A comparison of polymorphism of DQA genes in European bison belonging to two genetic lines: Lowland and Lowland-Caucasian

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Abstract: A comparison of polymorphism of DQA genes in European bison belonging to two genetic lines: Lowland and Lowland-Caucasian. The currently living population of European bison (Bison bonasus) is divided into two genetic lines: Lowland line (LB) and Lowland-Caucasian line (LC). In order to compare the genetic diversity in two above mentioned lines the polymorphism of the major histocompatibility complex (MHC) DQA1 and DQA2 genes was analyzed. MHC genes are highly polymorphic and can therefore be used to assess genetic diversity in different species including endangered ones. The genetic polymorphism of exon 2 DQA1 and DQA2 genes was examined in 200 individuals of Lowland line and 56 individuals of Lowland-Caucasian line. The SSCP analysis revealed two alleles and two genotypes of exon 2 DQA1 gene and three alleles and four genotypes of exon 2 DQA2 gene. Frequency of genotypes of exon 2 DQA2 gene was significantly different (chi-square test $p \leq 0.05$) between two genetic lines of European bison. Four haplotype configurations of DQA genes – DQA1, DQA2, DQA1/DQA2 and DQA1/DQA2_2 were observed. DQA1 haplotype configuration was observed only in individuals belonging to LC and DQA1/DQA2_2 only in European bison from LB. Differences of frequency of haplotypes in two genetic lines were highly significant (chi-square test $p \leq 0.01$).

Key words: European bison, Bison bonasus, polymorphism, SSCP, DQA, MHC.

INTRODUCTION
Historically, the European bison was distributed through western, central and south-eastern Europe. In the beginning of twentieth century, free-ranging populations became extinct. Only 54 (29 males and 25 females) individuals with documented pedigrees survived in zoological gardens and reserves. The currently living population of European bison descended from only 12 founders (Olech, 2009) and is divided into two genetic lines: a Lowland (Białowieża) line and a Lowland-Caucasian line. The Lowland line i.e. pure lowland subspecies (Bison bonasus bonasus), originates from only 7 founders (4 males and 3 females). The gene pool of the Lowland-Caucasian line contains genes from all 12 founders including male “Kaukasus” originating from subspecies caucasicus (Bison bonasus caucasicus) (Slatis, 1960; Olech, 1987; 2003).

The species went through an extreme bottleneck and consequently, currently living populations of European bison are highly inbred. Previous studies using various markers: allozyme (Hartl and Pucek, 1994), blood groups (Sipko et al.,
1995), mitochondrial DNA (Tiedemann et al., 1998; Burzyńska et al., 1999) microsatellites (Gralak et al., 2004; Nowak and Olech, 2008), and highly polymorphic MHC genes (Udina et al., 1994a; 1994b; Udina and Shaikhaiev, 1998; Łopieńska et al., 2003; Radwan et al., 2007) confirmed low genetic diversity in both genetic lines of European bison.

Studies of Major Histocompatibility Complex (MHC) genes in European bison are conducted based on existing knowledge about equivalent region in cattle. The bovine Major Histocompatibility Complex (MHC) called Bovine Lymphocyte Antigens (BoLA) is located on the short arm of chromosome 23 (BTA23) and contains three classes I, II and III (Andersson and Davies, 1994). Class III products have a wide range of activities from cell cytotoxicity (tumor necrosis factor TNF-α and TNF-β) to enzymatic activity (21β-hydroxylase) (Andersson and Davies, 1994). The product of class III genes is also Heat Shock Protein (HSP70) (McShane et al., 2001). The genes of class I and class II encode cell-surface glycoproteins whose function is to recognize, bind in their antigen binding groove, and present antigenic peptides to T lymphocytes. The antigen binding groove is encoded by the second exon of the gene which demonstrates high polymorphism and this determines the capability of MHC products to bind to a wide range of peptides (Sharif et al., 1998). MHC genes are extremely polymorphic in cattle and other vertebrates.

The BoLA class II is divided by gene inversion event into two regions designated IIa and IIb. The IIa region contains genes DOA, DMA, DMB, DYA, DYB. In IIb region are situated DRA, DRB, DQA and DQB genes which encode MHC class II molecules (Ballingall et al., 2004; Takeshima and Aida, 2006). A feature unique in cattle and sheep is certain variability in the number of DQ loci in their haplotypes. Each cattle class II haplotype expresses a single DR product and one or more DQ products due to duplication of the DQ genes and this duplication occurs in about half haplotypes (Ellis and Ballingall, 1999; Lewin et al., 1999; Russell, 2000). Cattle haplotypes carry either a single DQA1 locus, or single DQA1 locus together with a single DQA2 locus or two DQA2 loci (Gelhaus et al., 1999). All DQA loci in cattle and sheep are polymorphic with approximately 50 different alleles identified in both species (http://www.projects.roslin.ac.uk/bola, www.ncbi.nlm.nih.gov). Highly polymorphic genes of MHC are used as markers for cattle and other many species (Lewin et al., 1999) and also can be used to assess diversity in endangered species (Haig, 1998).

The aim of this study was to perform the first examination of variability in DQA loci in European bison and compare genetic diversity in exon 2 of DQA1 and DQA2 gene in two genetic lines: Lowland line and Lowland-Caucasian line.

MATERIALS AND METHODS

Samples (whole blood, liver and kidney tissue and hair roots) were collected from 200 European bison belonging to Lowland line (LB) and 56 from Lowland-Caucasian line (LC) came from free-ranging and captive herds from Poland and other European countries.
DNA isolation

The total DNA was extracted from whole blood using the standard phenol-chloroform extraction protocol (Sambrook et al., 1989), from soft tissues by treatment with proteinase K and precipitation by sodium chloride (http://www.genomics.liv.ac.uk), and from hair roots using the Chelex 100 (Sigma) method (Walsh et al., 1991).

Amplification

Exon 2 DQA1 gene

Exon 2 of DQA1 gene was amplified by PCR method with primers described by Snibson et al. (1998) primer Forvard, and Zhou and Hickford (2004) primer Revers.

Amplification was carried out in 15 μl total volumes. The reaction mixture consisted of optimized concentrations of: 1xREDtaqPCRBuffer (Sigma), MgCl₂ (Polgen), dNTPs (Polgen), 8 pmol of each primers, 0.5 unit REDtaq DNA polymerase (Sigma), 100 ng DNA template and sterile water. The amplification profile consisted of 2 min at 94°C initial denaturation, followed by 30 cycles of 30 s at 94°C, 40 s at 56°C and 45 s at 72°C, with a final extension of 5 min at 72°C.

Exon 2 DQA2 gene

Exon 2 of DQA2 gene was amplified with polymerase chain reaction (PCR) using primers described by Scott et al. (1991). Amplification was carried out in 15 μl total volumes. The reaction mixture consisted of optimized concentrations of: 1xREDtaqPCRBuffer (Sigma), MgCl₂ (Polgen), dNTPs (Polgen), 8 pmol of each primers, 0.5 unit REDtaq DNA polymerase (Sigma), 100 ng DNA template and sterile water. The amplification profile consisted of 2 min at 94°C initial denaturation, followed by 30 cycles of 30 s at 94°C, 40 s at 56°C and 45 s at 72°C, with a final extension of 5 min at 72°C.

PCR-SSCP analysis

The amplified product of each sample was analyzed by Single Strand Conformation Polimorphism (SSCP) technique. PCR product was mixed with loading dye (Bromophenol blue, Xylene, Formamid, EDTA), denaturated at 90°C for 5 min and cooled on ice. Then product was resolved on 12% nondenaturing polyacrylamide gel. Electrophoresis was carried out on DCode™ Universal Mutation Detection System (BioRad) at constant conditions: 240 min. at 35 W, 10°C in 1XTBE buffer. Gels were stained with silver nitrate (AgNO₃) (Sambrook et al., 1989).

Cloning and sequencing

PCR products corresponding to different SSCP patterns were cloned and sequenced. Chosen PCR product was purified with QIAquick Gel Extraction (Qiagen) and cloned using pGEM™-TEasy vector (Promega). Purified PCR product was cloned into vector pGEM™-TEasy (Promega). Component cells of Escherichia coli JM109 (Promega) were transformed with recombinant plasmid and selected by ampicilin-blue/white screening. After growing in Louria-Bertani liquid
medium the positive clones (white) were prepared with Wizard® Plus Minipreps DNA Purification System (Promega) and sequencing in a reaction total volume 27 μl using Thermo Sequenase Cy5 Terminator Cycle Sequencing Kit (Amersham Bioscience). Nucleotide sequences were detected in automated sequenced ALFexpress II (Amersham Pharmacia Biotech).

**Statistical and sequence analysis**

The Popgen32 program (http://cc.oulu.fi/~jaspi/popgen/popdown.htm) was used to estimate the statistical parameters such as frequencies of genotypes and haplotypes.

Potential differences between two genetic lines of European bison were evaluated using chi-square test. The BLAST algorithm was used for the comparison of sequences obtained with homologous sequences from NCBI GenBank (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree showing relationships was constructed from the matrix of sequences similarities calculated with the UPGMA method using MEGA program (Kumar et al., 2004).

**RESULTS AND DISCUSSION**

In PCR reaction used were primers described previously for amplification of exon 2 DQA1 gene (Snibson et al., 1998; Zhou and Hickford, 2004) and exon 2 DQA2 gene (Scott et al., 1991) in sheep. The European bison belongs to the family Bovidae so primers used for amplification of exon 2 of DQA1 and DQA2 genes in sheep, could be applied for amplification equivalent region in European bison. The previous comparison analysis of the nucleotide sequence of exon 2 ovine DQA1 and DQA2 genes with equivalent fragments of cattle showed that the ovine sequences are more similar to their bovine counterparts then to each other (Snibson et al., 1998).

The second exon of DQA1 gene was successfully amplified by PCR reaction in 85 from 200 examined European bison belonging to Lowland line (LB) and in 39 from 56 in Lowland-Caucasian line (LC). The amplified fragment was 253 bp long. These primers did not amplified the analyzed fragment in 115 from LB and in 17 from LC.

The second exon of DQA2 gene was successfully amplified by PCR reaction in all animals from LB. In 12 animals belonging to LB amplified fragment of 282 bp was obtained and in 186 animals from this line product was 253 bp long.

Among European bison from LC PCR product 253 bp long was obtained in 43 from 56 examined, in the other 13 animals exon 2 DQA2 gene did not amplified.

On the basis of generated results, four different haplotypes were marked No1 (DQA1), No2 (DQA2), No3 (DQA1/DQA2) and No4 (DQA1/DQA2_2) in examined individuals. No4 haplotype occurred in animals with longer (282 bp) amplified fragment of exon 2 DQA2 gene. No1 haplotype was observed only in individuals from LC, and No4 haplotype was occurred only in LB line.

Frequency of different haplotypes in two investigated genetic lines of European bison describes Table 1. Differences of frequency of haplotypes in two genetic lines were highly significant (chi-square test $p \leq 0.01$).
Comparison of these results with previously described by other authors (Ballingall et al., 1997; Snibson et al., 1998; Hickford et al., 2000; 2004; Zhou and Hickford, 2004; Traul et al., 2005) showed that MHC class IIa haplotypes of European bison are akin to those identified in domestic cattle, sheep and American bison. Those haplotypes carry nonduplicated and duplicated DQA genes, animals have haplotypes with single DQA1 and duplicated DQAnull/DQA2 or DQA1/DQA2. In addition some haplotypes with duplicated DQA gene in cattle and American bison have also gene named DQA3 in configuration DQA2/DQA3 (Ballingal et al., 1997; Traul et al., 2005). The most common haplotype in European bison from LB is No2 (DQA2) which was identified in 57% examined animals. In LC, individuals with haplotype No3 (DQA1/DQA2) were in the majority (42.8%). In LB were no animals with single DQA1 haplotype.

Previous investigation revealed that most of sheep (82–89%) contain DQA1/DQA2 haplotype which is extremely polymorphic with 8 DQA1 alleles and 16 DQA2 alleles (Escayg et al., 1996). In American bison examined by Traul et al. (2005) 25% individuals had a haplotype with duplicated DQA gene.

Those studies showed also the presence of two unique SSCP patterns of exon 2 DQA1 gene in both genetic lines of European bison. On the basis of obtained SSCP patterns, two alleles and two genotypes marked a and b were identified. Both of them were homozygous. The frequency of genotype a was 0.494 in LB and 0.513 in LC. Frequency of genotype b was respectively 0.506 and 0.487. SSCP analysis detected three unique patterns of exon 2 DQA2 gene. On the basis of SSCP patterns, three alleles and four genotypes were identified. Identified genotypes were marked c, d, e, f. Three of them were homozygous (c, d, e), and one was heterozygous (f). Genotype e was observed in animals with longer PCR product (282 bp). Frequency of observed genotypes was respectively 0.540, 0.215, 0.060 and 0.185 in LB and 0.442, 0.186, 0.00, and 0.72 in LC. Frequency of genotypes of exon 2 DQA1 gene did not differ between two genetic lines of European bison (chi-square test p = 0.981). Whereas the frequency of genotypes of exon 2 DQA2 gene was significantly different (chi-square test p ≤ 0.05).

More SSCP patterns of exon 2 DQA2 gene suggested highest polymorphism then in the exon 2 DQA1 gene in European bison. The analogous results described Snibson et al. (1998) in sheep. They stated that the second exon of the OLA-DQA2 gene is more polymorphic than the equivalent region of the OLA-DQA1.

Those studies revealed polymorphism of the second exon of the DQA1 and DQA2 gene in European bison. However polymorphism in this region is not high while compared to cattle and sheep. Lower number of DQA1 and DQA2 alleles in

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>LB</th>
<th>LC</th>
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<tbody>
<tr>
<td>No1 (DQA1)</td>
<td>0</td>
<td>0.232</td>
</tr>
<tr>
<td>No2 (DQA2)</td>
<td>0.425</td>
<td>0.429</td>
</tr>
<tr>
<td>No3 (DQA1/DQA2)</td>
<td>0.515</td>
<td>0.339</td>
</tr>
<tr>
<td>No4 (DQA1/DQA2_2)</td>
<td>0.060</td>
<td>0.0</td>
</tr>
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TABLE 1. Frequencies of observed haplotypes in European bison
European bison is probably resulting of extreme population bottleneck. Previous study of MHC genes including DQA genes in many wild bottlenecked species and populations showed low number of alleles in this region.

The nucleotide sequences of exon 2 DQA1 and DQA2 gene of European bison received in this study were submitted to the NCBI Gene Bank, and assigned the accession numbers EU153369.1 to EU153374.1. A comparison between obtained DQA sequences of European bison shows Figure 1. A UPGMA phylogenetic tree in Figure 2 was constructed from obtained nucleotide sequences of exon 2 DQA1, and DQA2 gene of European bison, and bovine and ovine nucleotide sequences published in the NCBI GenBank (http://www.ncbi.nlm.nih.gov). The tree revealed three clusters grouping sequences analyzed on two main branches. The cluster I contains sequences of exon 2 DQA1 and DQA2 gene of European bison, cattle and sheep. The similarity between two sequences of DQA2 and one of DQA1 gene from European bison genome grouped in cluster I with equivalent ovine and bovine sequences was 97 to 100% (average 98%). One sequence of exon 2 DQA1 gene of European bison (EU153370.1) was clustered together with sequences of exon 2 DQA2 gene and average nucleotide homology between them was 98%. Comparison of two sequences from this cluster EU153369.1 and L139A2 showed 100% nucleotide homology although there were two different PCR reaction products: EU153369.1 – exon 2 DQA1 gene, L139A2 – exon 2 DQA2 gene. In cluster II were grouped sequences of exon 2 DQA2 and one sequence of exon 2 DQA1 gene with ovine sequences of exon 2 DQA2 gene. The average similarity between sequences within the cluster II was 98% (97–98%). The analyzed sequences of European bison from cluster I and cluster II showed 87 to 90% (average 88.83%) of nucleotide homology in the exon 2 DQA1 and DQA2 gene. The sequence number EU153371.1 was clustered on another tree branch into group III together with ovine exon 2

FIGURE 1. A comparison between nucleotide sequences of exon 2 DQA1 and DQA2 in European bison
DQA1, and cattle sequences described just like DQA.

Previous studies on the relationship between ovine and bovine DQA nucleotide sequences revealed two main branches of phylogenetic neighbor-joining tree clustered sheep DQA1 with cattle DQA1, whereas sheep DQA2 sequences clustered with cattle DQA2, DQA3 and DQA4 sequences (Hickford et al., 2004). In this study European bison DQA1 sequences were grouped with sequences of DQA1 and DQA2 bovine and sheep as well as DQA2 sequences. Moreover, bovine DQA1 and DQA2 sequences from NCBI Gen Bank, which were used for a comparison were grouped together in cluster I. This may suggest that the nature of DQA gene is not yet well understood and requires further research. It also indicates the pertinence of isolating further DQA loci.

FIGURE 2. UPGMA tree constructed using DQA nucleotide sequences obtained and labeled with the symbols as follows; ■ – exon 2 of DQA1, ▲ – exon 2 of DQA2, ◆ – exon 2 DQA, 1 – nucleotide sequences obtained. Tree is constructed using the MEGA program (Kumar et al., 2004). Bootstrap statistical analysis (Felsenstein, 1985) of tree topology is shown above branches. Branch lengths are proportional to genetic distance.
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Streszczenie: Porównanie polimorfizmu genów DQA u żubrów należących do dwóch linii genetycznych: Białowieskiej i Białowiesko-kaukańskiej. Obecnie żyjąca populacja żubrów została podzielona na dwie linie genetyczne: białowieską (LB) i białowiesko-kaukaską (LC). W celu porównania zmienności genetycznej u żubrów należących do dwóch linii genetycznych badano polimorfizm genów DQA należących do głównego kompleksu zgodności tkankowej (MHC). Geny MHC cechuje wysoki polimorfizm i w związku z tym mogą być one wykorzystywane do szacowania zmienności genetycznej różnych gatunków, w tym zagrożonych wyginięciem. Analiza polimorfizmu eksonu 2 genów DQA1 i DQA2 objęła 200 żubrów należących
do linii białowieskiej i 56 z białowiesko-kaukaskiej. Analiza SSCP ujawniła występowanie dwóch alleli i dwóch genotypów eksonu 2 genu DQA1 oraz trzech alleli i czterech genotypów eksonu 2 genu DQA2. Frekwencje genotypów eksonu 2 genu DQA2 różniły się istotnie (test chi-kwadrat p \leq 0,05) pomiędzy dwoma liniami genetycznymi żubrów. Zaobserwowano występowanie czterech haplotypów DQA – DQA1, DQA2, DQA1/DQA2 i DQA1/DQA2_2. Haplotyp DQA1 był obecny jednie u osobników z LC a DQA1/DQA2_2 tylko u żubrów z LB. Analiza częstości występowania zidentyfikowanych haplotypów w dwóch liniach genetycznych żubrów wykazała wysoko istotne statystyczne różnice (test chi-kwadrat p \leq 0,01).

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