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Restriction fragment length polymorphism (RFLP) of exon 2 of the MhcBibo-DRB3 gene in European bison *Bison bonasus*

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Polymerase chain reaction (PCR) primers specific to exon 2 of the bovine Major Histocompatibility Complex (MHC) class II gene BoLA-DRB3 were used to amplify the homologous region (284bp) in 21 European bison *Bison bonasus* (Linnaeus, 1758). Generally, variation in patterns of restriction fragments was low and the patterns found in earlier studies on cattle were present also in this species. Restricted polymorphism of DRB3 genes of MhcBibo (European bison) and the earlier studied MhcBibi (American bison) is most probably due to the genetic bottlenecks having occurred in the respective herds of both species. This suggests that possibilities of recognizing foreign antigens and providing a successful immune response are reduced.

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Introduction

Breeding European bison *Bison bonasus* (Linnaeus, 1758) for its successful conservation requires data on genetic diversity of this endangered species. During the last years, present herds of European bison have been studied using various marker systems: allozymes (Hartl and Pucek 1994, Sipko *et al.* 1996), blood groups (Sipko *et al.* 1995), DNA polymorphism of nuclear genes (Udina *et al.* 1994, 1995, Burzyńska and Topczewski 1995), and mitochondrial DNA (Tiedemann *et al.* 1998). Animals from the pure Lowland line (Polish and Belorussian portions of Białowieza Forest, and Prioksko-Terrasny Reserve, Russia), from the Lowland-Caucasian line (Nadvornyansky Reserve, Ukraina, Prioksko-Terrasny and Oka Nature Reserves (Russia), and from a hybrid line between European and American bison (Caucasian Reserve and Nal'chik game farm, Russia) were studied. Very low estimates of heterozygosity were revealed. The study on RFLPs of mitochondrial DNA from the Polish part of Białowieża Forest revealed the absence of variation (Tiedemann

et al. 1998). Genetic diversity within and among lines of European bison was shown to be considerably lower than within and among cattle breeds. The lowest genetic variation, demonstrated in the Lowland line for all marker systems, is caused by a severe population bottleneck with only five (initially seven) founders of this line and an extremely large genetic impact of only two founders in the present population, which is estimated to range from 60 to 82% by different authors (Sipko *et al.* 1996, W. Olech, pers. comm.). Data on genetic polymorphism of the species were summarized in an IUCN report (Hartl *et al.* 1995).

However, there are still some problems of European bison genetics and conservation to be studied. The best way of preserving the remaining genetic polymorphism is the most important one because of the severe population bottleneck (only 12 founders) in the past and the high level of inbreeding ($F = 0.324$, estimated for the Lowland line by Olech 1989). Breeding programs of European bison would be directed towards minimizing inbreeding in captive populations and to ascertain the genetic representation of as many founders as possible in the establishment of free-ranging populations. Thereby, the preservation of a certain spectrum of immunogenetic variation is of paramount importance for preserving the possibility to interact with foreign antigens. Presently, a disease affecting male reproductive organs is known to occur in a number of herds of European bison. So far, no specific infectious agent causing the disease could be revealed by the various studies performed. However, it may be caused by a decrease in the immune resistance of the bison population due to a high level of homozygosity at Major Histocompatibility Complex (MHC) genes coding antigens which recognize the broad spectrum of foreign antigens (see Hartl *et al.* 1995). Extreme polymorphism at genes coding antigens of the MHC and their role in immune response make the study of these genes in endangered species very significant as to successful breeding for conservation, estimating genetic diversity, and assessing phylogenetic relationships (Schreiber *et al.* 1993). Therefore, in European bison the study of the MHC class II gene DRB3, which plays a key role in immune response, should be quite relevant for conservation of this species.

Earlier in cattle, over 30 alleles at BoLA-DRB3 were identified by PCR with subsequent restriction fragment length polymorphism (RFLP) analysis of exon 2 BoLA-DRB3 with restriction endonucleases RsaI, BstYI, and HaeIII (Van Eijk *et al.* 1992). In American bison *Bison bison*, the same procedure was used with primers specific to exon 2 BoLA-DRB3 for assessing heterogeneity between managed herds. RFLP-patterns proved to be quite scarce, and restriction sites demonstrated in cattle were found to be conserved (Morris *et al.* 1994).

To evaluate the power of the approach in estimating genetic relationships among animals from the pure Lowland and the Lowland-Caucasian line, and for comparing the peculiarities of DRB3 gene polymorphism within the Bovinae subfamily, in the present study we investigated genetic variation of DRB3 in European bison by PCR with bovine specific primers and subsequent RFLP-analysis using endonucleases RsaI and HaeIII.

Material and methods

We used blood samples from European bison of the Lowland line (Prioksko-Terrasny Reserve and Belorussian part of the Białowieża Forest, n = 11) and of the Lowland-Caucasian line (Prioksko-Terrasny and Oka Nature Reserve, Nadvornyansky Reserve, n = 10), from two American bison, and from four F_1-hybrids between European and American bison.

DNA was isolated from blood samples by a modified guanidine thiocyanate technique (Boom *et al.* 1990). PCR was performed with primers HL030 (5' ATCCTCTCTCTGCAGCACATTTCC 3') and HL032 (5' TCGCCGCTGCACAGTGAAACTCTC 3'), previously used to amplify bovine DNA (Van Eijk *et al.* 1992). Primer HL030, flanking the 5' end of exon 2 of BoLA-DRB3, is complementary to the 5' end region of exon 2 (7 nucleotides) and to the adjacent intron region. PCR was carried out in 25 µl of reaction mixture containing 67mM Tris-HCl, pH 8.8; 17 mM $(NH)_2SO_4$; 0.01% Tween-20; 2.5 mM $MgCl_2$; dNTPs, 100 µM of each; 0.5 µM of forward and reverse primers, 1 unit of Taq polymerase, and 10 ng of DNA. Two segment PCR was performed on thermal cycler "Techne" PHC-3 under the following conditions: 68°C – 20s and 95°C – 10s during 30 cycles; initial denaturation at 95°C – 1 min, and final extension at 68°C – 2 min. Since several copies of DRB genes with a high degree of homology may be present in a single animal (Van Eijk *et al.* 1992, Morris *et al.* 1994, Sulimova *et al.* 1995), "hot" start procedure was applied to increase the specificity of the PCR reaction (Mullis 1991). After amplification, 5–10 µl of the incubated mixture from each sample were analyzed by electrophoresis in 6% polyacrylamide gel. Between 5 and 10 µl of the PCR products were digested with 5 units RsaI and HaeIII under standard conditions during 2 h in 20 µl total volume. Digestion products were separated by electrophoresis in 9% polyacrylamide gel, stained with ethidium bromide, and analyzed under UV light.

Results

Restriction fragment patterns observed in European bison are summarized in Table 1. Digestion with HaeIII produced the following RFLP patterns present in cattle and also revealed in American bison: *a/a* and *b/b*, the heterozygote *a/b*, the pattern corresponding to cattle *c/c*, which was absent in American bison (Morris *et al.* 1994), and the heterozygote *b/c* (Fig. 1). Restriction fragment patterns

Table 1. Frequencies of RFLP patterns of exon 2 of MhcBibo-DRB3 in samples of European bison. L line – Lowland line, LC line – Lowland-Caucasian line. Designation of RFLP patterns follows Van Eijk *et al.* (1992).

RFLP pattern		L line (n = 11)	LC line (n = 10)	Total (n = 21)
RsaI	*a*	0.500	0.250	0.381
	f	0.091	0.350	0.214
	k	0.045	0.000	0.024
	m	0.045	0.000	0.024
	q	0.319	0.050	0.190
	s	0.000	0.350	0.167
HaeIII	*a*	0.181	0.700	0.429
	b	0.500	0.250	0.381
	c	0.319	0.050	0.190

corresponding to cattle *d*, *e*, and *f* patterns were not observed. In herds of American bison, only *a* and *b* patterns were revealed in the earlier study. In two American bison from our sample the following patterns were observed: *a/b* and *b/b*. In four first geneneration hybrids of European bison × American bison, one heterozygote *a/b* and three homozygotes were observed.

In cattle, RFLP analysis for RsaI revealed 19 different patterns (Van Eijk *et al.* 1992). In European bison, only 6 patterns, *a* (78, 54, 50, 39, 33, 30 bp), *f* (141, 54, 50, 39 bp), *k* (156, 78, 50 bp), *m* (111, 104, 69 bp), *q* (141, 90, 50 bp), and *s* (141, 93, 50 bp) were detected. Patterns *k* and *m* were present in a single animal from the Lowland line. In American bison, ten patterns were formed (Morris *et al.* 1994), three of which (*a*, *f*, and *m*) were also found in European bison. In

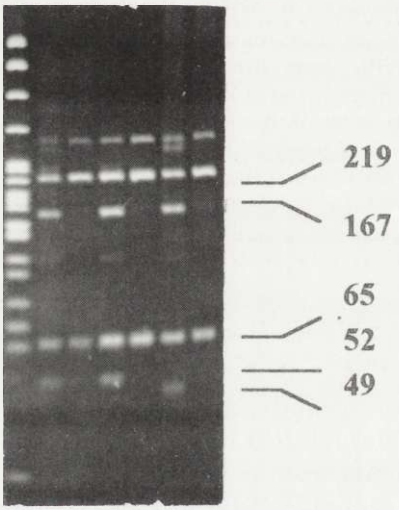

Fig. 1. Electrophoresis (9% polyacrylamide gel) of amplification products of exon 2 after digestion by restriction endonuclease HaeIII. MspI fragments of plasmid pBr322 (lane 1) were used as a molecular weight marker. In the other lanes, samples of animals studied are shown along with the designation of the respective patterns. Specimens: European bison from Lowland-Caucasian line (lane 2), European bison from Lowland line (lanes 3-7). The sizes of HaeIII fragments constituting the RFLP patterns shown are as follows: *a* (167, 65, 52 bp), *b* (219, 65 bp), *c* (167, 65, 49 bp). Positions of the respective HaeIII fragments are indicated at the right hand side of the figure.

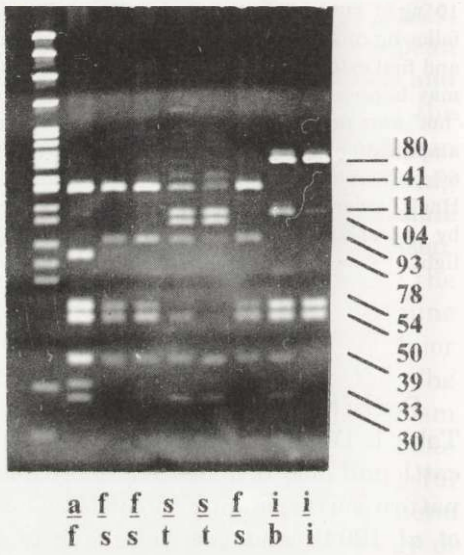

Fig. 2. Electrophoresis (9% polyacrylamidegel) of amplification products of exon 2 after digestion by restriction endonuclease RsaI. MspI fragments of plasmid pBr322 (lane 1) were used as a molecular weight marker. In the other lanes, samples of animals studied are shown along with the designation of the respective patterns. Specimens: European bison from Lowland line (lane 2), European bison from Lowland-Caucasian line (lane 3, 4, 7), F_1 hybrids American bison x European bison (lanes 5, 6), and American bison (lanes 8, 9). The sizes of RsaI fragments constituting the RFLP patterns shown are as follows: *a* (78, 54, 50, 39, 33, 30 bp), *b* (111, 54, 50, 39, 30 bp), *f* (141, 54, 50, 39 bp), *i* (180, 54, 50 bp), *s* (141, 93, 50 bp), and *t* (111, 104, 39, 30 bp). Positions of the respective RsaI fragments are indicated at the right hand side of the figure.

American bison, patterns *k*, *q* and *s* were not observed. In European bison, cattle patterns *b*, *g*, *h*, *i*, *l*, *n*, and *o* as well as three new RFLP patterns detected in the American bison (*t*, *w*, and *y*) were not present. In the two American bison studied, a homozygous *i/i*, and a heterozygous *i/b* pattern were observed. In the four hybrids, altogether two heterozygous (*s/t* and *f/s*) patterns were revealed (Fig. 2). RFLP pattern *i* was shown to be present in three American bison herds (Morris *et al.* 1994) and, in one of the herds, the frequency of the allele was estimated to be 0.540. Pattern *t* (111, 39, 30, and 104 bp), which was absent in cattle and present in five American herds studied by these authors, is identical to cattle pattern *m* (111, 69, and 104 bp) with an additional restriction site (Fig. 2).

We were not able to assess correspondence of all DRB3 alleles found in European bison with those previously found in cattle, because the majority of the bovine alleles were determined by using combinations of RFLP patterns of three enzymes (in our study we did not use BstYI), and sequencing. In fact, only sequencing would allow a final assessment of correspondence of all European bison alleles with the respective bovine alleles. However, we detected combinations of RFLP patterns for two endonucleases, which correspond to existing bovine alleles, such as the allele 30^c, a combination of the *q* pattern for RsaI and the *c* pattern for HaeIII. The allele 30^c is characterized by the presence of a deletion of 3 bp. We observed three homozygotes (*q/q* pattern for RsaI and *c/c* for HaeIII, probably corresponding to $30^c/30^c$) in animals from the Lowland line. We were able to detect virtually all the most probable combinations of RsaI and HaeIII patterns in 16 European bison and to compare them with those present in cattle (Table 2). This was possible, because one animal of the Lowland-Caucasian line and 7 animals of the Lowland line were homozygous as to RFLP patterns of both enzymes, several animals were homozygous as to HaeIII patterns, and two animals were *a/q b/c* (with respect to RsaI and HaeIII RFLP patterns, respectively). The difference both between *aq* and *bc* is a single three bp deletion in one fragment, most likely to be the same in both cases. According to RFLP patterns, two animals from the Lowland line and three from the Lowland-Caucasian line were double heterozygotes (*a/f*

Table 2. Frequencies of combinations of RsaI and HaeIII RFLP patterns in the samples of European bison studied (only animals included, where an unequivocal designation of haplotypes was possible). * – haplotype corresponding to bovine alleles with respect to RsaI and HaeIII RFLP patterns (for explanation see text), L line – Lowland line, LC line – Lowland-Caucasian line. ** – a similar combination of RFLP patterns for both enzymes is typical for the three bovine alleles considered.

RsaI pattern	HaeIII pattern	Haplotype*	L line (n = 9)	LC line (n = 7)	Total (n = 16)
a	*b*	new	0.500	0.143	0.344
f	*a*	8c-10^c**	0.000	0.286	0.125
k	*a*	new	0.055	0.000	0.031
m	*a*	22^c	0.055	0.000	0.031
q	*c*	30^c	0.390	0.071	0.250
s	*a*	new	0.000	0.500	0.219

a/b). Because only *ab* and *fa* combinations were observed in the rest of the sample, it was possible to determine the most probable variant of RFLP combinations for both endonucleases.

The observed frequencies of the restriction patterns of the two endonucleases and their combinations (haplotypes) are summarized in Tables 1 and 2. In the Lowland line, the sum of frequencies of the two most abundant RFLP patterns (*a* and *q*) was 0.819. In the sample from the Lowland-Caucasian line, three RFLP patterns (*a*, *f*, and *s*) were found to have a summarized frequency of 0.950. In the total sample of European bison, the summarized frequency of four RsaI patterns (*a*, *f*, *q*, and s) was 0.952. As demonstrated earlier for 5 American bison herds (Morris *et al.* 1994), HaeIII pattern *a* was the most frequent one also in European bison. Three combinations of restriction patterns found in our study which were not typical for cattle can be explained by single mutation events. For example the *a* (167, 65, and 52 bp) pattern of HaeIII differed from the *b* (219 and 65 bp) pattern by the presence of one additional HaeIII restriction site. Thus, haplotype *ab*, unusual for cattle, can be easily derived from *aa*. We calculated expected heterozygosity (considering the haplotypes in Table 2 as alleles) for the total sample studied and estimated the inbreeding coefficient of European bison (according to Li 1976) to be $F = 0.168$.

Discussion

The similarity of the length of amplified fragments and the restriction patterns obtained with those previously observed in cattle and American bison permits the conclusion that we have indeed amplified the European bison homologue to BoLA-DRB3 and MhcBibi-DRB3. In accordance with the existing nomenclature rules for MHC (Klein *et al.* 1990) we adopt the designation MhcBibo-DRB3. The conservation of restriction sites in three species of the subfamily Bovinae suggests that the primary structure of DRB3 genes is rather conservative. However, three haplotypes detected in our study were not found in cattle and may represent DRB3 alleles specific for the European bison.

We found variation in MhcBibo-DRB3 strikingly low when compared with that in BoLA-DRB3, and this probably reflects the severe bottleneck the species experienced in the recent past. Low variation may result in a reduced ability of European bison to recognize foreign antigens. As it is known, in a population exposed to multiple pathogens heterozyous individuals would be favoured because of their ability to bind more of the various foreign antigens (Hughes and Nei 1989). Due to overdominant selection, wild populations usually show an excess of heterozygotes in MHC genes. Especially high heterozygosity was demonstrated for those parts of MHC antigen molecules, which are involved in the interaction with peptides (antigen-binding site). In European bison, a very high percentage of homozygotes was found, sharply contrasting the distribution of DRB3 genotypes

in cattle (cf Van Eijk *et al.* 1992, Sulimova *et al.* 1995). However, it should be mentioned that the European bison in total might harbour more genetic variation than revealed in the present study, because in our samples the majority of animals were from the Prioksko-Terrassny and the Oka Reserve, respectively, which may be more inbred than the total population of the species. We also assume that sequencing would reveal more variation, as it was demonstrated in the zebu (*Bos indicus*), where 14 new alleles were revealed by sequencing of exon 2 of the DRB3 gene (A. Gelhaus, unpubl. EMBL/Genbank/DDBJ databases). But reduced polymorphism at DRB gene, probably due to a quite recent population bottleneck, was found also at the sequence level in moose (*Alces alces*, Mikko and Andersson 1994).

The allelic spectrum at the DRB3 gene in European bison is lower than in American bison, the latter species also having experienced population bottlenecks but not as severe as the former. In American bison, herds were usually founded using several tens of animals, with the largest number of founders being 250 for one herd (Polziehn *et al.* 1996). By contrast, numbers of founder individuals were as low as 5 and 12, respectively, for the pure Lowland line and the Lowland--Caucasian line. According to our results we assume that DRB3 gene variation as revealed by PCR-RFLP analysis can serve as a useful molecular marker for assessing genetic variation in European bison populations. The approach may be also used for detecting rare allelic variants in free-ranging and captive populations, and, thus, contribute to the preservation of a certain spectrum of immune response. The differences in RFLP patterns among European and American bison may be useful for detecting cases of hybridization among the two forms. This is particularly relevant for purebred herds of the Lowland-Caucasian line which may interbreed with a line originating from hybridization of European and American bison in the Caucasus (cf Pucek 1991).

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References

Boom R., Sol C. J. A. and Salimans M. M. M. 1990. Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology 28: 495–503.

Burzyńska B. and Topczewski J. 1995. Genotyping of *Bison bonasus* – κ-casein gene following DNA sequence amplification. Animal Genetics 26: 335–336.

Hartl, G. B., Laikre L., Olech W., Udina I. G., Sipko T. P., Belousova I. P. and Krasochko P. A. 1995. Genetic status of European bison. [In: Population and habitat viability assessment for European bison (*Bison bonasus*). Z. Pucek, U. S. Seal and P. Miller, eds]. IUCN/SSC Conservation breeding specialist group, Apple Valley, Minnesota: 91–96.

Hartl G. B. and Pucek Z. 1994. Genetic depletion in European bison (*Bison bonasus*) and the significance of electrophoretic heterozygosity for conservation. Conservation Biology 8: 167–178.

Hughes A. L. and Nei M. 1989. Nucleotide substitutions at major-histocompatibility-complex class II loci: Evidence for overdominant selection. Procceedings of the National Academy of Sciences of the United States of America 86: 958–962.

Klein J., Bontrop R. E., Dawkins R. L., Erlich H. A., Gyllensten U. B., Heise E. R., Jones P. P., Parham P., Wakeland E. and Watkins D. I. 1990. Nomenclature for the major histo-compatibility complexes of different species: a proposal. Immunogenetics 31: 217–219.

Li C. C. 1976. First course in population genetics. The Boxwood Press, California: 1–550.

Mikko S. and Andersson L. 1994. Generation of polymorphism at the major histocompatibility complex in artiodactyl species. XXIV International Conference on Animal Genetics, Czech Republic, Prague 23–29 July 1994: 58.

Morris B. G., Spencer M. C., Stabile S. and Dodd J. N. 1994. Restriction fragment length polymorphism (RFLP) of exon 2 of the MhcBibi-DRB3 gene in American bison (*Bison bison*). Animal Genetics 25, Suppl. 1: 91–93.

Mullis K. B. 1991. The PCR in an anaemic mode: how to avoid oligodeoxyribonuclear fusing. PCR Methods and Applications 1: 1–4.

Olech W. 1989. The participation of ancestral genes in the existing population of European bison. Acta Theriologica 34: 397–407.

Polziehn R. O., Beech R., Sheraton J. and Strobeck C. 1996. Genetic relationships among North American bison populations. Canadian Journal of Zoology 74: 738–749.

Pucek Z. 1991. History of the European bison and the problems of its protection and management. [In: Global trends in wildlife management. B. Bobek, K. Perzanowski and W. Regelin, eds]. Transactions of the 18th IUGB Congress, Kraków, August 1987. Świat Press, Kraków–Warszawa, 1: 19–39.

Schreiber A., Kolter, L. and Kaumanns W. 1993. Conserving patterns of genetic diversity in endangered mammals by captive breeding. [In: Ecological genetics in mammals. G. B. Hartl and J. Markowski, eds]. Acta Theriologica 38, Suppl. 2: 141–151.

Sipko T. P., Rautian G. S., Udina I. G., Ukhanov S. V. and Berendyaeva Z. I. 1995. Investigation of blood group polymorphism in European bison (*Bison bonasus*). Russian Journal of Genetics 31: 78–84.

Sipko T. P., Rautian G. S., Udina I. G. and Rakitskaya T. A. 1996. Polymorphism of biochemical markers in European bison (*Bison bonasus*). Russian Journal of Genetics 32: 346–351.

Sulimova G. E., Udina I. G., Shaikaev G. O. and Zakharov I. A. 1995. DNA polymorphism at the BoLA-DRB3 gene of cattle in relation to resistance and susceptibility to leukemia. Russian Journal of Genetics 31: 1105–1109.

Tiedemann R., Nadlinger K. and Pucek Z. 1998. Mitochondrial DNA-RFLP analysis reveals low levels of genetic variation in European bison *Bison bonasus*. [In: Ecological genetics in mammals III. G. B. Hartl and J. Markowski, eds]. Acta Theriologica, Suppl. 5: 83–87.

Udina I. G., Sokolova S. S., Sipko T. P. and Sulimova G. E. 1994. Comparative analysis of DNA polymorphism for DQB and DRB loci of the Major Histocompatibility Complex in representatives of the family Bovidae. Russian Journal of Genetics 30: 313–317.

Udina I. G., Badagueva Y. N. Sulimova G. E. and Zakharov I. A. 1995. Distribution of alleles of the κ-casein gene in populations of European bison (*Bison bonasus*). Russian Journal of Genetics 31: 1452–1453.

Van Eijk M. J. T., Stewart-Haynes J. A. and Lewin H. A. 1992. Extensive polymorphism of the BoLA-DRB3 gene distinguished by PCR-RFLP. Animal Genetics 23: 483–496.

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