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Phylogeny and genetic variation of the European bison *Bison bonasus* based on mitochondrial DNA D-loop sequences

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Genomic DNA from 14 representative animals of 3 maternal lines of *Bison bonasus* (Linnaeus, 1758) was used for amplification of a 1026-bp fragment of mtDNA D-loop. Analysis of this mitochondrial control region demonstrated only four variable sites in the studied *B. bonasus* population. Nucleotide substitutions in the fragment studied were very unstable, suggesting that intralineage sequence variation can occur in *B. bonasus*. To estimate phylogenetic relationships within the *Bovinae* subfamily mtDNA control region was analysed. The phylogenetic analysis separated two species of *Bison,* and placed *Bison bison* most closely to *Bos grunniens*. The rate sequence divergence of the hypervariable region of the D-loop between *B. bonasus* and *B. bison* was calculated as 78.5% per Myr.

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Introduction

Recent developments in molecular genetics have provided many new and powerful methods valuable for ecologists. Molecular genetic data may be used for quantifying genetic difference or similarity and also can be helpful in the planning of breeding strategy of endanger species. Analysis of mitochondrial DNA (mtDNA) has been commonly used for studies of closely related species because mammalian mitochondrial DNA has the rate of nucleotide substitution five to ten times higher than that of nuclear DNA (Brown *et al.* 1979) and is maternally inherited. The animal mitochondrial genome contains structural genes and one control region, which includes the displacement loop (D-loop). Length variation and nucleotide substitutions in the control region have been found within and between species (Wilkinson and Chapman 1991, Loftus *et al.* 1994, Ishiba *et al.* 1995).

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All living European bisons *Bison bonasus* (Linnaeus, 1758), descend from 12 founder animals, among which those belonging to the lowland line or the Białowieża line stem from only seven founders (4 males and 3 females). This results in a very limited gene pool and high inbreeding within population (Olech 1989). In wild populations it is not possible to obtain pedigree information, so to assess genetic relationships among individuals, genetic markers are necessary. One such marker can be mtDNA. We used analysis of the mitochondrial control region to study genetic structure of the Białowieża *B. bonasus* population with special reference to differences between maternal lines. We report here the nucleotide sequence of the whole mtDNA control region of *B. bonasus* and polymorphic sites for different maternal lines. Genetic relationships in the Bovinae subfamily and rate of sequence divergence between *B. bonasus* and *B. bison* are also discussed.

Material and methods

Sample collection and total genomic DNA preparation

Fresh blood samples were collected from 14 animals of known pedigree from the Białowieża Primeval Forest Breeding Centre. These individuals descended from three maternal lines: Planta line (5 animals), Bilma line (6 animals) and Plavia line (3 animals).

Genomic DNA was isolated from leucocytes by SDS/proteinase K treatment followed by phenol/ /chloroform extraction and ethanol precipitation (Madisen *et al.* 1987). Approximately 20-50 μg DNA were isolated per ml of blood.

PCR primers and reactions

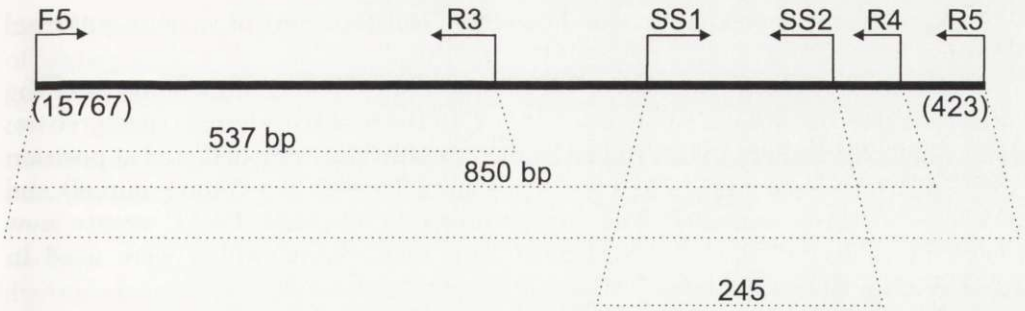
Primers F5 and R5 (see Fig. 1) were designed from the known bovine mtDNA sequence by Ron *et al.* (1993). They cover the conserved sequences of proline tRNA (F5) and phenylalanine tRNA (R5) genes. Each reaction (50 μl) contained 50 ng of DNA, 0.2mM of each dNTP (Amersham), 15 mM $MgCl_2$, 25 pmols of each primer, 2.5 units of Taq DNA polymerase and reaction buffer (Promega). After 30 cycles (94°C for 1 min; 61°C for 1 min; 72°C for 1 min) the presence of the expected 1026 bp product was checked on 1% agarose gel.

PCR cloning and sequencing

The amplified fragment was separated from primers and dNTPs by electrophoresis on a 6% polyacrylamide gel, then excised from the gel and eluted with 0.5 M ammonium acetate at 42°C overnight and precipitated with ethanol. The amplification products from 5 individuals (pedigree no: 7555, 7556, 7842, 7838, 7841) were cloned separately using pMOS*blue* (T-vector Kit, USBiochemical, Ohio, USA). At least three different clones from each individual were sequenced using the Sequenase™ Version 2.0 system (USBiochemical, Ohio, USA) and primers F5 and R5. Two additional primers, R3 and R4, were used to sequence the remaining portions of the fragments. The sequencing strategy and positions of the primers used are shown in Fig. 1. The sequence has been deposited in GenBank under the accession number U34294.

The 1026 bp PCR products from the other 9 individuals were purified by 6% polyacrylamide gel electrophoresis and then directly sequenced from SS1 and SS2 primers using Sequenase™ Version 2.0 system (USBiochemical, Ohio, USA).

The same PCR products were used for restriction analysis: 10ml of the PCR reaction mix was digested with 5 units of *Ssp*I or *Taq*I for 1h at 37°C and 60°C, respectively. Digested DNA was separated in 2% agarose gel in 1× TAE buffer, stained with ethidium bromide and visualised in UV light.



PCR Primers (Ron *et al.* 1993)

F5 (15737) 5' CTG CAG TCT CAC CAT CAA CCC CCA 3'

R5 (423) 5' GGA GTT GGG AGA CTC ATC TTG GCA T 3'

Sequencing primers:

R3 (16274) 5' CTG AAG AAG GAA CCA G 3'

R4 (176) 5' AGT GGT GGT AGA TAT TTT 3'

SS1 (103) 5' CCC GGA GCA TAT ATT GTA GCT GGA C 3'

SS2 (348) 5' GGG GTC GCG CTT ATA TAT TGA C 3'

Fig. 1. Primers used for PCR and sequencing of the *Bison bonasus* mtDNA D-loop. The mt sequence location of the first bp of each primer, according to the numbering system of Anderson *et al.* (1982), is given in parenthesis.

Data analysis

Sequence data were analysed using software DNASIS (DNASTAR Inc., Madison, USA). CLUSTAL (Higgins and Sharp 1989) was used for multiple sequences alignment. A phylogenetic tree was constructed by a composite alignment and distance matrix approach based on the neighbor-joining method of Saitou and Nei (1987). Approximately 650 bp fragments of mitochondrial D-loop from following species were used for phylogenetic analysis: *Bos taurus* (GenBank Accession no. L27719), *Bos indicus* (GenBank Accession no. L27733), *Bison bison* (GenBank Accession no. U12955), *Bison bonasus* (individual no. 7555, this work), *Bos gaurus* (GenBank Accession no. AF083371), *Bos grunniens* (GenBank Accession no. AF083355) and *Alces alces* (GenBank Accession no. AF016951).

Results and discussion

Initially we tried using conserved primers (Kocher *et al.* 1989) to amplify the D-loop region of *B. bonasus* mtDNA. As this approach did not succeeded, we used bovine primers, described by Ron *et al.* (1993). The 1026bp PCR products from five animals were cloned into plasmid pMOS*blue* and sequenced. The sequencing was repeated at least three times each, giving the same results. This sequence contains the full copy of the mt-DNA D-loop (890 nucleotides), flanked with tRNA-Pro from the 5' end and with tRNA-Phe from the 3' end. Only four variable sites in the studied fragment were found in contrast to the results obtained for cattle. For example, Ron and co-workers (1993) found 17 substitutions and 1 insertion in the

719 bp mitochondrial control region in selected Holstein cows of various maternal lineages.

Pedigree data and the number of polymorphic sites among individuals from the different maternal lines are shown in Table 1. In the first five animals (pedigree no: 7555, 7556, 7842, 7838, 7841) studied by us, substitutions were detected at position 232 G→ A (three animals), 736 T→ C (two animals), 897 T→ C (one animal) and 957 T→ C (three animals). The substitutions G232A and T957C create new restriction sites for enzymes *Ssp*I and *Taq*I, respectively, which were used in subsequent mt-DNA analysis.

In the PCR products from the remaining 7 individuals substitutions were identified by restriction analysis (substitutions at positions 232 and 957) and by direct sequencing with primers SS1 and SS2 (substitutions at positions 736 and 897). There were no additional variable sites in the sequenced DNA fragments, except for heteroplasmic sequences T/C and T/A at positions 889 and 890, which were found in two females, no. 6629 and 8010, both from the Planta line. The real number of heteroplasmic animals in the studied group may be higher than observed, because we used DNA isolated from leucocytes for amplification. Koehler *et al.* (1991) suggested that heteroplasmy may be maintained in certain organ tissues but not in the developmentally homogeneous population of leucocytes.

There is no apparent correlation between nucleotide substitutions and a particular maternal line of European bison. For example, in the closely maternally related individuals 7662 and 7385 from the Bilma line we have observed different substitutions. This can be due to a rapid fixation of mtDNA sequence variants. In

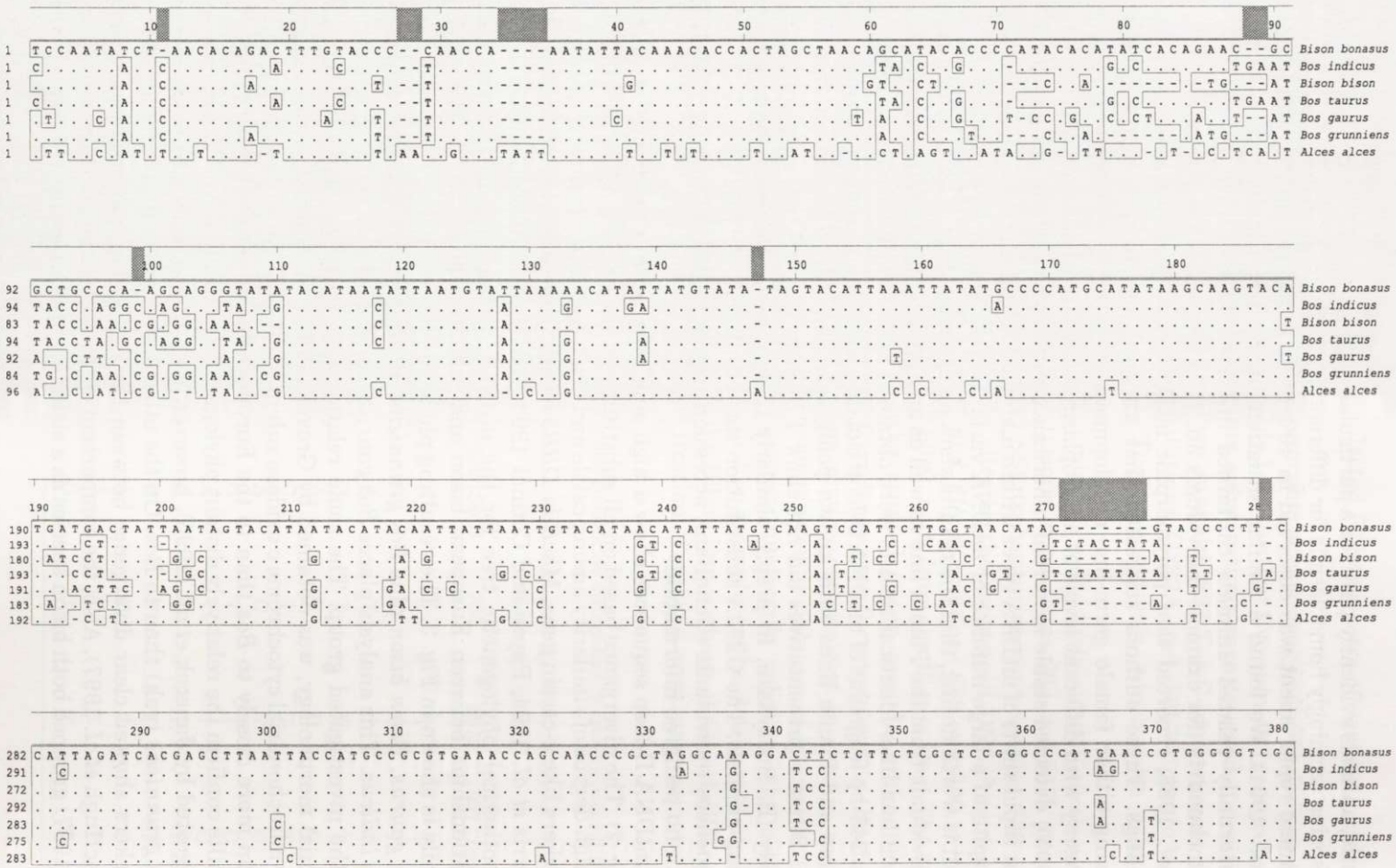
Table 1. Pedigree data of *Bison bonasus* individuals and nucleotide differences. Numbers according to the European Bison Pedigree Book.

Pedigree number	Maternal lineage	Pedigree number of		Polymorphic sites			
		Mother	Father	232 (*Ssp*I)	736	897	957 (*Taq*I)
7842	Planta	5853	4746	A	T	T	C
3042	Planta	1131	980	G	T	C	T
6629	Planta	3979	4736	A	T	T	C
7661	Planta	6603	Unknown	A	T	T	T
8010	Planta	6841	6343	A	T	C	C
7555	Bilma	6125	3967	G	T	T	T
7838	Bilma	6125	3967	A	C	T	C
6957	Bilma	4954	4746	G	T	T	C
7662	Bilma	5349	3967	A	T	T	C
7385	Bilma	5349	3967	A	T	T	T
8083	Bilma	5855	6605	G	T	T	T
7841	Plavia	6127	4746	A	C	C	C
8086	Plavia	7831	7122	A	T	T	T
7556	Plavia	5555	4746	G	T	T	T

cattle, animals evidently pure for a particular leucocyte mtDNA sequence produced offspring seemingly homoplasmic for different leucocyte sequences and mitochondrial genome replacement was observed in 40% of 32 mother-daughter pairs (Koehler *et al.* 1991). We found a similar situation in the investigated group (Table 1): maternally related animals contained different D-loop variants in leucocyte mitochondria (for example individuals no. 7555 and 7838 from Bilma line). Laipis *et al.* 1988, reported that polymorphic mtDNA may partition unequally among siblings. These authors suggested that unequal partitioning might take place during either female germ-line development or in early embryogenesis to yield progeny with different levels of heteroplasmy. On the other hand, Marklund *et al.* (1995), detected stable maternal inheritance in four horse lineages and suggested that rapid shifts of mtDNA types within maternal lineages is not common in horses, in spite of a large number of mtDNA variants observed in this species.

It is worth noting, that the results show an absence of the T736C substitution in individuals from the Planta line as well as an absence of the T897C substitution in the Bilma line. Identical results were obtained after amplification and sequencing of a 245-bp fragment of mtDNA D-loop isolated from two museum specimens of the Bilma and Planta lines with known pedigrees. The D-loop sequences from Plant and Bilma representatives did not show T736C and T897C substitutions, respectively (B. Burzyńska, in prep.), similarly to the results obtained with presently living animals. The G232A substitution was also found in two *B. bonasus* mtDNA sequences individuals (GeneBank accession numbers BBU12953 and BBU12954) with no pedigree information.

mtDNA D-loop sequences show a high similarity within the *Bovinae* subfamily (Fig. 2). The divergence based on all substitutions varied from 5.3 to 13% (Table 2). A high degree of similarity among cattle and bison was also shown for other genetic markers like κ-casein gene, *MhcBibi-DRB3* or SRY gene (Cronin and Cockett 1993, Morris *et al.* 1994, Payen and Cotinot 1994, Burzyńska and Topczewski 1995). A hypothetical phylogenetic tree for the mtDNA control region, showing genetic relationships between European bison and other members of the *Bovinae* subfamily is shown in Fig. 3. The resulting phylogenetic tree has four main branches: *Bos gaurus*, *Bison bison* plus *Bos grunniens*, *Bison bonasus* and *Bos taurus* plus *Bos indicus*. This analysis places *Bos gaurus* (gaur) as the most divergent member of the investigated group. The same relationship, based on cladistic analysis of cranial morphology, was obtained by Groves (1981). In contrast, a comparison of the mitochondrial cytochrome *c* oxidase subunit II (COII) gene placed the American bison more closely to *Bos* than to the European bison (Janecek *et al.* 1995). Our results confirm the relatively distant phylogenetic position of both *Bison* species as suggested by Janecek *et al.* (1995), however, they place American bison closer to *Bos grunniens* (yak) than to *Bos*. On the other hand, an analysis of microsatellite markers showed clear divergence between the *Bos* group and both bison species (MacHugh *et al.* 1997). Also, the comparison of the κ-casein gene fragment (Ward *et al.* 1997) grouped both bison species as a sister group. Generally, the data obtained


Bison bonasus
Bos indicus
Bison bison
Bos taurus
Bos gaurus
Bos grunniens
Alces alces

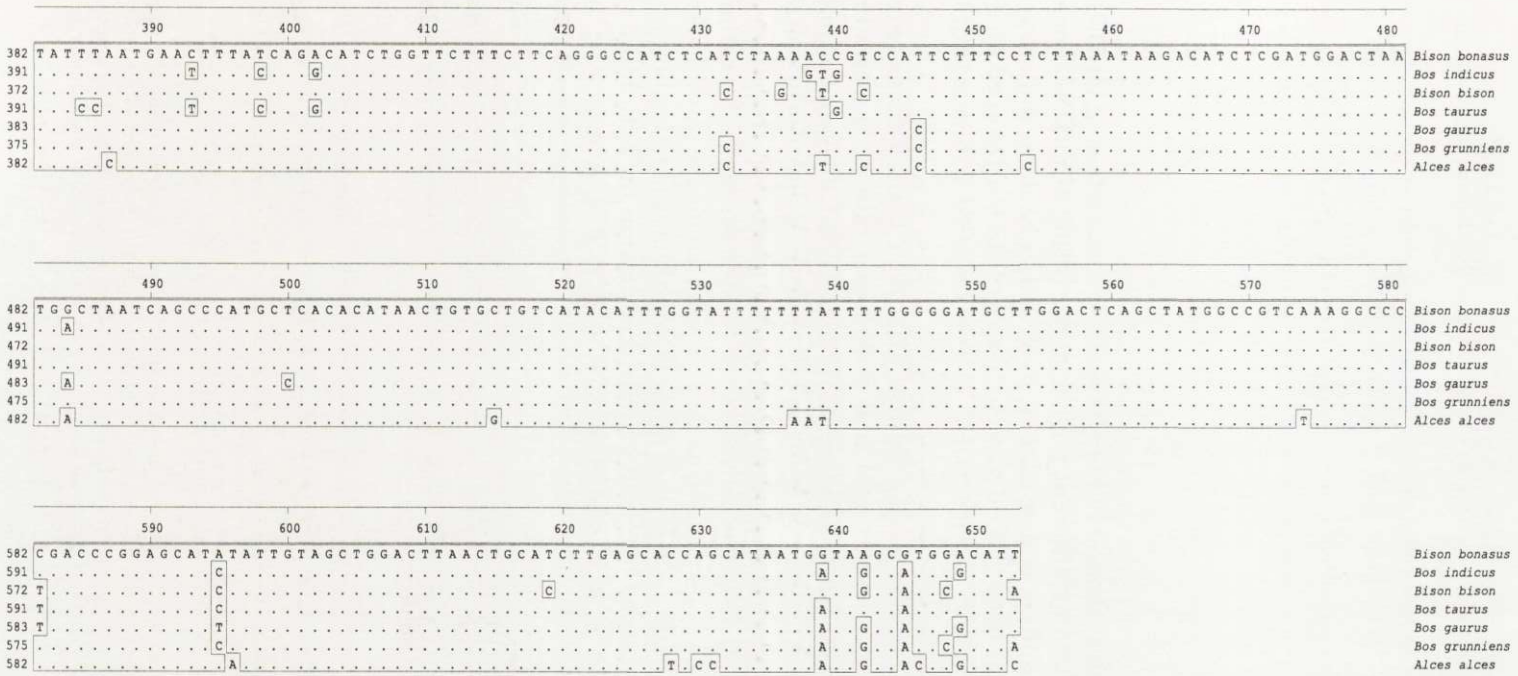


Fig. 2. Alignment of the mtDNA D-loop nucleotide sequences from selected species or breeds of *Bovinae*: *Bison bonasus*, *Bos indicus* Sahiwal strain, *Bison bison, Bos taurus* Friesian strain, *Bos gaurus, Bos grunions* and *Laces*. The *Bison bonasus* D-loop nucleotide sequence is shown at the top. Dots indicate identity with the first sequence and dashes indicate deletion.

Table 2. Pairwise divergence estimates of the 653 bp mt-DNA D-loop region for the six *Bovidae* species (in percentage).

	Bos indicus	*Bison bison*	*Bos taurus*	*Bos gaurus*	*Bos grunniens*	*Alces alces*
Bison bonasus	11.0	10.8	11.1	12.6	10.6	16.1
	Bos indicus	11.1	5.3	11.8	12.2	17.8
		Bison bison	10.7	10.6	7.0	15.7
			Bos taurus	11.0	13.0	19.1
				Bos gaurus	11.0	16.2
					Bos grunniens	16.7

by analysis of nuclear markers differ from those obtained using mt-DNA markers, which can be explain by different phylogenetic sorting of mtDNA lineages.

The *Bison/Bos* split is estimated to have occurred about one million years ago, as judged from paleontological evidence (Loftus *et al.* 1994). Detailed analysis of the hypervariable region of cattle mtDNA D-loop revealed 116 substitutions in the 370-bp fragment (Bradley *et al.* 1996). Two of them were transversions, giving an estimated transition/transversion ratio of 57:1. Four transversions between bison and cattle sequences were found and divergence rate of the two-lineage was estimated to be 62.8% per million years. We found five transversions in the 363-bp hypervariable region between *B. bison* and *B. bonasus*. Using transition/transversion ratio given above we estimate the corresponding number of transitions to be 285. This gives a calculated sequence divergence of 78.5% per Myr. This estimate should be taken as preliminary, because we used in our calculations the transition/transversion ratio obtained for cattle. Analysis of other DNA markers should allow more precise estimation of divergence time and genetic relationship for both bison species.

Fig. 3. A phylogenic tree for the *Bovidae* based on the sequences analysis using the neighbor-joining method. All substitutions in the 653 nucleotide position from the fragment of mt-DNA D-loop were analysed. Six *Bovinae* species and one outgroup (*Alces alces*) were included. The length of each pair branches represents the distance between sequence pair. Units indicate the number of substitution events.

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