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Low Major Histocompatibility Complex Class I (MHC I) Variation in the European Bison (*Bison bonasus*)

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Abstract

Variation in the major histocompatibility complex (MHC) class I of the European bison was characterized in a sample of 99 individuals using both classical cloning/Sanger sequencing and 454 pyrosequencing. Three common (frequencies: 0.348, 0.328, and 0.283) haplotypes contain 1–3 classical class I loci. A variable and difficult to estimate precisely number of nonclassical transcribed loci, pseudogenes, and/or gene fragments were also found. The presence of additional 2 rare haplotypes (frequency of 0.020 each), observed only in heterozygotes, was inferred. The overall organization of MHC I appears similar to the cattle system, but genetic variation is much lower with only 7 classical class I alleles, approximately one-tenth of the number known in cattle and a quarter known in the American bison. An extensive transspecific polymorphism was found. MHC I is in a strong linkage disequilibrium with previously studied MHC II *DRB3* gene. The most likely explanation for the low variation is a drastic bottleneck at the beginning of the 20th century. Genotype frequencies conformed to Hardy–Weinberg expectations, and no signatures of selection in contemporary populations but strong signatures of historical positive selection in sequences of classical alleles were found. A quick and reliable method of MHC I genotyping was developed.

Key words: *bottleneck, conservation, European bison, 454 sequencing, MHC class I, polymorphism*

Products of the major histocompatibility complex (MHC) genes present antigens derived from pathogens to the effector cells of the vertebrate immune system and are thus involved in triggering an effective adaptive immune response (Janeway et al. 2004). MHC class I proteins present mainly intracellular antigens derived from viruses, intracellular bacteria, and protists, whereas MHC class II molecules usually present antigens of extracellular pathogens. MHC harbors the most polymorphic vertebrate genes; their polymorphism is thought to be maintained by balancing selection either through negative frequency dependence or through heterozygote advantage (Snell 1968; Doherty and Zinkernagel 1975). Balancing selection is driven by the host–parasite interactions, with rare MHC alleles and/or heterozygosity at MHC loci conferring an advantage in fighting the pathogen assault (reviewed in Bernatchez and Landry 2003; Sommer 2005; Piertney and Oliver 2006). Distinguishing between these mechanisms may be very difficult in practice (Spurgin and Richardson 2010).

Each MHC allele may potentially bind and present to T cells a different set of pathogen-derived peptides, and thus population carrying a higher number of MHC alleles may respond to a broader spectrum of pathogens. This has led to suggestions that depletion of variation at the MHC genes may compromise the ability of populations to respond to pathogen assault and lead to an increased risk of extinction (O'Brien and Evermann 1988; Hughes 1991; Hedrick 2001). However, the evidence that the loss of MHC variation negatively affects population survival has been so far equivocal and difficult to separate from effects of general inbreeding (Radwan, Biedrzycka, et al. 2010). Bottlenecks lead to the depletion of genetic variation, with the scale of depletion dependent on the severity of the bottleneck: both the extent of population size reduction and the duration of bottleneck. Models show that balancing selection may not be able to maintain variation during the bottleneck but leads to a faster recovery of variation following the bottleneck (Ejsmond and Radwan 2011). A study of bottlenecked populations of island foxes,

however, demonstrated retention of polymorphism at MHC genes while variation was lost throughout the rest of the genome (Aguilar et al. 2004). On the other hand, there are several well-documented examples of species with exceptionally low MHC variation, some of them known to have recovered from severe bottlenecks, and some known to have lost much of MHC variation during the bottleneck (reviewed in Radwan, Biedrzycka, et al. 2010). These examples and commonly observed correlation between variation in neutral markers and MHC indicate that drift may indeed affect MHC variation, and that in at least some cases, populations with strongly reduced MHC variation may survive and expand. This seems to be the case of the European bison, a species that experienced an extreme population bottleneck, but due to intensive management was reestablished after World War II. Despite increase in number, the European bison population suffers from diseases (Pucek et al. 2004), which may indicate impaired immune function.

Historically, the European bison (*Bison bonasus*) ranged across western, central, and eastern Europe, inhabiting vast deciduous forests (Heptner et al. 1966). By the early 20th century, only 2 populations remained. The Lowland bison (*B. b. bonasus*) within Białowieża forest declined rapidly from 785 individuals in 1915 to extinction after World War I (April 1919), while a population of *B. b. caucasicus* in the northwest Caucasus met the same fate in 1927 (Pucek 1991). After World War I, the species survived only in a few European zoological gardens (Sztolcman 1924). Together, there were only 54 (29 males and 25 females) European bisons with documented pedigrees, originating from 12 ancestors (Slatis 1960). After a period of intensive captive breeding and a series of reestablishments, the species once again occupies some of its former range in Central and Eastern Europe. In this study, we have investigated individuals from a free-ranging population of the Lowland (or Białowieża) line. This line originates from only 7 founders (4 males and 3 females) and represents pure animals of the *B. b. bonasus* subspecies (the other line, Lowland-Caucasian, is also derived from the Białowieża population but has some genetic contribution from *B. b. caucasicus*). The descendants of these founders (14 males and 24 females) were released from the breeding reserve between 1952 and 1966 and created the contemporary free-living population (Krasiński 1983). The number of European bison of this line living in free-ranging herds is now close to 900, distributed between 12 populations over the territories of Poland, Belarus, and Lithuania (see Pucek et al. 2004). The largest free-ranging population (ca. 710 individuals); occurs in the Białowieża Forest in Poland and Belarus.

Previous studies of MHC variation in the European bison targeted the *DRB3* MHC class II (Udina and Shaikhaev 1998; Łopieńska et al. 2003; Radwan et al. 2007). A very limited variation was observed with only 4 *DRB3* alleles retained in the Białowieża population; these alleles, however, exhibit strong signatures of historical positive selection for amino acid replacements in regions involved in antigen binding (Antigen Binding Sites, ABS; Radwan et al. 2007). The association between the *DRB3* genotype and 2 types of infection was tested: posthitis, a disease of male reproductive organ of probably bacterial etiology, and the intensity of infection by an invasive nematode (Radwan et al. 2007; Radwan, Demiaszkiewicz, et al. 2010). No association between diseases and *DRB3* genotype or heterozygosity was detected, and it was suggested that the alleles that may have conferred resistance might have been lost during the bottleneck (Radwan et al. 2007).

There has so far been no data on the MHC class I structure and variation in the European bison. MHC class I genes involved in fighting intracellular pathogens are expressed on the surface of all nucleated cells and may also be involved in mate choice, suggesting additional mechanism that may maintain their polymorphism (reviewed in Penn and Potts 1999; Milinski 2006). MHC class I genes have been extensively studied in the domestic cattle (e.g., Ellis et al. 1999; Holmes et al. 2003; Di Palma et al. 2002; Birch et al. 2006, 2008; Babiuk et al. 2007), and sequences from the American bison are also available from GenBank.

Building on the resources available for these 2 close relatives, we set out to characterize MHC class I variation in the European bison. We devoted particular attention to the classical MHC I loci. Specifically, we wanted to: 1) determine the number of functional alleles in the population, their assignment to loci, arrangement in haplotypes, and to place this variation in the context of MHC I variation in cattle and the American bison, to evaluate the effect of the severe bottleneck on the MHC class I variation in the European bison; 2) look for signatures of historical positive selection in classical class I sequences; and 3) genotype a substantial number of animals to estimate frequencies of haplotypes and genotypes, provided that identification of haplotypes would be possible, and test their frequencies against Hardy–Weinberg expectations.

Materials and Methods

Samples, DNA and RNA Extraction, and Reverse Transcription

Genomic DNA (gDNA) was extracted from liver, heart, or kidney tissue of 99 bison using QIAamp DNA Mini Kit (Qiagen). The analyzed animals were culled by the National Park staff in Białowieża Primeval Forest, northeastern Poland, to control the bison population according to the Białowieża National Park guidelines, which aim to retain the natural age and sex structure of the population. For the analysis of expression, mRNA was obtained from spleens of 12 individuals (for which also gDNA samples were available). The spleen samples were preserved in RNAlater stabilization reagent (Qiagen). RNA was extracted with the RNeasy kit (Qiagen) from about 30 mg of the homogenized tissue, including the DNAse treatment step to completely remove the traces of gDNA from RNA preparations. Complementary DNA (cDNA) was obtained

Table 1 Sequences of novel or modified primers used in the present study

Primer	Sequence (5′ → 3′)
Bov7_Fusion	cgtatcgcctccctcgcgccatcagNNNNNNGGCT ACGTGGACGACACG
Bov11_Fusion	ctatgcgccttgccagcccgctcagNNNNNNCCCTC CAGGTAGTTCCT
Bov8deq_Fusion	cgtatcgcctccctcgcgccatcagNNNNNNGGCTG CNRCGTGGRGYCG
Bb03R_Fusion	ctatgcgccttgccagcccgctcagNNNNNNTCGCT CACCGGCCTCGCTCTG
Bb01F	ATGRGGCCGCGARCCCTC
Bb02R	TGMGAGACACATCAGAGCCC

Sequences of the 454 Titanium adaptors A and B are in lowercase; NNNNNN denotes a 6-bp sequence tag used for assigning reads to amplicons.

using Omniscript Reverse Transcriptase (Qiagen) in 20 μl reactions containing 6 μl template RNA, 2 μl Oligo$(dT)_{12\text{-}18}$ primer (0.5 μg/μl), 1 μl RNase inhibitor (10 u/μl), 1 μl RT, and 6 μl of RNase free water. The reaction was incubated at 37 °C for 60 min.

Sequencing and Genotyping

In this study, we used 2 sequencing approaches: Sanger sequencing followed by cloning and 454 sequencing. We analyzed cDNA and gDNA and targeted both full-length sequences, and the portion of the gene containing the antigen-binding region (exons 2 i 3), which was assayed both as a single fragment and as 2 separate amplicons (Table 1 and Supplementary Figure S1).

Amplification, Cloning, and Sanger Sequencing

For the characterization of the MHC class I variation in the European bison, we applied primers Bov7 and Bov11, designed to amplify from as many as possible functional class I sequences in cattle (Pichowski et al. 1996; Ellis et al. 1999; Birch et al. 2006). These primers amplify parts of the 2nd and 3rd exon and the intervening intron. Amplification was performed from both cDNA and gDNA. Polymerase chain reactions (PCRs) contained 15 μl of HotStar *Taq* Master Mix (Qiagen), 1 μM of each primer, 10–100 ng of cDNA or gDNA, and deionized water up to 30 μl. PCR cycling was as follows: 95 °C for 14.5 min followed by 35–38 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s and a final elongation at 72 °C for 3 min. PCR products were purified with MinElute PCR purification kit (Qiagen) and cloned using StrataClone PCR cloning kit (Stratagene). Inserts from multiple colonies per amplicon were amplified using standard M13 primers, sequenced with the Big Dye Terminator kit 3.1 (ABI), and run on a 3130xl genetic analyzer (ABI).

Preparation of Samples, 454 Sequencing, and Genotyping

Although characterization of MHC class I sequences through cloning provided valuable information, the low throughput and high cost would make this approach not feasible for large-scale genotyping. Our initial attempts of using the single stranded conformational polymorphism (SSCP) analysis for genotyping exons 2 and 3 proved unsuccessful due to coamplification of multiple sequence variants per amplicon and the resulting complexity of SSCP patterns. Therefore, we resorted to the 454 sequencing to genotype exons 2 and 3 in all samples. In the context of MHC genotyping, 454 sequencing is equivalent to a large-scale cloning of amplicons in a cell free system, followed by highly parallel sequencing of clones, which provides the desired number of sequencing reads per amplicon quickly and for a fraction of the cost of traditional cloning and Sanger sequencing (Babik et al. 2009; Babik 2010; Galan et al. 2010; Kloch et al. 2010). In 454 amplicon sequencing, samples are amplified with fusion primers, containing at the 5′ end the 454 adaptors used in sequencing and at the 3′ end the primer sequence specific for the target. Multiple amplicons may be sequenced in a single 454 analysis if a tag, short sequence specific for the sample, is introduced between adaptor and specific primer sequence during fusion primer synthesis. We sequenced 3 types of amplicons with 454 technology (Supplementary Figure S1):

1. cDNA samples were amplified with primers Bo7_Fusion-Bo11_Fusion, targeting the fragment of the MHC class I transcript of 375 bp (without primers).

gDNA samples were amplified with 2 pairs of fusion primers (Table 1, Supplementary Figure S1):

1. Bo7_Fusion-Bb03R_Fusion pair targeted the 165 bp fragment of exon 2 (full exon length 270 bp) and
2. Bo8deg_Fusion-Bo11_Fusion targeted a 153 bp fragment of exon 3 (full exon length 276 bp).

We did not attempt amplification from gDNA with primers Bo7_Fusion-Bo11_Fusion because the fragment would be too long for 454 Titanium sequencing in a single pass. Focusing on fragments of exons 2 and 3 for population scale genotyping is justified because our analysis of all available cattle and American bison full-length classical MHC class I alleles showed that 87% and 85% of alleles, respectively, were distinguished on the basis of this fragment. Exons 2 and 3 code for the domains alpha 1 and alpha 2 of the MHC I protein, which are involved in antigen binding and as such are the most variable part of the MHC I gene (Reche and Reinherz 2003). Therefore, this approach for identification of the entire allelic diversity appears more effective than cloning full-length alleles from a necessarily limited number of samples. Individuals with unique alleles, identified in the large-scale population screen may be then selected for the analysis of full-length sequences through cloning and Sanger sequencing.

We used 6 bp tags in the forward primer sequence; tag sequences differed by at least 3 nucleotides. The sequencing experiment was performed as a part of a single 454 Titanium run, which was divided into 8 sections, so that a single tagged primer could be used up to 8 times.

PCR conditions for amplification with primer pairs Bo7_Fusion-Bo11_Fusion and Bo7_Fusion-Bb03R_Fusion were identical. Fifteen-microliter reactions contained 1× Hot Start Buffer (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Fermentas), 1 μM of each primer, 0.75 U of Hot Start *Taq* polymerase (Fermentas), and 10–100 ng of DNA template. PCR cycling was as follows: 3 min 30 s at 94 °C followed by 33 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C and the final elongation step of 3 min at 72 °C. For amplification with primers B8deg_Fusion-Bo11_Fusion, we modified the amplification protocol by using 0.5 μM of each primer and increasing the number of amplification cycles to 35.

For each amplicon type, PCR products intended to be sequenced in the same section of the sequencing plate were pooled in approximately equimolar amounts, as assessed through gel-band intensity. Pools of amplicons were purified using MinElute PCR purification kit and were combined with other pools analyzed in the same experiment in quantities corresponding to the desired yield of sequencing reads. Pools were sequenced bidirectionally using the GS FLX Titanium MV emPCR kit for emulsion PCR and the GS FLX Titanium Sequencing Kit XLR70 in combination with GS FLX Titanium PicoTiterPlate Kit 70 × 75 for sequencing (Roche Applied Sciences). Sequencing reads were extracted and multifasta files were used for further manipulations using the jMHC software (Stuglik et al. 2011), available from http://code.google.com/p/jmhc/, and Microsoft Excel. Reads with the complete tag sequence, complete sequences of specific amplification primers, and without ambiguities in the target region were extracted and sorted according to the tags. For each amplicon type, the following procedure was applied on the level of the full data set (i.e., all analyzed amplicons of the given type) for distinguishing PCR chimaeras and other PCR/sequencing errors from true alleles (TAs). We use the term "allele" for unique sequence variants for simplicity, but assigning sequence variants to loci was not possible in the current study.

First, for each sequence variant only amplicons with the total coverage of >100 reads and at least 2 reads of the variant were considered. For each amplicon, the per amplicon frequency (percentage of all reads in this amplicon representing the variant) was computed, and variants were sorted according to their Maximum Per Amplicon Frequency (MPAF) in the entire data set. The rationale for this procedure is that multiple types of PCR and sequencing artifacts are expected but none of them should be very common on the individual basis, particular artifacts should be rarer among individual's reads than most TAs (Zagalska-Neubauer et al. 2010). The variants were then examined bottom-top starting with the MPAF value of 1% as follows:

- We selected 3 amplicons in which the given variant was most abundant (including the one on which the MPAF value was based).
- We checked whether in all 3 amplicons the variant could be explained as:

A chimera of more abundant variants within the same amplicon?

By a 1-bp indel introduced to the sequence of a more abundant variant in the same amplicon?

By a 1-bp substitution introduced to the sequence of a more abundant variant in the same amplicon?

If any of these criteria was fulfilled, we classified the variant as an artifact and all the remaining variants were classified as TAs.

This approach allowed distinguishing, on the level of the full data set, putative TA from various kinds of PCR and sequencing artifacts.

For these sequences obtained from gDNA, which were identical as transcribed sequences or showed very high similarity to cattle nonclassical class I genes, we applied the nomenclature rules as for the cattle (http://www.ebi.ac.uk/ipd/mhc/bola/nomen_rules.html). This assignment should be considered preliminary until the full-length sequences of all variants are obtained from cDNA, although only minor adjustments are expected in the future. For other sequences obtained from gDNA, we applied tentative names in the format *Bibo-ua** followed by consecutive numbers in the 2-digit format.

With the information about TA, we performed genotyping of 3 kinds of amplicons described above.

Obtaining Nearly Full Coding Sequences of Functional Alleles

On the basis of 454 genotyping, we selected 4 individuals possessing all presumably functional alleles present in the European bison population for the characterization of full-length (exons 1–7) coding sequences of the classical MHC class I alleles. For amplification, we used a mixture of primers Bov 21a/g and Bov 21 (forward) and a mixture of Bov 3 and Bov3-BSF (reverse) (Birch et al. 2006), which however did not amplify all alleles, therefore, from alignments of several cattle and bison alleles, we designed an additional pair of primers, Bb01F and Bb02R (Table 1), which ensured successful amplification of functional alleles. Both types of amplicons were cloned, and multiple clones were sequenced as described above.

Population Genetic Analyses

Inspection of the individual genotypes allowed preliminary distinguishing of several haplotypes, which appear to be stable, that is, not recombining in the bison population. We tested whether genotype frequencies conformed to random mating expectations according to the Hardy–Weinberg principle. Because for most individuals both MHC I and MHC II *DRB3* genotypes were available (Radwan et al. 2007), it was also possible to test for association (linkage disequilibrium) between MHC I and II haplotypes. Both kinds of tests were performed in Genepop 4 (Rousset 2008); for testing Hardy–Weinberg proportions, the exact test of Guo and Thompson (1992) was used and the log likelihood ratio test (*G*-test) was applied for testing linkage disequilibrium.

Detecting Signatures of Historical Selection and Recombination

For the 6 (nearly) full-length transcribed classical alleles, the average rates of synonymous (d*S*) and nonsynonymous (d*N*) substitutions were computed for full lengths of domains alpha 1 and 2 (encoding the variable part of MHC I molecule), as well as for positions encoding amino acids determining the specificity of peptide binding (ABS) and the remaining alpha 1 and 2 positions (non-ABS). The location of ABS was inferred from the human MHC I molecular structure (Reche and Reinherz 2003). Because 3 human class I genes, HLA-A, -B and -C have slightly different ABS, we conservatively classified as ABS only codons that occur in ABS of all 3 genes. Computations were performed in MEGA4 (Tamura et al. 2007).

The impact of historical selection on the MHC sequences was assessed through the *Z*-test of selection in MEGA and by fitting 5 models of codon evolution available in PAML (Yang 2007). These were: 1) M0: one ω (d*N*/d*S* ratio), 2) M1a: nearly neutral ($\omega \leq 1$), 3) M2a: positive selection (a proportion of sites evolving with $\omega > 1$), 4) M7: an extension of M1a, where the beta distribution approximates ω ($\omega \leq 1$) variation, and 5) M8: the beta extension of M2a. The best-fitting models were chosen on the basis of the value of the Akaike information criterion (AIC; Posada and Buckley 2004). Positively selected codons were identified through the Bayes empirical Bayes procedure (Zhang et al. 2005).

Six full-length sequences were tested for the presence of recombination with the GARD method pond (Pond et al. 2006) through a web-based routine (http://www.datamonkey.org/GARD/).

Results

Diversity of MHC I Sequences in the European Bison

Two techniques were used to assess MHC class I sequence diversity: 1) amplicon cloning followed by Sanger sequencing and 2) 454 amplicon sequencing. Most of the following description is based on the results of 454 sequencing because these provided high-quality data for a number of samples. Sequences of alleles generated in this study were submitted to GenBank (Accession Numbers JQ322982–JQ322996 and online supplementary material); all alleles were detected in multiple amplicons, thus they passed the 2 PCR criterion commonly applied in MHC studies.

454 sequencing enabled actual genotyping of a number of amplicons derived from both cDNA and gDNA; this technique identified most sequence variants. Sixteen amplicons spanning most of the exons 2 and 3 were derived from cDNA of 12 bisons (4 individuals were replicated) and sequenced using the 454 technology to the average coverage of 556 (SD = 240, range 72–834) reads per amplicon. Altogether 11 sequence variants (alleles) were identified. Their relationships in the context of the cattle and American bison MHC I variation are shown in Figure 1.

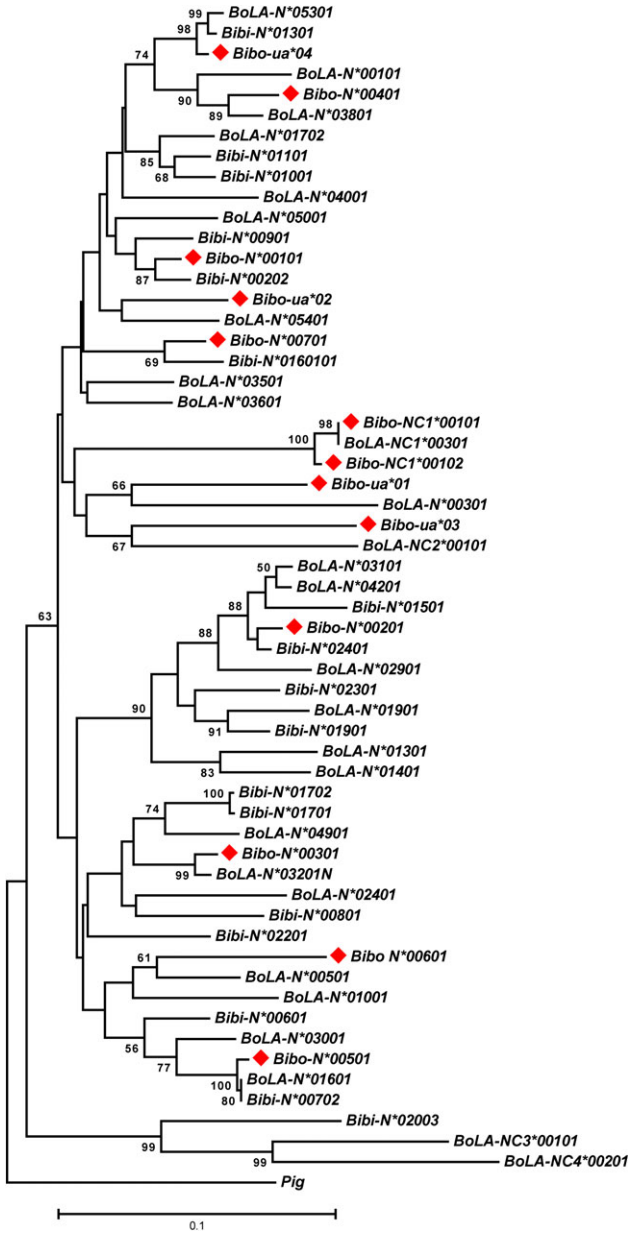

Figure 1. The relationships among the European bison (starting with *Bibo* and marked with red symbols) MHC class I sequences and representative sequences from cattle (*BoLA*) and the American bison (*Bibi*) based on a 375-bp fragment of exons 2 and 3; transcribed but apparently nonfunctional (see text) alleles *Bibo-N*00402N* and *Bibo-N*00702N* are not included in the tree. The tree was rooted with the pig sequence.

Five highly transcribed (MPAF 8.3–49.6%) alleles *Bibo-N*00101–Bibo-N*00501* were similar to classical cattle and American bison alleles. Another 2 alleles, *Bibo-N*00601* (MPAF 1.6%) and *Bibo-N*00701* (MPAF 1.1%) appear to be derived from classical MHC I genes as well, but were either transcribed at lower level or less efficiently amplified. Interestingly, 2 variants were much shorter than canonical 375 bp: *Bibo-N*00402N* (MPAF 1.9%) differed from

*Bibo-N*00401* only by a 74-bp deletion, and *Bibo-N*00702N* (MPAF 0.8%) differed from *Bibo-N*00701* only by a 70-bp deletion. Both shorter alleles always co-occurred with their longer "parental" forms and are most likely nonfunctional due to the presence of frameshifts and stop codons. Two alleles, *Bibo-NC1*00101* and *Bibo-NC1*00102* (MPAF 3.6 and 2.2%), were highly similar to the cattle nonclassical gene *NC1*: in the examined fragment *Bibo-NC1*00101* was identical to cattle *BoLA-NC1*00201* and **00301* alleles and *Bibo-NC1*00201* differed by only 1 bp from *BoLA-NC1*00401*.

One hundred and ten amplicons of a 165-bp fragment of exon 2 derived from gDNA of 99 bisons were sequenced to the average coverage of 349 (SD = 148) and the range of 88–884 reads per amplicon. Twenty-two variants with the MPAF range 4.0–17.2% were detected. Relationships among these sequences are shown in Supplementary Figure S2. All alleles detected in cDNA were present, except truncated *Bibo-N*00402N* and *Bibo-N*00702N* that have the part of sequence with the Bb03R primer binding sites deleted. However, 14 variants were not found in cDNA; 4 of these could be linked to exon 3 variants through longer cloned sequences obtained from gDNA (see below). Four variants not detected in cDNA exhibited similarity to transcribed alleles (*Bibo-ua*02, *04, *07, *09*), 2 were rather divergent (*Bibo-ua*01* and **08*), and 8 were similar to nonclassical sequences (Supplementary Figure S2). Five of these apparently nonclassical sequences were tentatively assigned to nonclassical genes on the basis of high similarity to cattle sequences: *Bibo-NC2*00101*, *Bibo-NC2*00102*, *Bibo-NC3*00101*, *Bibo-NC4*00101*, and *Bibo-NC4*00102*.

One hundred and four amplicons of a 153-bp fragment of exon 3 derived from gDNA of 94 (of a total of 99 sampled) bisons were sequenced to the average coverage of 199 (SD = 109) and the range of 34–552 reads per amplicon. Fifteen variants with MPAF 2.4–47.8% were detected. All but one transcribed variants were present. The lack of the *Bibo-N*00601* allele among fragments amplified with Bo8deg_Fusion and Bo11_Fusion primers was expected because this allele has a G → A mutation at the 3′ end of the Bo8deg binding site preventing its amplification. All cattle and the American bison alleles have G in this position. Twelve variants were, if present in a given amplicon, quite abundant (MPAF 10.8–49.0%), whereas 3 variants were always rare, with MPAF 2.4–4.5%. Because samples were amplified from gDNA, then if we exclude the gene copy number effect, these low MPAF values may indicate less efficient amplification. The lack of sequences, which could have been assigned to nonclassical loci *NC3* and *NC4* (Supplementary Figure S3), was expected because these have a mutation A → C at the 3′ end of the Bo11 primer. Of 3 variants with low MPAF, one (*Bibo-ua*10*) was clearly related to the nonclassical *NC2* gene (100% bootstrap support) and 2 remaining similar sequences (*Bibo-ua*11 and *12*) grouped with a substantial bootstrap support of 74% with the *BoLA-N*01001* allele; these 3 variants could not have been unambiguously linked with exon 2 sequences although there is little doubt that *Bibo-ua*10* is contiguous with *Bibo-NC2*00101* or *Bibo-NC2*00102* (or both, if they do not differ in the assayed part of the third exon).

Cloning and Sanger sequencing of a subset of individuals augmented the results of 454 sequencing by validation of variants, linking nonoverlapping sequences from 454 analysis and characterization of (nearly) full-length coding sequences of functional alleles. Amplicons spanning most of the exons 2 and 3 were cloned from cDNA of 6 individuals, which constituted a subset of 454-sequenced cDNA samples; 79 clones (13.2 per individual on average) were Sanger sequenced. Only 3 alleles: *Bibo-N*00101*, **00201*, and **00301* were detected in clones. Apparently the number of clones was too small to detect other transcribed alleles. Other alleles detected in 454 cDNA sequences were, however, confirmed in 228 Sanger sequenced clones derived from gDNA of 14 individuals (16.3 per individual on average); for 5 of these individuals, sequences derived from cDNA were available as well. Sequence variants found in clones derived from gDNA and not detected in cDNA facilitated interpretation of the results of 454 amplicon sequencing of individual exons from gDNA. Nonoverlapping sequences from exons 2 and 3, which were identical to a confirmed variant obtained by cloning, could be safely assumed to represent the same allele and were joined and named accordingly.

Full or nearly full-length (exons 1–7) coding sequences of 6 of 7 classical alleles (Accession Numbers JQ322984-87, JQ322989-90) were revealed through Sanger sequencing of 112 clones derived from 7 amplicons obtained from 4 individuals, which represented the entire MHC class I diversity detected in the European bison as evidenced by 454 sequencing. Despite substantial effort, we were not able to obtain the full-length sequence of the *Bibo-N*00701* allele which may indicate that this allele is indeed transcribed at low level or is a transcribed gene fragment lacking some exons.

Identification of Haplotypes, Testing Hardy–Weinberg Proportions and Linkage Disequilibrium with MHC Class II

The number of genetic patterns in both cDNA and gDNA was limited, and 6 patterns were observed in multiple individuals. Three simple patterns were interpreted as homozygous genotypes and 3 complex patterns could be unambiguously derived from these as their pairwise combinations and were thus consistent with the presence of 3 common heterozygotes. Thus, preliminary identification of MHC class I haplotypes was possible (Table 2). Three haplotypes were inferred from cDNA: 1) hapI with 3 classical genes represented by alleles: *Bibo-N*00301*, **00401*, **00701*, and 2 truncated, most likely nonfunctional genes, 2) hapII has 2 transcribed classical genes (alleles *Bibo-N*00201* and *N*00501*), and 3) hapIII with one classical gene (allele *Bibo-N*00101*) (Table 2). Each haplotype has also an allele of the nonclassical *NC1* gene. Allele *Bibo-N*00601* was present in some but not all individuals possessing the

Table 2 Preliminary identification of the MHC class I haplotypes in the European bison

	hapI Observed	hapII Observed	hapIII Observed	hapIV Inferred	hapV Inferred
Transcribed					
Classical functional					
*Bibo-N*00101*			+		
*Bibo-N*00201*		+		+	
*Bibo-N*00301*	+				
*Bibo-N*00401*	+				
*Bibo-N*00501*		+		+	
*Bibo-N*00601*			+/−		
*Bibo-N*00701*	+				
Classical nonfunctional					
*Bibo-N*00402N*	+*				
*Bibo-N*00702N*	+*				
Nonclassical					
*Bibo-NC1*00101*	+	+		+	
*Bibo-NC1*00102*			+		+
Transcription not confirmed					
Similar to classical					
*Bibo-ua*02*					+
*Bibo-ua*04*	+				
*Bibo-ua*07*					+
*Bibo-ua*09*	+	+	+	?	?
Similar to nonclassical					
*Bibo-NC2*00101*	+		+	+	?
*Bibo-NC2*00102*		+			
*Bibo-NC3*00101*		+			
*Bibo-ua*03*	+	+	+	?	?
Highly divergent					
*Bibo-ua*01*		+		+	+
*Bibo-ua*08*					+

Haplotypes I–III were observed as homozygotes, haplotypes IV and V were observed only as heterozygotes and had to be inferred. +/−, this allele was observed only in some individuals with hapIII and may indicate either the presence of 2 haplotypes here pooled as hapIII or unequal amplification (see text); *, These alleles were observed only in individuals genotyped from cDNA, as they possessed a large deletion preventing their amplification from gDNA in exon 3 primers used in the present study; ?, presence or absence of an allele could not be validated in the inferred haplotype.

hapIII, which may indicate that this haplotype is actually an aggregate of 2 haplotypes; however, evaluating this possibility is beyond the resolution of the present study. Further insights into the haplotype structure of the bison MHC I was provided by the analysis of gDNA. For exon 2, 17 of 22 sequence variants could be assigned to haplotypes. The remaining 5 variants have notable features. One of them *Bibo-N*00601* was discussed above and 4 other sequences show similarity to the nonclassical genes *NC3* and *NC4*, which in the cattle are separated by approximately 0.5 Mb from the classical loci and nonclassical *NC1* gene. Thus, recombination could have broken associations between alleles in these 2 subregions of MHC class I. Exon 2 and 3 sequences from gDNA allowed assignment of additional alleles to the 3 haplotypes identified already in cDNA and suggested the presence of 2 additional haplotypes. These haplotypes, hapIV and hapV, were present only in heterozygotes and thus their composition is uncertain. The tentative nature of haplotypes IV and V notwithstanding, they were very rare, each with frequency of 0.02. Thus, we can conclude that there are 3 major frequent MHC I haplotypes in the lowland line of the European bison, with frequencies 0.348 (hapI), 0.328 (hapII), and 0.283 (hapIII). Each major haplotype was observed in multiple homozygotes.

Genotype frequencies (Supplementary Table S1) were in an excellent agreement with Hardy–Weinberg expectations ($P = 0.88$); observed heterozygosity $H_O = 0.717$, expected $H_E = 0.693$.

We observed an extremely strong linkage disequilibrium between MHC class I genotypes and MHC class II *DRB3* genotypes ($P < 10^{-6}$, Supplementary Table S2).

A Simple Genotyping Method for Distinguishing Major Haplotypes

Having identified the classical class I alleles present on particular haplotypes, we devised a simple method for genotyping MHC class I. The method uses 4 specific forward primers, which are used in multiplex together with labelled Bb03R primer (Supplementary Table S3). Products of the multiplex reaction are then run on an automated genetic analyser and alleles are distinguished by length. All 6 genotypes may be unambiguously distinguished using this method (Supplementary Figure S4); details are presented in the online Supplementary Material.

Table 3 Average nucleotide distances among the cattle, American bison, and European bison classical MHC class I alleles

	Cattle (64 alleles)	American bison (29 alleles)	European bison (7 alleles)
All	0.121 (0.011)	0.106 (0.011)	0.121 (0.013)
ABS	0.290 (0.050)	0.227 (0.038)	0.291 (0.045)
Non-ABS	0.092 (0.010)	0.084 (0.010)	0.093 (0.011)

The distances were computed for the 375-bp fragment of exons 2 and 3 amplified with primers Bo7_Fusion-Bo11_Fusion using the Kimura 2 parameter model. Only cattle and American bison alleles distinguishable in this part of sequence were included. All, all nucleotide positions. In parentheses, standard errors obtained through 500 bootstrap replicates.

Table 4 Synonymous and nonsynonymous divergence of the 6 European bison classical MHC class I alleles (*Bibo-N*00101–Bibo-N*00601*) computed for the full length of the alpha 1 and alpha 2 domains

	dN	*dS*	*P*
ABS	0.380 (0.071)	0.089 (0.035)	5×10^{-5}
Non-ABS	0.078 (0.013)	0.114 (0.023)	0.14

All, all sites; ABS, common to all 3 human classical genes (Reche and Reinhertz 2003); non-ABS, remaining sites. Synonymous and nonsynonymous distances were computed using the Nei–Gojobori method with Jukes–Cantor correction for multiple substitutions; standard errors given in parentheses were obtained through 500 bootstrap replicates. *P*, *P* value of the *Z* test of selection testing the hypothesis $dN/dS = 1$.

MHC Class I Diversity in the European Bison in Comparison to Cattle and the American Bison

We detected only 7 classical class I MHC alleles in the large population sample of the European bison. This is a low diversity compared with the cattle and the American bison. There have been 71 full-length classical alleles characterized in cattle and 33 classical alleles characterized in the American bison. The average divergence of the cattle, American bison, and European bison MHC class I sequences was very similar (Table 3), so the alleles retained in the European bison are not particularly divergent.

Signatures of Historical Selection and Recombination in the MHC Class I Sequences

Codon-based tests of natural selection performed on the 6 (nearly) fully sequenced classical alleles showed evidence for the action of historical positive selection. The M8 model of codon evolution allowing for positive selection produced a much better fit to the data than one dN/dS ratio (M0) or nearly neutral (M7) models (deltaAIC = 57.8). Identical results were obtained in a M1a–M2a comparison, regarded as more conservative. The Bayes Empirical Bayes procedure identified 9 codons as evolving under positive selection (all posterior probabilities > 0.99). All these codons were located in the alpha 1 and alpha 2 domains and 7 were located in ABS common to all human MHC class I classical genes, indicating a strong overrepresentation of positively selected sites in ABS ($P < 10^{-5}$, Fisher's exact test). There was a highly significant excess of nonsynonymous substitutions in ABS but not in non-ABS (Table 4). Of 7 detected recombination breakpoints, 5 were located in alpha 1 and alpha 2 domains. Although recombination may have affected identification of individual codons under selection, it is unlikely that it distorted the overall picture of pervasive positive selection in the alpha 1 and 2 domains.

Discussion

The present study demonstrated a low variation in the European bison MHC class I region. Apparently only 7 transcribed, presumably functional classical alleles on 3 frequent haplotypes are present. Additional 2 haplotypes possibly occur in low frequencies. Despite limited variation, obtaining more detailed information about the MHC class I organization, such as the overall number of functional genes and the assignment of alleles to genes, have not been possible. This is due to the apparently complex architecture of MHC class I in the European bison, which may resemble that in cattle. There are several, possibly 6, expressed classical MHC class I genes in cattle (Birch et al. 2006). Various combinations of 6 genes, 3 of which appear highly polymorphic are transcribed on different haplotypes (Birch et al. 2006). Apparently no haplotype expresses all 6 genes. In addition to the classical class I genes, there are 4 nonclassical genes characterized by low polymorphism. One of these genes is closely linked to classical MHC class I loci, and the remaining nonclassical loci are located >0.5 Mb apart, close to MIC genes (Birch et al. 2008). Pseudogenes and gene fragments are also present in the cattle MHC class I region (Birch et al. 2008). Despite the availability of the cattle genomic sequence, additional mapping (Brinkmeyer-Langford et al. 2009), and extensive previous work (e.g., Ellis et al. 1999; Di Palma et al. 2002; Holmes et al. 2003; Birch et al. 2006, 2008; Babiuk et al. 2007), the understanding of haplotype organization and variation of the bovine MHC class I is still incomplete, which indicates that obtaining such information for the European bison may be challenging.

The low variation observed in the European bison remains in a stark contrast with data on MHC class I variation in cattle, where more than 70 expressed classical class I alleles have been described (http://www.ebi.ac.uk/ipd/mhc/bola/index.html), and the dynamics of the discovery of new MHC class I alleles has been high (Birch et al. 2006; Babiuk et al. 2007), indicating that the actual variation is higher than described so far. Another close relative of the European bison, the American bison, also exhibits higher variation with 33 full-length alleles deposited in GenBank.

The low variation in the European bison is not a consequence of limited sampling, as our population sample was large, and it constituted a sizeable part of the total population of the Lowland line, which contains most of the bison genetic variation. Because large-scale genotyping was limited to parts of the second and third

exons, we may have missed some variation present in other parts of the gene. We show, however, that in the cattle and the American bison at least 85% described alleles may be distinguished solely on the basis of the assayed region, so most of MHC I variation present in the analysed population was likely identified. Thus, because the species closely related to the European bison show much higher MHC variation, the strong bottleneck experienced by the European bison in the beginning of the 20th century has probably been responsible for the depletion of variation in the MHC region. Low variation is also evident in other parts of the genome (Hartl and Pucek 1994; Wójcik et al. 2009; Tokarska, Kawałko et al. 2009; Tokarska, Marshall, et al. 2009; Pertoldi et al. 2010). Radwan et al. (2007) showed that the drastic bottleneck could easily reduce variation to levels observed for the MHC II (4 retained alleles), and it is not surprising that in the tightly linked class I, a comparable diversity is found (3–5 haplotypes retained). Moreover, Radwan et al. (2007) showed that the estimates of the number of alleles expected after the bottleneck did not differ much for a wide range of prebottleneck diversity (15–100 alleles).

Low MHC variation has been invoked as a major factor increasing extinction risk in endangered vertebrates (O'Brien and Evermann 1988; Hughes 1991). In at least one case, the Tasmanian devil, there is substantial evidence that a low MHC class I variation may indeed increase the probability of extinction (Siddle et al. 2007). However, the evidence linking the risk of extinction with low MHC variation remains equivocal and the effects of the loss of variation in MHC are difficult to distinguish from the effects of general inbreeding (Radwan, Biedrzycka, et al. 2010). It is very difficult to assess whether the low MHC variation in the European bison increases the risk of extinction through infectious diseases. Two serious threats for the viability of the European bison populations emerged during last decades. First is posthitis, a disease of unclear, perhaps bacterial origin, affecting the reproductive organs of bulls (Jakob et al. 2000; Lehnen et al. 2006). Second, more recent, is an invasive blood-sucking helminth *Ashwortius sidemi*, which has been spreading rapidly in recent years across the bison population (Demiaszkiewicz et al. 2008, 2009). No association between susceptibility to the pathogen and MHC class II genotype was detected in either case (Radwan et al. 2007; Radwan, Demiaszkiewicz, et al. 2010). Because of the tight linkage between classical MHC class I genes and MHC class II DRB3 gene detected in the present study, it is unlikely that MHC class I would correlate with susceptibility. It was hypothesized that alleles, which would confer resistance, may have been lost during the bottleneck (Radwan et al. 2007), but this hypothesis is extremely difficult to test. We cannot rule out the possibility that the limited variation in MHC I may pose a particular threat for the bison population if a novel viral pathogen appears.

Sometimes selection is invoked as a force that retains MHC variation during the bottleneck (e.g., Aguilar et al. 2004). It has been also hypothesized that more divergent alleles are more likely to survive the bottleneck due to the advantage they confer in heterozygotes in terms of a broader antigen-binding range (Hedrick et al. 2002; Hedrick 2003; Lenz 2011). There are however problems with both hypotheses. First, a simulation model explicitly addressing the dynamics of MHC variation maintained by selection during the bottleneck (Ejsmond and Radwan 2011) has shown that directly after the bottleneck the negatively frequency dependent selection actually reduced variation below the level expected under drift alone (the possibility of MHC-dependent mating was not included in the model). Second, the same study showed that alleles retained after the bottleneck were not more divergent than expected by chance (but see Lenz 2011). In the context of the European bison MHC variation, selection seemed to play not a significant role in maintaining allelic diversity. First, as detailed above, the number of MHC alleles in the postbottleneck population is consistent with neutral expectations given the bottleneck the bison experienced. Second, the retained class I alleles are not more divergent than the average for cattle and American bison.

An excellent agreement between the frequencies of genotypes expected on the basis of allele frequencies supports correct genotyping but also indicates the lack of strong ongoing viability selection on MHC class I. We cannot, however, rule out that a weaker selection has been operating because for detecting modest departures from Hardy–Weinberg proportions samples of several hundreds would be needed (Apanius et al. 1997). The 3 common haplotypes have similar frequencies and their frequency distribution is very even, which is commonly considered a signature of balancing selection; the Ewens–Watterson test, comparing the distribution to that expected under neutrality was only marginally nonsignificant ($P = 0.06$). However, more even distribution of allele frequencies may result from the departures from drift-mutation equilibrium caused by the bottleneck. Indeed, too even distribution of allele frequencies has been proposed as a signature of the bottleneck (Luikart and Cornuet 1998), because rare alleles are more prone to extinction during the bottleneck. Moreover, frequency dependent selection, the major form of balancing selection invoked to explain the maintenance of MHC variation, would not typically produce even allele frequency distribution detectable by the Ewens–Watterson test (Ejsmond et al. 2010). Therefore, we conclude that, similarly as in MHC II (Radwan et al. 2007), no signal of ongoing selection may be inferred from the distributions of MHC I halotype and genotype frequencies.

We detected, however, unequivocal signatures of the historical positive selection in the classical class I genes, with codons under positive selection overrepresented in the putative ABS. The lack of the evidence for contemporary selection in populations, accompanied by the overwhelming evidence for historical selection are common patterns seen in many vertebrate species that have experienced reduction in population size (reviewed in Radwan, Biedrzycka, et al. 2010).

In the present study, we used primers designed to amplify as much MHC class I variation as possible, to

quickly and comprehensively characterize variation in a species, which has not been studied in this respect to date. Because amplification was performed from gDNA, multiple products coamplified, including functional alleles of classical and nonclassical genes, possible pseudogenes and gene fragments, known to occur also in cattle MHC region (Ellis 2004; Birch et al. 2008). Such strategy enabled a comprehensive screen for genomic sequences similar to MHC class I genes, but due to the number of various sequence variants amplified, reliable genotyping with cloning and SSCP, standard techniques used in MHC studies (Babik 2010) was not possible. Therefore, we resorted to 454 pyrosequencing. This technique proved rapid, reliable, and relatively cost efficient, enabling a single-step genotyping of a large population sample, which in turn facilitated selection of a representative sample of individuals for characterization of the MHC variation through cloning and sequencing of full or almost full coding sequences. The inferred arrangement of alleles into haplotypes and apparent stability of haplotypes allowed us to design a very simple and reliable genotyping method, which utilizes haplotype-specific primers and fluorescent detection of amplicons differing in size. This method enables rapid and cost-efficient genotyping of the MHC class I in the European bison, which might be used for studying the MHC class I based mate choice in free-living populations, maternal–foetal interaction, or associations with emerging pathogens.

Supplementary Material

Supplementary Material can be found at http://www.jhered.oxfordjournals.org/.

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