also argue that the lower number of MHC II DRB alleles in ruminants, compared to primates, is due to the fact that they are evolutionarily younger.

It is also interesting in the context of the moose population studies to note that MHC diversity increases with temperature, as a result of which species living in higher latitudes may retain lower genetic diversity within MHC (Weber et al., 2013). Van Den Bussche et al. (2002) also suggest that Arctic ungulate mammalian species may be exposed to fewer pathogens and parasites than those living in areas close to the equator, and that we should therefore expect less variation in MHC in ungulates in northern areas than in lower latitudes. Interspecies studies confirm the hypothesis of Van Den Bussche et al. (2002) that latitude may influence the level of MHC DRB diversity in wild ungulates by the likely relationship between MHC diversity and pathogen diversity (Mainguy et al., 2007).

The low diversity in the DRB locus in moose and reindeer is also likely to be related to the limited exposure of these species to boreal parasites (Mikko and Andersson 1995; Ellegren et al., 1996; Mainguy et al., 2007). The limited diversity of MHC genes can also be explained to some extent by the impact of factors associated with the social organisation of the species (Mainguy et al., 2007). These factors include the lonely lifestyle of the moose, and its monogamy (Ellegren et al., 1996; Sommer et al., 2002), which reduce the incidence of intra-species contacts. Thus the reduced risk of transmitting infectious diseases may favour low levels of MHC polymorphism. Irrespective of the fact that the number of MHC DRB II alleles in moose is low, it should be noted that their frequencies in the studied moose populations were remarkably similar. Thus, it could not be ruled out that their frequencies within populations reflect selection pressures based on the heterozygote advantage principle (Ejsmond et al., 2010).

It should also be noted that hunting has a significant impact on the ecology and genetics of vertebrates. By reducing genetic diversity, it may lead to a short-term decrease in the levels of fitness components (Altizer et al., 2003; Fernández-de-Mera et al., 2009). In a bighorn sheep population, selective trophy hunting resulted in a significant decrease in average horn weight and size in males (Coltman et al., 2003). Analyses of moose in Canada have shown that the relatively higher diversity in the MHC II DRB gene in populations from protected areas (national parks) was due, among other things, to the lack of hunting in these areas (Wilson et al., 2003). Interestingly, three Polish moose

populations in national parks: Biebrza Valelly, Kampinos and Polesie National Parks, in our study also showed the highest number of MHC II DRB loci. Wilson et al. (2003) suggest that the higher density of moose in national parks creates unusual conditions for the definitely lonely lifestyle of this species (Ellegren et al., 1996). The increasing number of contacts and the associated increase in the number of transmitted pathogens increases the pressure of natural selection on populations living in protected areas.

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