

Taxonomic position of *Bison bison* (Linnaeus 1758) and *Bison bonasus* (Linnaeus 1758) as determined by means of *cytb* gene sequence

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Based on the analysis of the *cytb* gene of mtDNA the taxonomic position was determined of two closely related *Bovidae* species: American bison (*Bison bison*) and European bison (*Bison bonasus*). Reference sequences were determined for both species. In the American bison two variants of *cytb* gene were identified. Both of them are novel sequences, so far not published in GenBank. For comparison the reference sequences in four another representatives of *Bovidae* (Polish Red cattle, Zebu cattle, Merino sheep and Polish White Improved goat) were determined. Phylogenetic reconstructions were obtained by the neighbour-joining method (N-J) on all mutations and on changes with removed mutations in third position. Topology of phylogenetic trees showed that American bison and European bison form a separate nodes. The molecular data presented corroborate the results of earlier studies showing that although the two species in question are very closely related, their classification as subspecies is rather questionable. The fact that nodes for American as well as European bison show relatively high bootstrap scores supports this hypothesis.

KEY WORDS: bison / cytochrome b / gene sequence / molecular phylogeny

Species Bison belongs to the order *Artiodactyla*, suborder *Ruminantia*, family *Bovidae*, subfamily *Bovinae*, tribe *Bovini*, and genus *Bison* [Belousova *et al.* 2002]. There are two species within the genus: the American bison (*Bison bison*, Linnaeus 1758) and the European bison (*Bison bonasus*, Linnaeus 1758). There are two subspecies of American bison: the plains (prairie) bison (*Bison bison bison*) and the wood bison (*Bison bison athabascae*). Within European bison three subspecies are recognized: the

lowland bison (*Bison bonasus bonasus*), the Caucasus bison (*Bison bonasus caucasicus*) extinct in 1925 [Heptner et al. 1966], and Hungarian (Carpathian) bison (*Bison bonasus hungarorum*) that is also extinct.

American bison and European bison are both recognized as endangered species and are included in the IUCN Red List of Threatened Species [2000].

The taxonomic relationship between the American and European bison is still unclear. The former is close to its European relative morphologically, and especially genetically. They easily interbreed, producing fertile offspring in all combinations.

It is commonly assumed that *Bison* as a genus originates from southern Asia. According to Flerov [1979] both the European bison and the American wood bison (*B. bison athabascae*) come from the palearctic *Bison priscus*, while the American plains (pre-riparian) bison (*B. bison bison*) arose from the autochthonous *Bison antiquus*. Some authors claim [e.g. Mc Donald 1981] that the present-shape American bison comes from the *Bison antiquus* line, and European bison may be derived from Pleistocene re-emigrants from North America. Craniological similarity between Holocene and late Pleistocene bison of Eurasia and North America [Skinner and Kaisen 1947, Bohlken 1967] makes this hypothesis very interesting. At the end of the Pleistocene the formation of an ice bridge between Alaska and Siberia allowed to enter America by Asian bison (*Bison occidentalis*), and Asia by re-emigrants from Alaska (http://www.fermeborealis.com/html/e/bison/L1_historique.html).

It seems that molecular comparisons of the *cytb* gene sequence can help solving the problem of phylogenetic relationships between American and European bison. We choose the cytochrome b gene as a phylogenetic probe because it is easier to align a conservative protein-coding sequence among close related species.

Cytochrome b is one of best-known proteins that make up complex III of the mitochondrial oxidative phosphorylation system [Hatefi 1985] and is the only one of them encoded by mitochondrial genome. Cytochrome b is a transmembrane protein, consisting of eight alpha helices, and is involved in the oxidation of ubiquinol or plastoquinol. It is believed that it contains both redox centres Q_o and Q_i [Hatefi 1985]. All eukaryotic organisms require this class of redox enzymes, and consequently cytochrome b, for energy conservation [Widger and Cramer 1991].

In this study the sequences are compared of the 273 bp-long fragment of *cytb* gene of American and European bisons, as well as of the Polish Red cattle, Zebu cattle, Merino sheep and Polish White Improved goats.

Material and methods

Biological material

The material covered hair, liver tissue and blood. Hair samples of four American bisons were obtained from the herd kept at the Horse Stud, Kurozwęki, Poland. Hairs of European bisons originated from two animals shut in Borecka and Białowieża Primeval Forests, and from another two maintained at the Forestry Inspectorate, Smardzewice,

Poland. Hairs of Zebu cattle were obtained from unidentified private herd in India. Liver samples originated from seven European bisons shut in Poland during 1995/1996 and 1996/1997 seasons. Blood samples were collected from five Polish Red cows included in the National Rare Livestock Breeds Preservation Programme, one Merino sheep, and three Polish White Improved goats, all kept at the Institute of Genetics and Animal Breeding, Jastrzębiec, Poland.

DNA extraction, PCR, and cyclic sequencing

Total genomic DNA from hair and liver was extracted according to the standard organic procedure [Wilson *et al.* 1995]. For each source of samples specific conditions of extraction were applied. Genomic DNA from blood samples was extracted using Wizard® Genomic DNA Purification Kit (PROMEGA).

The DNA amplification was performed using primers described by Parson *et al.* [2000]. The sequences of the forward (F) and reverse (R) primers were modified in such a way that one oligonucleotide in a pair was extended by an universal primer sequence (-21)M13 at the 5' end as shown below.

**F(-21)M13: 5' TGT AAA ACG ACG GCC AGT CCA TCC AAC ATC TCA
GCA TGA TGA AA 3'**

R: 5' CCC CTC AGA ATG ATA TTT GTC CTC A 3'

For sequencing the second strand the following primers were used for amplification:

F: 5' CCA TCC AAC ATC TCA GCA TGA TGA AA 3'

**R(-21)M13: 5' TGT AAA ACG ACG GCC AGT CCC CTC AGA ATG ATA
TTT
GTC CTC A 3'**

The PCR reaction was conducted in a GenAmp PCR System 9600 Thermal Cycler (AB), according to the following parameters: 94°C for 2 min. (denaturation) and next 94°C for 30s, 50°C for 45 s, 72°C for 45s – 35 cycles.

The PCR amplification was carried out in two steps. First, in a volume of 50 µl, with the following composition of the reaction mixture: 50-100 ng of genomic DNA, 200 µM of each dNTP, 1 × PCR buffer (AB), 1.5 mM MgCl₂, 1µM of each primer, 1.5 U DNA Taq Gold polymerase (AB). In the second round of amplification used were 4 µl of 1000× diluted PCR products under the same conditions except the quantity of primers (0.1 µM), and polymerase (1.0 U).

The PCR products were purified using ultrafiltration with Microcon 100 microconcentrators (AMICON). The quantity and quality of products was tested in 4% NuSieve 3:1 agarose gel (FMC) in relation to the DNA mass ladder standard (pUC 19, INGEN, Sieradz, Poland). Purified PCR products (273 bp-long) were sequenced with ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (AB) according to the user's manual in a GenAmp PCR System 9600 Thermal Cycler (AB). The sequencing products were separated in a DNA sequencer ABI PRISM 377 (AB). The electrophoretic data were collected by the Data Collection v.2.1. software (AB) and analysed by the Sequencing

Analysis version 3.0. software (AB).

Phylogenetic analysis

The mtDNA sequences were analysed of American bison, European bison, and, for comparison, of four other species belonging to *Bovidae* (Polish Red cattle, Zebu cattle, Merino sheep and Polish White Improved goats). Sequences were compared using standard nucleotide-nucleotide Blast software. Analyses were conducted based on all mutations as well as after removing mutations at third position. Phylogenetic reconstructions were obtained with the neighbour-joining method (N-J) using MEGA II [Kumar *et al.* 2001] packages. For calculating the distance between sequences the p-distance model [Nei and Kumar 2000] was applied showing the proportion of nucleotide sites at which the two sequences compared are different. Bootstrap resampling (based on 1000 resampling events) was also conducted to estimate the stability of the tree topologies [Felsenstein 1985]. Strict consensus trees were constructed for all arrangements.

Results and discussion

A 273 bp fragment was obtained of cytochrome b gene sequence for all species analysed. In American bison (*Bison bison*) sequences of two types (I and II) were found differing in one nucleotide position: substitution T→C at position 15039 according to Anderson's bovine reference sequence [Anderson *et al.* 1982]. This substitution was silent, with no changes in amino acid composition. For American bison *cytb* gene sequences types I and II the 99% similarity with the sequence published by GenBank (AF036273.1) occurred. For all individuals of European bison one type of sequence was obtained. Sequences for Polish Red cattle, Zebu cattle, sheep and goats were also determined as single haplotypes. Sequences in text format are shown in Figure 1.

Results of earlier research by Hsieh *et al.* [2001, 2003] showed that size of the fragment analysed is sufficient to discriminate between even closely related species. Comparisons of sequences revealed significant differences between European and American bison type I and II (22 and 23 differences, respectively). There are several potential explanations of the differences observed. One of them is within-species variation. However, when analysing differences between other *Bovini* species (Polish Red cattle vs American bison type I and II: 20 and 19 differences, respectively; Polish Red cattle vs European bison: 24 differences) and between *caprini* species (sheep vs goat: 26 differences) so wide disagreement between two „subspecies” within one species – American and European bison – is astonishing. Sequence of *cytb* gene is considered as one of the most conservative gene sequences where within-species variation is very low, if any [Prusak 2003, Irvin *et al.* 1996]. This is associated with the fact that cytochrome b is one of the proteins that make up complex III of the mitochondrial oxidative phosphorylation system [Hatefi 1985]. Furthermore, the metabolic respiration is one of the oldest attributes of life and thus different organisms during evolution tended to

maintain their *status quo* in a way to produce energy needed for metabolic processes. In this context another possible explanation of differences observed between American and European bison is species position for each of them. In order to test this hypothesis we have constructed phylogenetic trees using neighbour-joining method on p-distance [Nei and Kumar 2000]. The major feature of the consensus tree constructed based on

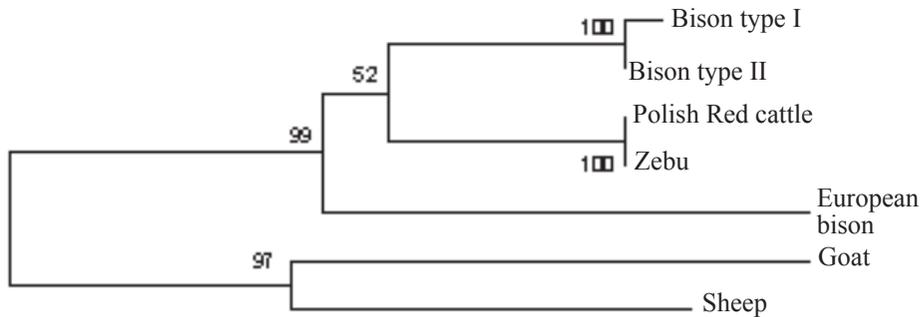


Fig. 2. Neighbour-joining tree constructed on p-distance for mutations in three codon positions.

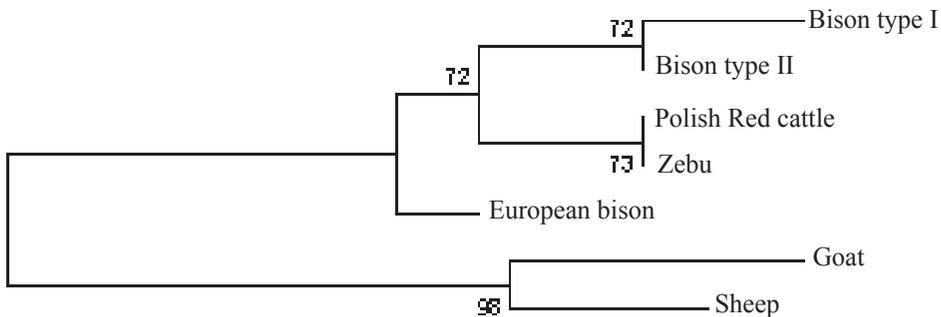


Fig. 3. Neighbour-joining tree constructed on p-distance for mutations in first and second codon position.

mutations at three codon positions is shown in Figure 2.

The phylogeny presented illustrates that mtDNA sequences are separated into two major groups: tribe *bovini* and tribe *caprini* (both supported by high bootstrap scores). American and European bison both create the separate nodes in the tree, supported by high bootstrap value. Sequences of the former cluster with those of domestic cattle rather than with sequences of European bison. However, the bootstrap value for such nodding

appears low (52%). It is noteworthy that similar observations on grouping American bison with domestic cattle have been made by Janecek *et al.* [1996] who reported that the phylogenetic analyses based on cytochrome c oxidase subunit II (COII) gene suggest the American bison to be closer to *Bos*. They also concluded that the genus *Bison* could be paraphyletic, with the American bison closely related to *Bos* species rather than to the European bison. To determine whether silent sites affect the sequence similarities, changes in the third position were removed. In this position non-random distribution of bases is strong and the highest differences in base composition occur among species [Irvin *et al.* 1991, Prusak 2003]. The resulting tree (Fig. 3) is similar to that in Figure 2, but is less supported by bootstrap values, suggesting that the bias at third positions does not significantly affect the phylogeny.

The taxonomic position of American and European bison is unclear. Many studies have been done, yet none have led to a clear definite conclusion. The close genetic similarity of the American and European bison was recently confirmed by Ryskov *et al.* [1994] and Sipko *et al.* [1997]. European and American bison easily breed with each other and some authors consider them as subspecies of the *Bison* species [Bohlken 1958, Haltenth 1963]. Van Zyll de Jong [1986] suggested that with respect to size and several morphological traits *Bison bonasus* is closer to *Bison bison* than either of these is close to *B. b. athabascae*. But the latter form occupies a specific position within the present *Bison* species and only slightly differs from prehistoric Asiatic form – *B. b. occidentalis*. However, some authors, on the basis of markedly different morphotypes and extreme disjunctive geographical distribution continue to give the European bison and American bison the statuses of a separate species, disregarding their mentioned ability to interbreed [Sokolov 1959, Geptner *et al.* 1961, Wilson and Reeder 1993]. This hypothesis is significant when considering problems of maintaining genetic purity and species diversity of European bison and its crosses with Caucasian subspecies as well as with American bison.

In summary, based on the *cytb* gene sequence analysis it is confirmed that although American bison and European bison are very closely related, they should be treated as separate species (*Bison bison* and *Bison bonasus*) of a *Bison* genus rather than subspecies of a *Bison* species.

Moreover, the results presented here showed that derived sequences of *cytb* gene can be considered as representative for investigated species and be used for species identification when the source of biological material available is unidentified.

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Taksonomiczna pozycja bizona (*Bison bison* L.) i żubra (*Bison bonasus* L.) określona na podstawie sekwencji w genie *cytb*

Streszczenie

Na podstawie analizy sekwencji genu cytochromu b mitochondrialnego DNA określono pozycję systematyczną bizona (*Bison bison*) i żubra (*Bison bonasus*). Dla obu gatunków ustalono referencyjne sekwencje genu *cytb*. U bizona wyodrębniono dwa typy sekwencji (I i II) różniące się podstawieniem T→C w pozycji 15039 według bydlęcej sekwencji referencyjnej Andersona. Obie są sekwencjami nowymi, nie zarejestrowanymi dotąd w bazie GenBank. Określono również sekwencje genu *cytb* dla czterech innych przedstawicieli rodziny *Bovidae* (bydło polskie czerwone, bydło zebu, owca i koza), które wykorzystano jako materiał porównawczy w analizie filogenetycznej. Drzewa filogenetyczne zbudowano metodą najbliższego sąsiada (*neighbour-joining* – N-J), uwzględniając zmiany dla trzech pozycji w kodonie oraz zmiany w pierwszej i drugiej pozycji kodonu. Analiza filogenetyczna wykazała, że bizon i żubr tworzą w obu drzewach odrębne gałęzie. Takie umiejscowienie bizona i żubra w topologii drzew potwierdził test wiarygodności wysokimi wartościami „bootstratu”. Uzyskane wyniki są zgodne z wcześniejszymi badaniami, które wskazują, że aczkolwiek bizon i żubr są ze sobą blisko spokrewnione i wykazują wiele podobieństw morfologicznych, to jednak powinno się je traktować jako oddzielne gatunki.

