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MHC-DRB3 variation in a free-living population of the European bison, *Bison bonasus*

J. RADWAN,* A. KAWAŁKO,† J. M. WÓJCIK† and W. BABIK‡
*Institute of Nature Conservation, Polish Academy of Sciences, A. Mickiewicza 33, 31-120 Kraków, Poland, †Mammal Research Institute, Polish Academy of Sciences, Waszkiewicza 1c 17-230 Białowieża, Poland, ‡UFZ-Centre for Environmental Research Leipzig-Halle, Department of Community Ecology, Theodor-Lieser-Strasse 4, 06120 Halle (Saale), Germany

Abstract

MHC genes play a crucial role in pathogen recognition and are the most polymorphic genes in vertebrates. Loss of variation in these genes in bottlenecked species is thought to put their survival at risk. We examined variation at the MHC II DRB3 locus in the European bison, *Bison bonasus*, a species that has undergone an extreme bottleneck: the current population originated from only 12 founders. We also tested for the association of DRB3 genes with the incidence of posthitis, a disease affecting the reproductive organs of bulls and posing a new threat to the survival of the species. We found very limited MHC diversity, with only four alleles segregating in a sample of 172 individuals from a free-ranging Białowieża population. The alleles were highly divergent and revealed the hallmark of positive selection acting on them in the past, that is, a significant excess of nonsynonymous substitutions. This excess was concentrated in putative antigen-binding sites, suggesting that selection was driven by pathogens. However, we did not observe departures from Hardy–Weinberg equilibrium, an indicator of strong ongoing selection. Neither have we found a significant association between DRB3 alleles or genotypes and susceptibility to posthitis. Alleles conferring resistance to males may have been lost during the extreme bottleneck the species had undergone.

Keywords: *Bison bonasus*, bottleneck, conservation, MHC class II, posthitis

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Introduction

Infections, also those transmitted from domesticated animals, may significantly increase the risk of extinction of endangered species (Meagher 1999; Work *et al*. 2000; Hedrick *et al*. 2001; Haydon *et al*. 2002; van de Bildt *et al*. 2002; Naugle *et al*. 2004). In particular, depletion of variation at the major histocompatibility complex (MHC) genes is thought to compromise the ability of populations to respond to pathogen assault (O'Brien & Evermann 1988; Hughes 1991; Hedrick 2001).

MHC genes code for peptides that present antigens to lymphocytes, thus initiating the adaptive immune response. This function seems to be the reason for high diversity of MHC genes, the most polymorphic genes described in vertebrates (Garrigan & Hedrick 2003). Fast-evolving parasites may adapt to the most common host genotype and escape presentation of their antigens to the adaptive immune system of the host. Rare allelic variants of MHC genes, to which parasites are unlikely to adapt, would thus be favoured by negatively frequency-dependent selection (Snell 1968; Borghans *et al*. 2004). This would result both in the preservation of allelic lineages over long evolutionary timescales, and in the spread of novel alleles that arise via mutation or microrecombination. Positions in MHC sequences coding for residues involved in binding antigens consistently show an excess of nonsynonymous over synonymous substitutions, which is the hallmark of positive selection (Garrigan & Hedrick 2003; Sommer 2005). There is also some evidence that rare alleles confer resistance to parasites (Paterson *et al*. 1998; Trachtenberg *et al*. 2003; Froeschke & Sommer 2005). Additionally, heterozygote advantage in resistance to parasites can contribute to polymorphism at MHC loci, because heterozygotes at MHC should be able to present a broader range of antigens

Correspondence: W. Babik, Fax: +49 345 55 85 329; E-mail: wieslaw.babik@ufz.de

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(Doherty & Zinkernagel 1975). This hypothesis was supported by recent empirical work (Thursz *et al*. 1997; Carrington 1999; Penn *et al*. 2002).

Due to the strong action of genetic drift during population bottlenecks, rare MHC alleles, even advantageous, may be lost, thus compromising the ability of a population to adapt to fast-evolving parasites. Furthermore, the evolution of the ability of a parasite to invade the host would be facilitated by the presence of only a limited number of host genotypes. It has been suggested that high susceptibility of cheetah and other bottlenecked species to diseases results from the loss of MHC variation (O'Brien & Evermann 1988). The importance of MHC variation, and strong selection for its maintenance, has also been implicated in a recent report on an endangered island fox which retained some MHC variation in spite of the virtual lack of neutral variation (Aguilar *et al*. 2004 but see Hedrick 2004). Nonetheless, direct demonstration of an association between MHC diversity and population-wide or species-wide susceptibility to pathogens, in spite of the popularity of the idea, remains elusive. On the other hand, some studies cast doubt on the importance of MHC for species viability. The susceptibility of bighorn sheep to epidemics from pathogens transmitted from domestic animals could not be attributed to low MHC diversity, as 21 alleles were found in this species (Gutierrez-Espeleta *et al*. 2001). Furthermore, some species, such as elk, roe deer (Mikko *et al*. 1999) or beaver (Ellegren *et al*. 1993; Babik *et al*. 2005) expand their range and experience fast demographic growth in spite of very low or even a lack of MHC variation. Clearly, there is a need for more data on MHC diversity and its association with population viability before it is implemented in conservation programmes.

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 two populations remained. The lowland bison (*Bison bonasus bonasus*) within Białowieża forest declined rapidly from 785 individuals in 1915 to extinction after World War I (April 1919), while a population of *Bison bonasus 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; 25 females) European bison with documented pedigrees, originating from 12 ancestors (Slatis 1960).

After a period of intensive captive breeding and a series of re-establishments, 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 line (or Białowieża line). This line originates from only 7 founders (4 males; 3 females) and represents pure animals of the *B. b. bonasus* subspecies. 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 2004). The largest free-ranging population (*c.* 710 individuals); occurs in the Białowieża Forest in Poland and Belarus. The Polish part of the forest is inhabited by about 400 bison (Z.A. Krasiński, personal communication).

The survival of the species has been recently threatened by the spread of posthitis, a disease affecting the reproductive capacity of bulls. The disease affects the male reproductive organs and is manifested by the inflammation of the prepuce and penis, leading to diphtheroid-necrotic lesions. The disease was detected at the beginning of the 1980s in Białowieża Forest (Piusiński *et al*. 1997; Jakob *et al*. 2000), although similar symptoms had been reported earlier in Russia and the Ukraine (see Pucek 2004). The disease was also sporadically observed in other regions of Poland, such as Gołuchów, Borecka Forest, and Bieszczady. At the end of the 1990s, similar symptoms were observed in five young European bison from Bayerischer Wald National Park, Germany (Wolf *et al*. 2000). Despite many years of study, its pathogenesis has not yet been fully elucidated. Recently, two bacteria species were isolated from the prepuce of bulls suffering from posthistis (Lehnen *et al*. 2006). It has been hypothesized that the loss of genetic variation at MHC is a factor contributing to the spread of this disease (Udina & Shaikhaev 1998).

Variation of MHC class II in European bison was previously explored for the DRB3 locus using restriction fragment length polymorphism (RFLP) (Udina & Shaikhaev 1998; Łopieńska *et al*. 2003). DRB3 is the most polymorphic class II gene in cattle, and its orthologues show substantial polymorphism in other ruminants as well (Mikko *et al*. 1997). Due to its high variation and close linkage with DQ loci (Lewin *et al*. 1999; Traul *et al*. 2005), DRB3 diversity should be representative of the diversity of the entire class IIa region. RFLP analysis in European bison revealed a small number of restriction patterns (Udina & Shaikhaev 1998; Łopieńska *et al*. 2003), but more variation could possibly be detected through sequencing of larger samples. Here, we investigate sequence variation of MHC DRB3 exon 2 on a large sample from a free-ranging population in Białowieża. Using the sequences and genotype data, we test for past and present selection acting on DRB3. We also investigate whether susceptibility to posthitis is associated with MHC DRB3 genotype.

Methods

Samples

We analysed 172 individuals culled by the National Park staff in Białowieża Primeval Forest, northeastern Poland,

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between 1992 and 2006. Sixty-nine males were culled because they were diagnosed with posthitis on the basis of malformations of their genital region. The presence of posthitis was confirmed post mortem by an expert veterinarian. Twenty-five animals were culled because they suffered injuries (e.g. broken legs, horns). The remaining animals ($n = 78$) were culled 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. Males from the last two groups constituted the unaffected sample in our analyses. The average age of males diagnosed with posthitis (affected males) was 41.1 months (range: 5–216, $n = 69$), unaffected males 37.2 months (range: 3–228, $n = 63$), and females 78.4 months (range: 4–312, $n = 40$). The proportion of affected males did not differ significantly between years of sampling ($\chi^2 = 19.43$, d.f. = 13, $P = 0.111$).

Laboratory procedures

DNA was extracted using the Chelex method (Walsh *et al*. 1991) from liver, heart or kidney tissue. We used primers HL030 (5′-ATCCTCTCTCTGCAGCACATTTCC-3′) and HL032 (5′-TCGCCGCTGCACAGTGAAACTCTC-3′) (Van Eijk *et al*. 1992; Udina & Shaikhaev 1998) to amplify a 236-bp (excluding primers) fragment of the 2nd exon of the DRB3 MHC gene in the European bison. Amplifications were carried out in 5-μL volumes; the reaction mixtures consisted of: 2.5 μL of HotStar*Taq* Master Mix Kit (QIAGEN), 10 pmol of each primer 5′-labelled with 6-FAM (HL030) and HEX (HL032) and 25–100 ng of genomic DNA. The polymerase chain reaction (PCR) cycling scheme was as follows: 95 °C for 15 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final elongation step at 72 °C for 3 min. The single strand conformational polymorphism (SSCP) technique was used for genotyping. The PCR product was diluted 50 times, 1.2 μL of the diluted product was mixed with 0.4 μL of GeneScan ROX-400HD marker (ABI), 0.75 μL of 0.3 M NaOH and 12.65 μL formamide, denatured at 95 °C for 5 min, cooled on ice for 2 min and separated on an ABI PRISM 3100 Genetic Analyser. We used 5% GeneScan nondenaturing polymer (ABI) with 10% (w/v) glycerol and 1× TBE running buffer with 10% (w/v) glycerol. Samples were injected for 22 s, electrophoresis was carried out at 15 kV and 20 °C. GENEMAPPER 3.5 software (ABI) was used for size calling and allele scoring.

Multiple (at least three) individuals representing each SSCP pattern–putative genotype, were directly sequenced. For sequencing, PCR was carried out as described above, but in 20-μL total reaction volume and using nonlabelled primers. PCR products were purified with the Clean-Up kit (A&A Biotechnology) and sequenced on both strands with the BigDye 3.1 sequencing kit (ABI). Sequencing reaction products were purified with the ExTerminator kit (A&A Biotechnology) and separated on an ABI PRISM 3100 Genetic Analyser. A subset of sequenced heterozygotes were cloned using the pGEM-T Easy vector system (Promega) to determine the sequence of individual alleles. Recombinant clones were detected by blue/white screening; plasmid DNA minipreps were prepared with the Plasmid Mini Kit (A&A Biotechnology). Multiple clones (6.4 per individual on average) containing inserts were sequenced from each putative heterozygote using the T7 or SP6 primer. Sequences were checked and aligned in SEQSCAPE 2.1 (ABI). Clones with recombinant inserts or substitutions, which were artefacts of PCR or cloning, were identified by comparison with sequences from directly sequenced PCR products. All such clones could be easily identified and were excluded from further analyses. New alleles were named in accordance with the guidelines established for naming bovine (BoLA) class II alleles (Davies *et al*. 1997).

To confirm that the assayed locus is expressed in the European bison, mRNA was obtained from spleens of two males (2- and 3-year-old bulls) culled in February 2006. The samples of the spleen were preserved in RNAlater stabilization reagent (QIAGEN). RNA was extracted with the RNeasy kit (QIAGEN) from about 30 mg of the homogenized tissue sample. Complementary DNA was obtained using Omniscript Reverse Transcriptase (QIAGEN) in 20-μL reactions containing 6-μL template RNA, 2 μL Oligo(dT)_{12-18} primer (0.5 μg/mL, Invitrogen), 1 μL RNase inhibitor (10 U/μL) and 1 μL RT and 6 μL of RNase free water. The reaction was incubated at 37 °C for 60 min. Primers for amplification of mRNA were designed on the basis of conservative regions of the cattle and American bison mRNA sequences. The forward primer *born1L* (5′-AGGGAGATCCAACCACATT-3′) spans the boundary between exons 1 and 2, and the reverse primer *born1R2* (5′-GTCACTGTAGGCTCCACTCG-3′) is at exon 3, next to the boundary with exon 2. PCR products were sequenced as described above.

Population genetic and phylogenetic analyses

We tested conformance of the allele frequencies with Hardy–Weinberg expectations, both within each of the three groups (females, affected and unaffected males) and overall, using the complete enumeration algorithm of Louis & Dempster (1987) as implemented in GENEPOP 3.5 (Raymond & Rousset 1995b). Tests addressing specific hypotheses of heterozygote excess and deficit, more powerful than the overall probability test (Rousset & Raymond 1995), were performed. Differences in allele and genotype frequencies between groups were assessed with Markov chain Monte Carlo approximations of Fisher exact tests (Raymond & Rousset 1995a) in GENEPOP. The sequential Bonferroni procedure was applied where appropriate to keep the Type I error level at $\alpha \leq 0.05$ (Rice 1989).

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In addition to comparing allele and genotype frequencies between sexes and between affected and unaffected males, we carried out a logit analysis that allowed us to control for age of the sampled individuals. In the generalized linear model, health status was a dependent variable with a binomial error distribution, the genotype was a categorical factor, and male age was a continuous predictor. Heterozygosity, or the presence or absence of a given allele, were also used as predictors in a similar framework. In models with genotype as a predictor, only the five frequent genotypes were entered into the model. Likewise, only the three frequent alleles were considered in the allele presence/absence model.

The average pairwise nucleotide distances (Kimura 2-parameter model, K2P), Poisson-corrected amino acid distances as well as the average rates of synonymous (d_S) and nonsynonymous (d_N) substitutions per site, using the Nei & Gojobori (1986) method with the Jukes-Cantor correction for multiple substitutions, were computed in MEGA3 (Kumar *et al*. 2004). Standard errors of the estimates were obtained through 1000 bootstrap replicates.

We tested if positive selection shaped the evolution of the assayed fragment of the DRB3 second exon using two approaches. First, the one-tailed *Z*-test, as implemented in MEGA3, compared the rates of synonymous vs. nonsynonymous substitutions at all amino acid positions, antigen-binding sites (ABS) and non-ABS. The location of the putative ABS and non-ABS was inferred from the human MHC II molecule structure (Brown *et al*. 1993). Second, we tested for positive selection by comparing likelihoods of three codon-based models of sequence evolution available in PAML 3.15 (Yang 1997): (i) M0: one ω (dN/dS) ratio (ii) M1a: nearly neutral, p_0 sites under purifying selection ($\omega_0 < 1$) and the rest neutral ($\omega_1 = 1$) (iii) M2a positive selection, p_0 sites under purifying selection ($\omega_0 < 1$), p_1 neutral ($\omega_1 = 1$) and the rest under positive selection ($\omega_2 > 1$). Models M7 & M8, extensions of M1a and M2a that include variation in the ratio of d_N/d_S according to the beta distribution did not explain the data better and therefore the results for these models are not presented. The best-fitting model was chosen on the basis of the lowest value of the Akaike Information Criterion (AIC, Posada & Buckley 2004; Sullivan & Joyce 2005). Positively selected codons were identified through the Bayes empirical Bayes procedure (Zhang *et al*. 2005).

To elucidate the position of the European bison DRB3 alleles among the alleles from other bovid species and to test if the *trans*-specific mode of sequence evolution applies to the contemporary European bison alleles we constructed phylogenetic trees including all the European bison alleles and the representative collection of other bovid DRB3 alleles. Human DRB sequence was included as an outgroup to root the trees. The best-fitting model of sequence evolution was chosen using MODELTEST 3.5. Both the likelihood ratio test and AIC, supported the best fit of the Felsenstein (1981) model with a nonzero proportion of invariable sites and gamma-distributed rate variation (F81 + I + Γ) with the following parameter values: nucleotide frequencies 0.216 (A) 0.234 (C) 0.345(G) 0.205 (T), proportion of invariable sites I = 0.484 and gamma shape parameter $\alpha = 0.479$. The neighbour-joining tree was constructed from the matrix of distances computed according to the model. The robustness of the obtained tree topology was tested with 1000 bootstrap replicates. Another tree was constructed under the Bayesian approach with MRBAYES 3.1 (Ronquist & Huelsenbeck 2003). The likelihood settings corresponded to the F81 + I + Γ model with the parameter values estimated from the data; priors were set to default values. Four Metropolis-coupled Markov chain Monte Carlo (three of them 'heated', temperature = 0.20) were run for $1 * 10^6$ generations and sampled every 1000 generations. The first 10% of trees were discarded as burn-in, resulting in 900 sampled trees. To calculate the posterior probability of each bipartition, the majority-rule consensus tree was computed from these 900 sampled trees.

Results

We detected four alleles in the Białowieża population of European bison, two of which have already been reported for the species (Tables 1 and 2). Sequences of new alleles were deposited in GenBank (Accession nos DQ785799 and DQ785800). Analysis of PCR products obtained from spleen mRNA shows that the locus is expressed: one assayed individual had alleles *Bibo-DRB3*0101* and *Bibo-DRB3*0201*, and the other was a homozygote **0201/*0201*.

Allele frequencies in three groups: females, affected and unaffected males, as well as the overall values, are given in Table 1. We did not detect departures from Hardy–Weinberg equilibrium neither overall nor within the groups, with the exception of a marginally significant heterozygote deficit ($P = 0.040$) in the group of unaffected males, insignificant after Bonferroni correction. We did not detect significant differentiation in allele or genotype frequencies among the three groups, neither overall nor in any of the pairwise comparisons. The generalized linear model with age and alleles as predictors showed that neither significantly affected the incidence of posthitis. Similar analyses with genotype or heterozygosity as predictors also failed to show significant results (heterozygosity: $\chi^2 = 0.36$, d.f. = 1, $P = 0.54$; genotype: $\chi^2 = 3.90$, d.f. = 5, $P = 0.56$).

Pairwise K2P nucleotide distances ranged from 7.6% to 10.6%, Poisson-corrected amino-acid distances ranged between 15.2% and 21.7%. Synonymous and nonsynonymous distances computed according to the Nei-Gojobori method ranged from 0.9% to 6.7% and from 9.8% to 12.0%, respectively (Table 3). One of the alleles (*Bibo-DRB3*0101*) has a deletion of three nucleotides in codon 57 of the

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Table 1 MHC II DRB3 allele frequencies in the Białowieża population of *Bison bonasus*. The overall frequencies are given as well as those in three groups: females, males with diagnosed posthitis (p+) and males free of posthitis (p–)

	N individuals	*Bibo-DRB3*0101*	*Bibo-DRB3*0201*	*Bibo-DRB3*0301*	*Bibo-DRB3*0401*
Females	40	0.312	0.268	0.398	0.022
Males p+	69	0.325	0.300	0.350	0.025
Males p–	63	0.397	0.254	0.333	0.016
Overall	172	0.346	0.270	0.363	0.020

Table 2 Amino acid alignment of four *Bison bonasus* MHC II DRB3 alleles. Shaded columns represent putative antigen binding sites (ABS) (Brown *et al*. 1993); sign '–' denotes codon deletion; signs 'x', '*' and '**' denote amino-acid positions under positive selection with posterior probability > 50, 95 and 99%, respectively, as identified by the Bayes empirical Bayes procedure (Zhang *et al*. 2005)

```
                      10        20        30        40        50        60        70
                ....|....|....|....|....|....|....|....|....|....|....|....|....|...
                x x x         x x   x    xx                xx xx      xx  xx  x  xx      xx
                                    *                     * *       *  **  *  **       *
                                    *                     * *          **  *  *
Bibo-DRB3*0101  EYRKSECHFFNGTERVRFLERYFYNGEEFVRFDSDWGEYRAVTELGRPDAKYWNSQKDLLERKRANVDTYCRHNYGVG
Bibo-DRB3*0201  ..............Y.D...H....Y.................QV.E.....EI..Q.....A.......GV
Bibo-DRB3*0301  Q.H.G............D.......TL.................S.EH....-EI...A..A..........V
Bibo-DRB3*0401  ..H............D.......R.................R.....EI..ER..E..RV.......
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Table 3 Pairwise divergence between four European bison MHC II DRB3 alleles. (A) below diagonal the average pairwise nucleotide distances (Kimura 2-parameter model, K2P), above diagonal Poisson-corrected amino acid distances; (B) below diagonal synonymous (d_S) and above diagonal nonsynonymous (d_N) substitutions per site, using the Nei–Gojobori method with the Jukes–Cantor correction for multiple substitutions. All values are given as percentages

(A) Nucleotide and amino acid distances

	**0101*	**0201*	**0301*	**0401*
*Bibo-DRB3*0101*		18.2	20.1	15.2
*Bibo-DRB3*0201*	7.6		21.7	19.8
*Bibo-DRB3*0301*	10.6	9.1		18.5
*Bibo-DRB3*0401*	8.5	9.5	9.6	

(B) Synonymous and nonsynonymous distances

	**0101*	**0201*	**0301*	**0401*
*Bibo-DRB3*0101*		8.2	12.0	9.8
*Bibo-DRB3*0201*	5.8		12.0	11.7
*Bibo-DRB3*0301*	6.7	0.9		12.0
*Bibo-DRB3*0401*	4.8	2.9	2.8	

assayed fragment (Table 2), representing codon 65 of the 2nd exon of the DRB3 gene.

The rate of nonsynonymous substitutions was significantly higher than the rate of synonymous substitutions when all sites were included in the analysis (*Z*-test of positive selection, $P = 0.006$) and when only ABS sites were included (*Z*-test, $P = 0.002$). The rate of nonsynonymous substitutions was not elevated at non-ABS sites (*Z*-test, $P = 0.199$).

The model of codon evolution assuming that a fraction of codons has been affected by positive selection fit the data substantially better than the uniform ω and nearly neutral models (Table 4). Of 10 codons identified by the Bayes empirical Bayes procedure as positively selected (posterior probability > 95%), six were at putative ABS. This relative excess of selected codons at ABS was significant ($\chi^2 = 7.37$, d.f. = 1, $P = 0.007$). When codons with pp of being under positive selection > 50% were included, as many as 14 of 22 these codons were located at ABS ($\chi^2 = 25.54$, d.f. = 1, $P < 10^{-6}$) (Table 2).

The phylogenetic tree of representative bovine DRB3 alleles is generally poorly resolved and the majority of branches lack substantial support (Fig. 1). However, each European bison allele is most closely related to different bison or cattle alleles. The *Bibo-DRB3*0401* allele is identical to the American bison *Bibi-DRB3*0602* allele.

Discussion

We have found four DRB3 alleles in the European bison (*Bison bonasus*) population of Białowieża. This number is small compared to DRB diversity reported for domestic cattle (105 alleles, BoLA Nomencalture Committee), American bison (*Bison bison*, 15 alleles, Traul *et al*. 2005) and other ruminants such as red deer, goat or sheep (Mikko *et al*. 1999), apparently reflecting an extreme bottleneck at the beginning of the 20th century. Other ruminant species that

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Table 4 Evaluation of the goodness of fit for different models of codon evolution and estimated parameter values

Model	ln L	δAIC	parameters
M0—one ω	−500.98	36.05	$\omega = 1.494$
M1a—nearly neutral ($\omega_0 < 1$, $\omega_1 = 1$)	−492.09	20.27	$p_0 = 0.589$, $p_1 = 0.411$
M2a—positive selection ($\omega_0 < 1$, $\omega_1 = 1$, $\omega_2 > 1$)	−479.95	best	$p_0 = 0.841$, $p_1 = 0.000$, $p_2 = 0.159$

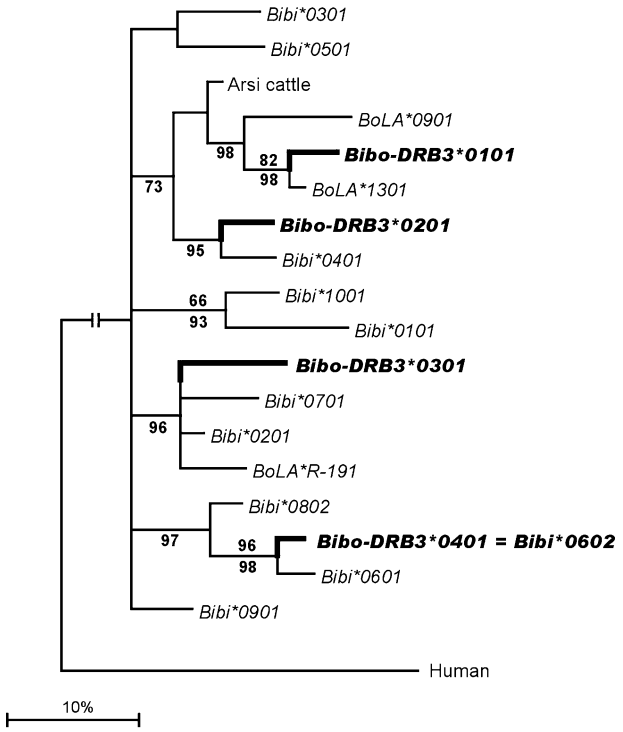

Fig. 1 Phylogenetic relationships of the European bison MHC II DRB3 alleles (bold) with a representative set of other bovid alleles. *Bibi, Bison bison* alleles; *BoLA, Bos taurus;* Arsi, *Bos primigenius* Arsi bred. Note that the *Bibi-DRB3*0602* is identical to the European bison Bibo-*DRB3*0401* allele. Fifty percent majority-rule tree from the Bayesian analysis is shown. Bootstrap values above 50% are shown above respective branches, Bayesian posterior probabilities above 70% are shown below respective branches. The tree was rooted using human DRB sequence.

had undergone population bottlenecks, e.g. moose or roe deer (Mikko *et al*. 1999), also show limited MHC DRB diversity. No MHC variation was found in musk ox and fallow deer, species that show little variation overall (Mikko *et al*. 1999).

It has been suggested that selection may retain MHC diversity even when population bottlenecks lead to the loss of genetic variation elsewhere in the genome, exemplified by an endangered island fox which retained some MHC variation in spite of the virtual lack of neutral variation (Aguilar *et al*. 2004). It is therefore worth considering whether the number of alleles that have been maintained in the European bison is larger than expected under the assumption of neutrality. The census sizes since the beginning of 19th century report a few hundred individuals, with the lowest number (200 individuals) present at the beginning of the 19th century (Krasińska & Krasiński 2004). This is akin to the bottleneck in the American bison (Traul *et al*. 2005), and thus it can be assumed that the number of alleles present in the European bison prior to the recent bottleneck was comparable to that found at present in the American bison. Under the assumption of neutrality, the number of alleles retained following a single generation bottleneck is $A = n - \Sigma_{i=1\ldots n}(1 - p_i)^{2N}$, where n is the number of alleles before the bottleneck and p_i is the frequency of the ith allele (Frankham *et al*. 2002). A pedigree analysis showed that N_e of the Białowieża population at the recent bottleneck was seven individuals (Olech 1989, 1999). Assuming 15 alleles with equal frequencies before the bottleneck, an average generation time of 10 years, and population sizes after the bottleneck based on available estimates (from 7 adult individuals in 1929 to 540 in 1999 with a 0.52 proportion of adults, Krasińska & Krasiński 2004), we obtained a rough estimate of 7.4 alleles expected to be retained under neutrality. Because of the strong effect of the bottleneck, a higher number of alleles assumed prior to the bottleneck does not substantially change the estimate, for example, 8.5 alleles are expected if there were 100 alleles prior to the bottleneck. Thus, we find no indication that selection has lead to the retention of a higher number of alleles than expected under neutrality. This inference is confirmed by the data from neutral markers. Luenser *et al*. (2005) reported an average of 2.3 alleleles/locus at 18 microsatellite loci in a sample of 38 individuals, but a recent survey (M. Tokarska and J.M. Wójcik, unpublished data) of a larger sample (n = 178 individuals, 21 loci) found 7.8 alleles per locus on average. Thus, the number of alleles at presumably neutral microsatellite markers is higher than the number of MHC alleles.

The alleles retained in the European bison are highly divergent, with the pairwise Poisson-corrected amino-acid distances ranging from 15.2% to 21.7%. The variation was concentrated in ABS sites, suggesting that alleles with different antigen-binding properties have been maintained by selection. This inference was further supported by an excess of nonsynonymous substitutions in ABS. Thus, as in a number of other species investigated so far (rewieved in

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Sommer 2005; Piertney & Oliver 2006), MHC diversity seems to be shaped and maintained by positive selection. Moreover, high divergence between alleles suggests that each represents an old allelic lineage that has been retained despite the bottleneck. Interestingly, in cases when only a few MHC II alleles are retained in species known to have undergone severe bottlenecks, these alleles were consistently highly divergent (Hedrick *et al*. 2000, 2001; Van der Walt *et al*. 2001; Sommer 2003; Babik *et al*. 2005). This pattern is in line with the mechanism of divergent allele advantage (Wakeland *et al*. 1990; Richman *et al*. 2001), posing that individuals expressing divergent alleles, coding for dissimilar MHC II molecules, have a selective advantage because they are able to trigger an immune response against a broader spectrum of antigens.

The lack of departures from Hardy–Weinberg equilibrium, both overall and within three groups implicates a lack of strong selection on MHC II genes. However, strong selection affecting viability would be manifested in departures from Hardy–Weinberg equilibrium detectable only with samples sizes of the order of hundreds (e.g. Apanius *et al*. 1997). Thus, the lack of detectable contemporary selection reported in several other large surveys (Gutierrez-Espeleta *et al*. 2001; Landry & Bernatchez 2001; Aguilar *et al*. 2004; Seddon & Ellegren 2004) is not conclusive and may indicate that either selection is too weak to be detectable in current populations with data typically available, or that selection varies in space and time.

Balancing selection tends to maintain more even allele frequencies as compared to neutral expectations (Ewens 1972), which forms the basis for tests detecting selection on an intermediate timescale (Watterson 1978; Slatkin 1994). However, the critical assumption of these tests, the mutation–drift equilibrium, or, in other words, a long-term stable effective population size, is violated in the European bison, making the tests unwarranted.

It has been suggested that novel pathogens threaten the survival of species whose MHC variation was depleted due to bottlenecks (O'Brien & Everman 1988). This is in contrast to species with high diversity, in which the novel antigen is more likely to be recognized by one or more MHC variants present in the population. We found no significant association between MHC alleles or genotypes and susceptibility to posthitis, and thus it is possible that the alleles with a binding motif capable of presenting antigens produced by an infectious agent causing posthitis were lost in the European bison. However, we cannot exclude the possibility of a weak association between DRB3 and posthitis. With our sample size, we should have been able to detect a heterozygote advantage of the order of 15–20% with 90% power (Zar 1984). Such high selection coefficients are not unusual in some populations (e.g. Sauerman *et al*. 2001; Lohm *et al*. 2002), but more often selection coefficients are lower than 10% (reviewed in Edwards & Hedrick 1998; Bernatchez & Landry 2003). Experimental infestations (e.g. Arkush *et al*. 2002) can be used in the future in order to increase the power of the tests as well as to contribute to an understanding of the aetiology of the disease.

The close phylogenetic relationship of the bacteria found in the prepuce of affected bulls to pathogenic *Arcanobacterium pyogenes* (Lehnen *et al*. 2006) suggests MHC class II is likely to be involved in the immune response; however, the causal relationship has yet to be established. A study of MHC class I would be necessary if the disease turns out to be caused by an intracellular pathogen.

Phylogenetic analyses indicated that European bison alleles were closer to American bison or cattle alleles than to each other. Such *trans*-species MHC polymorphism has been reported for a number of species (reviewed in Piertney & Oliver 2006) and is thought to result from a long-term maintenance of allelic lineages by balancing selection (Klein 1987; Garrigan & Hedrick 2003). This explanation seems especially plausible for the recently diverged *Bos* and *Bison* (about 1 million years; Bradley *et al*. 1996). In spite of the morphological similarity of American and European bison, analysis of mitochondrial sequences and amplified fragment length polymorphism (AFLP) profiles groups cattle (*Bos taurus* and *Bos indicus*) in one clade with the European bison, whereas the American bison represents a separate clade that includes yak (Hassanin & Ropiquet 2004; Verkaar *et al*. 2004). However, the analysis of Y-chromosomal DNA segments yields a phylogeny grouping both bison species close together (Verkaar *et al*. 2004). The explanation of this incongruence advocated by Verkaar *et al*. (2004) is the hybrid origin of the European bison, with American bison bulls introgressing into other species, which would have led to the formation of hybrid species, the European bison. The discrepancy between phylogenies derived from different markers may also reflect the process of lineage sorting (Verkaar *et al*. 2004). Due to their potential for adaptive evolution and frequent persistence of allelic lineages through multiple speciation events, MHC genes probably will not resolve this discussion, as they are poor indicators of phylogenetic relationships. However, the sequence of the rarest allele we have found in European bison was identical to an allele reported for the American bison. This suggests recent common ancestry of the two species or recent introgression.

One of the common European bison alleles, *Bibo-DRB3*0301*, has a deletion of codon 65 (codon 57 in the assayed fragment, Table 2), representing an ABS. This, however, is not a characteristic unique to the bison, as there is a group of common cattle alleles also harbouring this deletion. A postulated independent origin of a similar allele in the roe deer may suggest its adaptive significance (Mikko *et al*. 1997).

In conclusion, we have found a limited diversity of MHC DRB3 in the European bison, a species that has undergone

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a recent, extreme population bottleneck. Positive selection has shaped the evolution of European bison DRB3 in the past, and four divergent alleles were retained despite the extreme bottleneck. We have not found evidence supporting strong selection acting in a contemporary population. Neither have we found a significant association between DRB3 genotype and the occurrence of posthitis, a disease posing a new threat to the survival of the species. However, it is possible that the bottleneck led to the loss of alleles that could potentially give resistance to the disease.

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J. Radwan is a Professor at the Institute of Nature Conservation, Polish Academy of Sciences and at the Institute of Environmental Sciences, Jagiellonian University. His interests focus on sexual selection, its evolutionary-genetic background and conservation-genetic implications; A. Kawałko is a research assistant and J.M. Wójcik is a Professor at the Mammal Research Institute PAS in Białowieża; their work focuses on genetic diversity in mammals, especially in the European bison; W. Babik uses molecular tools to study evolutionary processes at and below the species level.

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