THE ANALYSIS OF CYTOCHROME B SEQUENCES FOR *BISON BONASUS* (ARTIODACTYLA: BOVIDAE) INDIVIDUALS FROM VÂNĂTORI-NEAMȚ AND NEAGRA-BUCȘANI NATIONAL PARKS

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Received: 5 January 2015 / Revised: 23 January 2015 / Accepted: 19 February 2015 / Published: 5 April 2015

Keywords: mitochondrial DNA, cytochrome b, *Bison bonasus*

Abstract: Mitochondrial DNA (mt-DNA) has proven to be a useful tool in molecular phylogenetic studies, because evolutionary relationships can be inferred in depth, between recently divergent groups, populations, species, and even individuals. In this experiment we investigated the genetic variability of *Bison bonasus* individuals from Vânători Neamț and Neagra Bucșani National Parks, by analyzing *cyt b* nucleotide sequences. The study allowed us to identify the haplotypes, the nucleotide frequencies, mt-DNA divergence and phylogenetic relationships within studied populations. In this study we observed 3 haplotypes at Vânători Neamț population and 2 haplotypes at Neagra Bucșani population.

INTRODUCTION

DNA-sequence data from the mitochondrial genome are frequently used to estimate phylogenetic relationships among animal taxa. The advantage to using DNA-sequence data is that many of the processes governing the evolution and inheritance of DNA are already understood (Brown et al. 1979). DNA data, however, do not guarantee the correct phylogenetic tree because of problems associated with shared ancestral polymorphisms and multiple substitutions at single nucleotide sites (Simon et al. 1994). Another characteristic of mt-DNA, generally accepted as a real advantage for populational genetic studies, is the lack of recombination (Wilkinson and Chapman 1991, Loftus et al. 1994, Ishiba et al. 1995). The majority of the animals have sexual reproduction. After the recombination process and the independent segregation of chromosomes during the first meiosis, genes from both parents are transmitted to the daughter cells, but because organelle genes (such as mt-DNA) are uniparentaly transmitted, these don’t recombine (Birky, 2001).

The biggest mammal species in Europe, the European bison (*Bison bonasus* L.), went through a severe bottleneck at the beginning of the 20th century. Reintroduction of wild populations started in the 1920s and it was based on a few animals from private and public collections. After World War I, the survival of this species was secured only in a few European zoological gardens (Sztolcman 1924). In total, only 54 individuals with proved pedigrees remained (29 males and 25 females), descending from 12 animals (Slatis 1960; Raczynski 1978; Pucek 1991).

After an intensive breeding period in the zoological gardens, the first animals were released in the Bialowieza National Park, in 1952, which is the core of the Bialowieza Forest, stretching across eastern Poland and western Belarus. With approximately 540 animals, the Bialowieza bison population is globally the largest (Kita et al. 2003; European Bison Pedigree Book - EBPB 2001). In 2000, 2864 individuals were registered worldwide (EBPB 2001; Hilton-Taylor 2000).

Tokarska et al. in 2011 suggest that European bison has been the subject of extensive genetic studies. The low level of genetic variability has been widely confirmed by genetic analyses using a variety of methods. However, there is no evidence that this will inevitably lead to extinction. In 2006 Anderung et al. developed a study based on ancient mt-DNA of just four European bison from the 13th to 14th centuries and revealed two haplotypes. Another analysis conducted by Wojcik et al. in 2009 revealed three haplotypes for 200 individuals of a 1429 base pair of control region. An earlier study based on the analysis of the variability of mt-DNA sequence in the European bison by Burzynska et al. (1999) revealed eight distinct haplotypes of a 1026 base pair fragment of control region.

The Vânători Neamț Natural Park and Neagra Bucșani Reservation are two protected areas established in 1999, as a site of the Nature 2000 ecological network, with both communitarian and avi-faunistic protection importance and one of the objectives is the release of the bison in its natural milieu. The bison is the symbol of the Carpathians and the most impressive herbivorous animal in Europe (800-1000 kg/ex. the male and 500-700 kg/ex. the female).

The aim of this study was to investigate the genetic variability of two Romanian bison populations Vânători Neamț and Neagra Bucșani, in order to provide new information on the genetic diversity of this species. Mitochondrial *cyt b* analysis was used for 23 individuals. We identify the total number of haplotypes, the nucleotide diversity, and phylogenetic relationships within studied populations.

MATERIAL AND METHODS

Biological material:

The studied individuals of *Bison bonasus* are originated from two different Romanian protected areas: Vânători Neamț National Park and Neagra Bucșani Reservation. Sixteen European bisons, with random age, were sampled during 2010 to 2014. From both areas the same number of individuals was analyzed: eight from Vânători Neamț National Park (seven adults and one death calf) and another eight adults from Neagra Bucșani Reservation.

The analyzed samples consist of blood or muscle tissue. The blood samples have been collected through puncture of the jugular vein, after the animals were tranquilized for translocations, 2 ml Vacutainer-type tubes being employed. One muscle tissue sample has been collect from a death calf, fresh blood sample being unavailable. The muscle tissue was sampled by cutting with a bistoury a small fragment from the leg. The blood samples were stored in Queen's Lysis buffer (Seutin et al., 1991) and muscle tissue in 98% ethanol.

For analyzing the phylogenetic relationships and quantify genetic diversity within the studied populations, DNA had to be isolated, amplified and sequenced.

DNA extraction and polymerase chain reaction:

Total genomic DNA was isolated from biological material by proteinase K treatment followed by phenol chloroform extraction (Gorgan, 2007, 2008). DNA was eluted in TE buffer (pH=8.0) and kept at -20° C. Polymerase chain reaction (PCR) was used to amplify the entire *cyt b* gene.

Two specific primers were used (Watanobe et al., 1999), and their sequences were: $mitL_1$ 5'-ATCGTTGTCATTCAACTACA-3', $mitH_2$ 5'-CTCCTTCTCTGGTTTACAAG-3'. The cycling conditions consisted of an initial denaturation step at 94°C for 4 min followed by 40 cycles of 94°C for 30 sec, 50°C for 45 sec, 72°C for 1 min and a final extension step at 72°C for 10 minutes.

The sequencing process was based on the dye-terminator Sanger method (Sanger et al. 1977) using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Switzerland) and process according to manufacturer protocol.

Sequences analysis: For this study we used 16 *cyt b* sequences obtained from our individuals and another 7 sequences were downloaded from GenBank (accession numbers are listed in Table 1) and included in this analysis. The phylogenetic relationships were determined by the Neighbour-Joining method (Saitou 1987) and the evolutionary history was inferred using the Maximum Composite Likelihood method. The complete *cyt b* sequences were aligned using Clustal W (Thompson et al., 1994) in the MEGA 5.0 (Tamura et al., 2011) phylogenetic package.

Table 1 Accession numbers from GenBank

GenBank accession	Species	Location	Reference	Length (bp)
AY079126	*Bison bonasus*	Artis Zoo, Amsterdam	[1]	1140 bp
HM045017	*Bison bonasus*	Wrocław Zoo, Poland	[2]	1140 bp
NC014044	*Bison bonasus*	Wrocław Zoo, Poland	[2]	1140 bp
JN632602	*Bison bonasus*	Paris, France	[3]	1140 bp
AY689186	*Bison bonasus*	Paris, France	[4]	1140 bp
Y15005	*Bison bonasus*	Frankfurt zoo, Germany	[5]	1140 bp
HQ223450	*Bison bonasus*	-	[6]	1140 bp

[1]Verkaar,E. L. et. al., (2004);[2]Zeyland J et. al., (2004);[3]Hassanin A. et. al., (2012);[4]Hassanin A.et. al., (2004);[5]Zimmermann S. et. al, (1998);[6]Derr, J. N.-(2010), Direct submission to NCBI.

RESULTS AND DISCUSSION

Haplotype data

After the sequence analysis, we identified the existence of 6 different haplotypes. Haplotype frequencies were determined using DnaSP 4.50.3 software (Rozas et al., 2003).

For Vănători-Neamț population 3 haplotypes were observed. Haplotype 1 (H1) is the most common, and it was observed at four male individuals (*Bb01m, Bb02v Bb04m,Bb05m, Bb03m*) and at two females (*Bb04f* and *Bb06f*). Haplotype 2 (*Bb03f*) and haplotype 3 (*Bb02f*) were found only in the Vânători Neamț population.

Because we not found other cyt b sequences on GenBank for comparison, we can affirm that so far both of them are local haplotypes. Haplotype diversity (Hd) for this population is 0.46. The average number of differences (K) is 1.75.

For Neagra Bucşani population we identified only 2 haplotypes. Haplotype 1(H1) has a high frequency, and it was detected at seven individuals: four males (*Bb02m, Bb03m, Bb06m,* and *Bb07m*) and 3 females (*Bb06f, Bb07f* and *Bb08f*). A single individual (*Bb01f*) presents haplotype 4 (H4) so we can conclude that this is a local haplotype.

Haplotype diversity in this population is 0.25, a much lower value compared to haplotype diversity from Vânători Neamț, where the same parameter is 0.46. This means that at Neagra Bucşani, the population presents a lower genetic diversity, with only two haplotypes in comparison to Vânători Neamț population, where three haplotype were identified.

For the sequences from GenBank, 3 haplotypes were found (H1, H5 and H6). Similar to the other two populations, haplotype 1(H1) is the most common one, being found at 5 individuals (AY079126, HM045017, NC014044, JN632602, AY689186). The fifth haplotype is Y15005, and it was identified at an animal in Frankfurt zoological garden (Zimmermann et al. 1998). The sixth haplotypes is HQ223450.

In Romanian National Parks, haplotypes H5 and H6 were not found. A possible cause may be the existence of two separate genetic lines of *Bison bonasus* species (Lowland and Lowland-Caucasius). In Romanian natural reservations, all the bisons originate from the Lowland Caucasius genetic line.

Nucleotide diversity

To calculate the nucleotide diversity (Pi) for both populations we used DnaSP 4.50.3 software (Rozas et al., 2003).We can observe (Figure 1) that the alignment of *cyt b* sequences for population 1 (P1) contains a great nucleotide variability. Pi increases at the beginning of the sequence, and after nucleotide position 800, it remains constant. The number of polymorphic sites for P1 is 7 and the average number of nucleotide differences is k=1.750. We calculated nucleotide diversity for P1 and obtained Pi= 0.00149. The total number of nucleotides differences for population 1 (P1= Vânători Neamț population) is 7.

For the second population, Pi varies at the beginning of alignment, until nucleotide position 100. After this, the nucleotide diversity is invariable but it increases again near the end of the sequences. The number of polymorphic sites for P2 is 2, the average number of nucleotide differences is k=0.500 and the nucleotide diversity is Pi=0.00043. The total number of mutation for P2 is 2. (P2 = Neagra Bucşani population).

Total data: Regarding the nucleotide diversity we can say that the total number of polymorphic sites for both populations is 9. The nucleotide diversity Pi(t) is 0.00096. The average number of nucleotide differences between populations is k=1.125. The average number of nucleotide substitution per site between populations is Dxy= 0.0009.

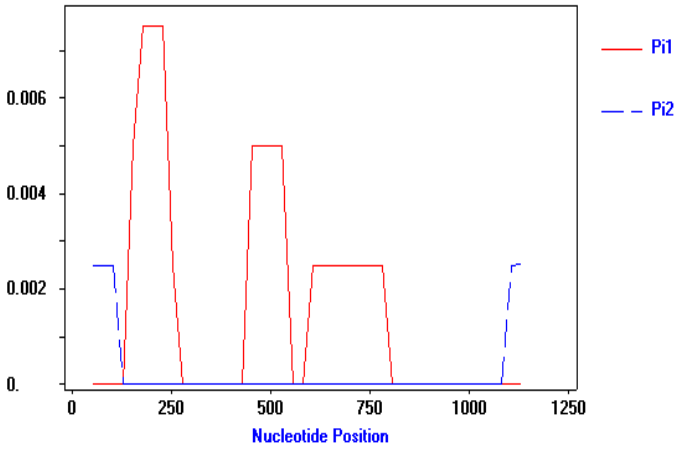

Figure 1. Nucleotide diversity for P1 and P2 (Pi1= nucleotide diversity for P1; Pi2=nucleotide diversity for P2

Network Haplotypes estimations

Using Network software, v.4.621 (Polzin et al., 2003) we could create the haplotype network (Figure 2). Five haplotypes were identified in our study; however, this surprisingly high variability was not confirmed in further studies. Only Burzyńska et al. 1999 observed eight haplotypes after developed a study on 14 specimens. They followed genetic variation on mitochondrial DNA D-loop sequences.

Historical studies on ancient mtDNA of just four European bison from the 13th to 14th centuries by Anderung et al. (2006) revealed two haplotypes. Extensive analyses of the 1429 bp sequence of the control region (D-loop) performed on nearly 200 individuals showed that there are just three haplotypes Wojcik et al. 2009.

Based on our study haplotype 1 has a high level of frequency for the analyzed individuals. It was identified for eighteen European bisons, thirteen of them belonging to the two Romanian populations (Vânători Neamț and Neagra-Bucșani). Also, haplotype 1 occupies a central position with the highest number of connections. It is likely to be the ancestor for the other 5 haplotypes during the expansion. H3, H4 and H6 are the closest to the H1, differing from it by the presence of a single nucleotide. H2 and H5 are two important haplotypes with a great number of transversions and transitions. For example between H1 and H2, we identified five substitutions, and between H1 and H5 seven substitutions and three indels were observed.

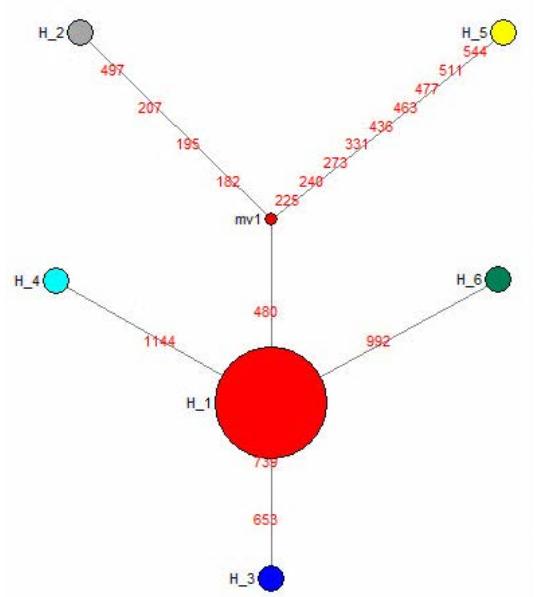

Figure 2. Maximum Likelihood Network Haplotypes estimations

Maximum Likelihood Estimate of Substitution Matrix

Each entry represents the probability of substitution (r) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei (1993). Rates of different transitional substitutions are shown in **bold** and those of transversional substitutions are shown in *italics* (Table 3).

Table 3. Maximum Likelihood Estimate of Substitution Matrix

From\To	A	T	C	G
A	-	*2.40*	*0.99*	**31.42**
T	*1.98*	-	**7.26**	*2.17*
C	*1.98*	**17.57**	-	*2.17*
G	**28.63**	*2.40*	*0.99*	-

Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 100.

The nucleotide frequencies are A = 26.24%, T/U = 31.83%, C = 13.14%, and G = 28.79%. For estimating ML values, a user-specified toplogy was used. The maximum Log likelihood for this computation was -2027.098. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated.

There were a total of 1172 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura-Nei., 2011).

CONCLUSIONS

The study of genetic variability in two *Bison bonasus* populations from Romania inferred from the cytochrome b gene revealed the following:

For the alignment of the 23 sequences of *cyt b*, six haplotypes were identified. There were identified 3 haplotypes in population of Vânători-Neamț, and 2 haplotypes in Neagra Bucşani population. We consider that the presence of one more haplotype in the Vânători Neamț population is probably due to the introduction of new individuals (received from Germany, Switzerland and France) in the last years.

For the sequences downloaded from GenBank, 3 haplotypes were observed.

Haplotype diversity (Hd) is higher for Vânători-Neamț population (0.46), comparative with Neagra Bucşani population, which is 0.25.

The most common haplotype is H1, observed at 18 individuals, so we can assume that it is probably the oldest haplotype. The identified haplotypes seems to radiate from H1 suggesting a conservation of this genetic form through the time and a starting point for other haplotypes. The convergence of H2 and H5 into a lost ancestral haplotype mv1, support our previous assumption that actual individuals of *Bison bonasus* evolved from a H1 like genetic group. Our point of view is also confirmed by historical records of European bison repopulation strategy which started from two genetic lines: one specific to Poland and another from Romania (Olech et al. 2008).

In the two studied populations we found 5 haplotypes, the fact which shows an important genetic variability taking into account the small number of individuals analyzed.

Nucleotide diversity is higher in Vânători-Neamț population (0.00149), comparative with Neagra-Bucşani populations (0.00043).

The nucleotide diversity between *cyt b* sequences shows a high similarity, but the presence of major differences and the presence of the substitutions and indels confirm that this species presents a high genetic variability.

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Acknowledgements: This work was supported by the strategic grant POSDRU/159/1.5/S/133652, co-financed by the European Social Fund within the Sectorial Operational Program Human Resources Development 2007 – 2013.

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