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Genetic polymorphism in selected gene *loci* in a sample of Białowieża population of European bison (*Bison bonasus*)*

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European bison (*Bison bonasus*) is a protected wild animal living free in woodland regions of Eastern and Southern Poland. The largest population lives in the primeval Białowieża forest in Poland and in Byelorussia (on the other side of the state border), the former belonging to the Białowieża National Park (BNP), Poland. Using DNA samples of 22 animals randomly selected from BNP population, 14 single nucleotide polymorphisms (SNPs) were analysed. Moreover, short tandem repeats (STRs) and insertion/deletion (InDel) polymorphisms were analysed in the growth hormone receptor (*GHR*) and signal transducer and activator of transcription 5A (*STAT5A*) genes. The studied sample of the European bison population appeared mostly monomorphic at the *loci* analysed. However, variable sequences were found within the genes coding for β-lactoglobulin (*LGB*), *GHR*, estrogen receptor α (*ERα*), and *STAT5A*.

KEY WORDS: European bison / gene polymorphism / single nucleotide polymorphism / short tandem repeat

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The European bison (*Bison bonasus*) occupies a unique ecological niche in Poland in that it is a protected wild animal roaming free in the woodland areas of Eastern and Southern Poland. The Polish largest *Bison bonasus* population lives in the Białowieża National Park (BNP). Another numerous population lives in Byelorussia (across the state border). BNP and Byelorussian populations amount to approx. 300 and 240 individuals, respectively.

Conservation of the European bison, especially in Poland, has a long history. The most recent estimate available for the world population of *Bison bonasus* is 3200 individuals (in Poland about 700 animals, including those from BNP). In the early 20th century European bison faced extinction due to the bottleneck phenomenon. The present population of Białowieża European bison was reconstituted from twelve animals and therefore its genetic variation is extremely low. It has been shown that increase in inbreeding of European bison is accompanied by the decrease in reproduction rate, decrease in lifespan, and increase in mortality – Luenser *et al*. [2005] and Krasiński A.K. (www.bpn.com.pl/zubr.htm). Moreover, the animals become extremely prone to parasitic diseases [Kita *et al.* 2003]. Related to the degree of inbreeding is the rate of genetic variation. An earlier study by Gębczynski and Tomaszewska-Guszkiewicz [1987] demonstrated that the variation in *Bison bonasus* was approximately the same as that in American *Bison bison.* Maintaining the genetic diversity within populations is one of the most important measures of reaching the success in the conservation of species.

Up to now known are only the following European bison cDNAs, or genomic sequences: testis specific Y-encoded protein (GenBank AY347592), *DRB* gene for MHC class II antigen (GenBank AJ532828), sex determining factor SRY (GenBank AY079134), prion protein (GenBank AF117328), κ-casein (GenBank AF030325, U10379), growth hormone (GenBank AY671801), mitochondrial cytochrome b (GenBankY1505), and cytochrome oxidase (GenBank AY689198).

The aim of this study was to evaluate the nucleotide sequence polymorphisms in selected gene *loci* in the sample of European bison from BNP population in order to provide new information on the genetic diversity within the species, and to compare sequences of fragments of European bison *STAT5A, GHR,* and *ER*α with the corresponding sequences of domestic cattle.

Materials and methods

Animals and DNA isolation

Blood samples of three European bison males and 19 females were used from the Polish Academy of Sciences Mammal Research Institute, Białowieża. The animals were from 4 months to 18 years old and originated from different parts of the 10 502 ha BNP. Most of them belonged to the free-ranging population. Genomic DNA was isolated from blood leukocytes as described by Kanai *et al*. [1994].

Genotyping

Altogether, eleven *loci* (18 polymorphisms) were genotyped. References, type of polymorphisms, primer sequences, and methods used for polymorphism analysis are given in Table 1. Primers and PCR conditions were the same as previously used for cattle (*Bos taurus*), and in all cases, the primer sequences perfectly matched European bison genomic DNA giving unique PCR products.

Restriction fragment length polymorphism (RFLP)

Fourteen RFLPs were determined using the conditions given below. The PCR reaction (12.5 µl) contained of 50-100 ng genomic DNA, 1.5 mM $MgCl_2$ in PCR buffer, 1.25 µl 1 × Q-Solution, 10 pmol of each primer, 0.25 mM of dNTPs, and 0.25 U of HotStart Taq DNA polymerase (QIAGEN, Germany). Ten µl of PCR products were digested at 37°C for 3 h with 10 U of relevant restriction endonucleases (New England BioLabs, USA) listed in Table 1. The PCR protocols were used, optimum for each primer pair and for amplified DNA fragments. The reactions were performed in MJ Research TETRAD thermal cycler. The resulting fragments were separated in 2% agarose gels using standard procedures. Results were documented with Molecular Imager FX apparatus (BioRad).

DNA sequencing

Sequenced were fragments of European bison genes coding for STAT5A, GHR, and ERα. Overlapping fragments of the *STAT5A* gene, including a promoter fragment, exon 1 and intron 1 were amplified using primers as reported earlier [Flisikowski *et al*. 2005] or given in Table 1. The amplified and sequenced fragment of the *GHR* gene encompassed promoter P1 and exon 1A (primer sequences are shown in Table 1). Seven overlapping fragments of the 5' region of the European bison *ERα* gene were amplified using domestic cattle primers and then sequenced. These fragments were combined to create the 2904-bp sequence. All PCR products were purified with QIAquick® PCR Purification Kit (QIAGEN) and DNA samples were sequenced with the ABI Prism™ BigDye™ Terminator Cycle Sequencing kit, using an ABI377 sequencer (APPLIED BIOSYSTEMS, USA) and Sequence Analyser 2.01 programme.

Microsatellites

Cy5-labelled PCR primers of sequences given by Lucy *et al*. [1998] were used to amplify a fragment of the 5'-flanking region of the domestic cattle *GHR* gene. Amplification was carried out for 35 cycles: 95°C for 20 s, 66°C for 30 s, and 72°C for 40 s. The fluorescent PCR products were separated in 6% denaturing polyacrylamide gels using an ALFexpress DNA Sequencer (AMERSHAM BIOSCIENCES Corp., Piscataway, NJ, USA). The PCR products were analysed after 5 min. denaturation in a 50% formamide solution containing blue dextran. In each lane 1 µl of PCR products was analysed together with the size standard. The genotyping was performed with the AlleleLinks 1.01 software (AMERSHAM BIOSCIENCES). After automated allele

Table 1. Genes analysed and techniques used for the polymorphism detection in European bison genome

Locus	Polymorphic site, position of mutation	Method	Primers	Amplified fragment (bp)	Reference
κ-casein (*CSN3*)	Exon IV, intron IV	RFLP-*Hind*III	F: GAAATCCCTACCATCAATACC R: CCATCTACGCTAGTTTAGATG	273	Kamiński and Figiel [1993]
β-lacto-globulin (*LGB*)	Exon IV, intron IV	RFLP-*Hae*III	F: TGTGCTGGACACCGACTACAAAAAG R: GCTCCCGGTATATGACCACCCTCT	247	Medrano and Aquilar-Cordova [1990]
	Exon IV	RFLP-*Sma*I	F: CTACAAAAAGTACCTGCTCTTCTGCCCG R: GGTATATGACCACCCTCTCCTGG	226	Prinzerberg and Erhardt [1997]
Growth hormone (*GH*)	Exon V	RFLP-*Alu*I	F: CCGTGTCTATGAGAAGC R: GTTCTTGAGCAGCGCGT	428	Lucy *et al*. [1993]
	Intron III	RFLP-*Msp*I	F: AGAATGAGGCCCAGCAGAAATC R: GTCCGTCACTGCGCATGTTT	705	Høj *et al*. [1993]
Growth hormone releasing hormone (*GHRH*)	Intron I	RFLP-*Hae*III	F: TTCCCAAGCCTCTCAGGTAA R: GCGTACCGTGGAATCCTAGT	297	Moody *et al*. [1995]
Growth hormone receptor (*GHR*)	5'-noncoding region; -226, C/T	RFLP-*Stu*I	F: CTGGCGTATGGTCTTTGTCA R: TGGTCTTGCTGCTTTCCTAT	318	Aggrey *et al*. [1999]
	5'-noncoding region; -154, A/G	RFLP-*Nsi*I	F: CTGGCGTATGGTCTTTGTCA R: TGGTCTTGCTGCTTTCCTAT	318	Ge *et al*. [2003]
	Detection of LINE1 element - 5'-noncoding region; promoter P1 and exon 1A	sequencing	F: TGCGTGCACAGCAGCTCAACC R: GAGCGAGGTGGGAGGAGAAGA	990	Maj *et al*. [2005]
	5'-noncoding region; -120, TG repeats	microsatellite	F: Cy5-CTGGCGTATGGTCTTTGTCA R: TGGTCTTGCTGCTTTCCTAT	318	Lucy *et al*. [1998]

Table 1 continued

Locus	Polymorphic site, position of mutation	Method	Primers	Amplified fragment (bp)	Reference
Estrogen receptor α (*ERα*)	5'-noncoding region; -2429, transition A/G	RFLP-*Bgl*I	F: TTTGGTTAACGAGGTGGAG R: TGTGACACAGGTGGTTTTTC	242	Szreder and Zwierzchowski [2004]
Leptin (*LEP*)	Unknown	RFLP-*Sau3A*I	F: GTCACCAGGATCAATGACAT R: AGCCCAGGAATGAAGTCCAA	1820	Pomp *et al*. [1997]
Pit1	Intron V	RFLP-*Hinf*I	F: CAATGAGAAAGTTGGTGC R: TCTGCATTCGAGATGCTC	1355	Moody *et al*. [1995]
Myostatin (*MSTN*)	Exon III, *nt821(del11)*	sequencing	F: TCTAGGAGAGATTTTGGGCTT R: CGTTTTCATAGGAGTATGGGTAG	188	Grobet *et al*. [1997]
Signal transducer and activator of transcrip-tion 5A (*STAT5A*)	5'noncoding region, A/G pos. -258	RFLP-*Sgr*AI	F: TCCTCCTCCCTTTTGACAGA R: CAGCGATTTCCTCCTCAAAG	361	Flisikowski *et al*. [2005]
	5'noncoding region, $CA_{8/9}$ pos. -480	sequencing	F: TGACTGCCAAGGTTCTTTCC R: TCTGTCAAAAGGGAGGAGGA	361	Flisikowski and Zwierzchowski (unpublished)
	Exon VII, C/T, pos. 6853	RFLP-*Ava*I	F: CTGCAGGGCTGTTCTGAGAG R: TGGTACCAGGACTGTAGCACAT	215	Flisikowski *et al*. [2003]
	Intron I, $CCG_{3/4}$	sequencing	F: TCAATACGGCTTTGCCTCTG R: CCACTATCCAGTCCGACAGC	880	Flisikowski and Zwierzchowski (unpublished)
Prolactin (*PRL*)	Exon III	RFLP, *Rsa*I	F: CGAGTCCTTATGAGCTTGATTCTT R: GCCTTCCAGAAGTCGTTTGTTTTC	156	Mitra *et al*. [1995]

calling, individual genotypes were checked manually to identify the peaks before exporting the genotypes to Excel.

Results and discussion

Alignment of the 978-bp sequence of European bison *STAT5A* gene 5' region [GenBank, acc. no AY280368] with the relevant fragment of the *Bos taurus STAT5A* gene (AJ242522) showed 99.3% identity. The sequenced two fragments of *Bison bonasus GHR* gene – 990-bp of the 5'-flanking region and 395-bp encompassing exon 4 and fragments of introns 3 and 4 – were deposited in GenBank under accession nos. AY243962 and AY739711. It was shown that unlike most European domestic cattle, the *Bison bonasus GHR* gene had no LINE-1 element inserted in the 5' non-coding region. In this respect it resembles the *GHR* gene of Zebu (*Bos indicus*) cattle and those few representatives of *Bos taurus* species with no retrotransposon at this location [Maj *et al*. 2005]. Alignment of the European bison *GHR* 5'-flanking sequence with the sequences of the corresponding fragment of the bovine *GHR* gene (GenBank U15731 and AY243962) showed 99.0% and 99.5% identity, respectively [Maj and Zwierzchowski 2006]. The sequence of *Bison bonasus* exon 4 and of the adjacent intron fragments appeared 100% identical with that of the domestic cattle gene.

A 2904-bp sequence of the 5' region of the European bison *ERα* gene was deposited in the GenBank [AY340597]. By analogy to the domestic cattle gene [Szreder and Zwierzchowski 2004] it was assumed that the sequenced fragment included noncoding exons A, B, C, their putative promoters, and a part of the coding exon 1. Alignment with the relevant region of the bovine *ERα* gene (GenBank, AY340597) showed 98.4% identity (47 differing nucleotides out of 2853).

A total of 18 European bison sequences, previously detected as variable in domestic cattle, were analysed within 11 gene *loci*. Fourteen of them were SNPs identified with RFLP, two were InDel polymorphisms – nt821 (del11) in *MSTN*, and LINE1$^{+/-}$ in the *GHR* gene – and three were STR polymorphisms – TG repeat in *GHR* and CA and CCG repeats in *STAT5A*. The full list of the *loci* and polymorphisms studied is given in Table 1.

In this study variable sequences were only found within the European bison genes coding for β-lactoglobulin (*LGB*), estrogen receptor α (*ER*α), growth hormone receptor (*GHR*) and *STAT5A*. Three genotypes were found in the RFLP-*Hae*III site in exon 4 of the β-lactoglobulin gene. In the sample of 22 European bisons 13 were AB heterozygotes, three – AA homozygotes, and six – homozygotes BB. As concerns the estrogen receptor α (*ERα*) gene, two AG heterozygotes and 20 GG homozygotes were found as resulting from the RFLP-*Bgl*I analysis.

In the European bison *GHR* gene the TG microsatellite sequence of variable length was found. Two genotypes and two alleles were identified. Among the 22 *Bison bonasus* individuals, 18 had only the long 19-TG-repeat allele, while four were heterozygotes containing one TG_{19} and one TG_{14} allele.

Two polymorphic tandem repeats (microsatellites) were found in the promoter region and in intron 1 of the European bison *STAT5A* gene. In the promoter (position -258) among 22 animals analysed, 13 were of the $CA_{8/8}$ genotype, seven were $CA_{8/9}$ heterozygotes, and two were $(CA)_{9/9}$ homozygotes. As concerns the CCG_n microsatellite polymorphism in *STAT5A* intron 1, among 22 animals 16 were $CCG_{2/2}$ homozygotes, four were $CCG_{2/3}$ heterozygotes, and two were homozygotes with three CCG repeats ($CCG_{3/3}$).

So far, polymorphism of protein-coding sequences has not been thoroughly studied in European bison. An exception is the polymorphism in κ-casein gene in several populations of the species. In domestic cattle the κ-casein gene exists in A, B, C, and E variants. Sipko *et al.* [1994] reported the predominance of the B allele of κ-casein in the Byelorussian population of European bison and, in particular, in the Białowieża-Caucassus line. The results of our study presented here and obtained for the sample of the Polish European bison population from Białowieża National Park showed the lack of allele A, the most abundant in domestic cattle. Also Burzyńska and Topczewski [1995] found only the BB κ-casein genotype in European bison from Poland. However, sequencing the PCR products revealed some other polymorphisms in the κ–casein gene. The T/C and A/G transitions were detected at positions 184 and 296, respectively. The former does not change the amino acid, but the latter results in the substitution of tyrosine (ACA codon) by alanine (GCA). Moreover, in two animals the deletion of nucleotide A was found at position 419.

Using the PCR-RFLP method, allelic polymorphisms of κ-casein, DRB (exon 2), and DQB (5'- flanking region) genes were studied in different European bison populations by Udina *et al*. [1994, 1995]. Variants A and B of κ-casein (similar to those in domestic cattle) were found with frequencies of 22.4% and 77.6%, respectively. The tendency towards the loss of the allele A was revealed to be a result of high level of inbreeding. Only few polymorphic variants in the DQB and DRB *loci* were found, some of them unique to European bison.

Polymorphisms in the non-coding sequences have been reported in different populations of *Bison bonasus* species. Semenova *et al*. [2000] described the genetic variability in American bison (*Bison bison*), European bison (*Bison bonasus*), and Grey Ukrainian cattle (*Bos taurus*) using DNA fingerprinting techniques with the M13 phage DNA, $(TTAGGG)_4$ synthetic oligonucleotide and three arbitrary primers as hybridization probes. They showed that the genetic variability of minisatellite and telomeric sequences in Grey Ukrainian cattle was higher than those in American bisons and European bisons. The genetic relationsship between European bison individuals appeared much higher than those in American bison or Grey Ukrainian cattle.

The polymorphisms of class II markers – short tandem repeats (STRs) and microsatellites – were studied in the Polish BNP population of European bison by Gralak *et al*. [2004] who identified 40 alleles at 21 microsatellite *loc*i in 22 animals. In the study by Luenser *et al*. [2005] carried out on 38 animals, nine microsatellites out

of 18 were found polymorphic, with the mean number of 2.3 alleles per *locus*. In both papers low level of genetic diversity was reported.

In the present work, studied were the single nucleotide sequence polymorphisms (SNPs) in a sample of 22 *Bison bonasus* individuals from BNP at selected class I marker *loci* (protein coding sequences) with polymorphism known for domestic cattle (*Bos taurus*). Although less polymorphic, and thus less informative then type II markers (microsatellites), SNPs have gained high popularity in genetic studies due to their high accuracy and reproducibility [Vignal *et al*. 2002]. However, variable sequences (SNPs) were only found within the European bison genes coding for β-lactoglobulin (*LGB*) and estrogen receptor α (*ER*α). In the sample of European bison studied in the present work more numerous were animals carrying the RFLP-*Hae*III variant B of *LGB*. Moreover, polymorphic tandem repeats (microsatellites) were found in the promoter region and in intron 1 of the European bison *STAT5A* gene. As concerns the CA_n repeat – the $CA_{9/9}$ genotype, *i.e*. the most frequent in Friesian cattle (89.9%) – appeared the least frequent in European bison (9.09%).

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Polimorfizm genetyczny wybranych *loci* białowieskiej populacji żubra (*Bison bonasus*)

S t r e s z c z e n i e

Na próbie 22 zwierząt losowo wybranych z białowieskiej populacji żubra (*Bison bonasus*) analizowano polimorfizm pojedynczych nukleotydów (SNP) i polimorfizm typu InDel, które w *loci* bydła domowego uznawane są za polimorficzne. Nadto, w genach kodujących receptor hormonu wzrostu (GHR) i czynnik transkrypcyjny STAT5A badano polimorfizm krótkich powtórzeń nukleotydów (STR). W odniesieniu do większości badanych *loci* zwierzęta okazały się monomorficzne. Polimorfizm stwierdzono tylko w genach: β-laktoglobuliny (*LGB*), *GHR*, receptora estrogenu α (*ERα*) i *STAT5A*.