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Assessment of interspecific and intrapopulational polymorphism at Bison bonasus specie

- Summary of the doctoral thesis-

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INTRODUCTION

The European bison (*Bison bonasus* Linnaeus, 1758) is the largest herbivore from Europe. Until the end of the XIX century there were only two populations of bison left in the wild: one in Bialowieza forest (*B. bonasus bonasus*) and one in the west, in the Caucasus Mountains (*B. bonasus caucasius*), according to the studies made by Pucek (1991 and 1994), which has made a short description about their disappearance. All the European bison of pure breed are descendants from a group of 12 individuals and they represent a combination of 12 diploid sets of genes (Slatis, 1960). Eleven from the twelve founders (all *Bison b. bonasus*) originate from Bialoweza forest, from the zoos of Berlin, Budapest and Pszczyna. Only one male of *Bison b. caucasius*, born in 1907 in the Caucasus Mountains was brought in Germany in 1908. There were noticed genetic problems regarding inbreeding, since considering a small number of founders, issues which resulted with a powerful influence onto the conservation measures for this species. The lowland genetic line was a subject for extensive genetic studies. In the past few years, it has been sustained that the species is inevitably on its way to extinction despite the conservation efforts made, the main cause being the loss of genetic diversity and implicitly the high inbreeding risk (Gill, 2002). The low genetic variability was confirmed on large scale with a series of genetic analysis and with a variety of methods. However, there is no clear evidence that proofs that this will unavoidable lead to the extinction of this species.

PURPOSE AND RESEARCH OBJECTIVES

A. Research purpose

In the study called "The identification of the interspecific and intrapopulation polymorphism of *Bison bonasus* species" the aim was to quantify the genetic variability of *Bison bonasus* species using mitochondrial and nuclear genetic markers that are relevant for genetic diversity, phylogeny and molecular phylogeography research. Also, the survey pursued the identification of the taxonomic relationships of the representative species belonging to Bovinae subfamily. Based on the results, future recommendations can be made regarding the gene flow and the control of future breeding of existing bisons within the two populations, thus monitoring the increase of genetic variability and the diminishing of the inbreeding effects. This research complements the existing data with solving some taxonomic uncertainties, but also with new information about the life history of representative species of Bovinae subfamily.

B. Research objectives

In order to achieve the research purpose, the following objectives were established:

- Genetic variability quantification of the main bison populations from Romania – Neagra Bucşani (Dâmboviţa County) and "Dragoş Vodă"Vânători Neamţ Reservation (Neamţ County).
- Phylogenetic relationships identification within Bovinae subfamily.

CHAPTER 1. *BISON BONASUS* SPECIES – BIOLOGICAL DESCRIPTIONS

1.1 *Bison bonasus* taxonomy

Kingdom: *Animalia*
Phylum: *Chordata*
Subphylum: *Vertebrata*
Class: *Mammalia*
Order: *Artiodactyla*
Family: *Bovidae* (Gray, 1872)
Subfamily: *Bovinae* (Gray, 1821),
Genera: *Bison*

Bison bonasus (European bison), is characterized by hypermetric body development (size 170 – 190cm, body mass of 500 – 800 - 1000kg, body length - 2,5 - 3m), large body, pronounced sexual dimorphism, specific conformation (Murariu, 1993).

1.2 Distribution

The European bison was limited throughout its history to the continental Europe area (Anonymous, 1981; Sokolowski, 1983). Initially it occupied the western, central and southeastern Europe and the Caucasus region. Yet, by the end of the XIX century, only two populations of European bison remained: *B. b. bonasus* in Białowiezaforest and *B. b. cucasius* in the west region of Caucasus Mountains (Figure 1).

Figure 1. *Bison bonasus* distribution during Holocene (mustard colored), before the extinction (dark green) and at the beginning of the XX century (red).

1.3 *Bison bonasus* genetic structure

Eleven of the twelve founders (*Bison b. bonasus*) come from Bialoweza forest, from the zoos of Berlin, Budapest and Pszcyna. Considering the small number of founders some genetic issues appeared regarding the inbreeding, these problems having a high impact in the conservation of this species.

There are 2 genetic lines for the species:

●**Lowland lineage** that has 7 base founders: 4 males and 3 females, pure breed individuals from *Bison b. bonasus* line.

●**Lowlan Caucasius lineage** (or the Caucasian lineage), (*B.b.bonasus X B.b. caucasius*) that has 5 base founders (1 male from Caucasius and 4 females from Lowland line).

The last recordings of a Caucasian bison herd, dates in 1907, on the territory of the former Soviet Union. The only representative of this species was the Kaucasus male, which was taken to Germany in 1908 and survived until 1925. For keeping the Lowland-Caucasius lineage, this one was paired with 4 females which belonged to the plain lineage. The Caucasian lineage had as a beginning point a number of 5 individuals. The initial genetic contribution of the Kaucasus male was estimated as 10% but currently decreased to about 6%, thus being considered an open lineage.

CHAPTER2. MATERIALS AND METHODS

To achieve the paper goals, the following steps were made: total DNA isolation and purification from different types of samples, total DNA quantification, amplification of the nuclear and mitochondrial markers using PCR, the amplicons verification in agarose gel and DNA purification, sequencing and acquiring the targeted sequences from GenBank international database. All these stages were performed in the Molecular Genetics Laboratory (B344) of the “Alexandru Ioan Cuza” University of Iaşi.

2.1 Biological material

In order to complete the study, the used biological material was blood, muscle tissue, hair and bone, sampled from *Bison bonasus* individuals. The samples from the viable specimens belong to "Dragoș Vodă" Vânători Neamț Natural Reservation population and Neagra Bucșani Natural Resevation population. Also, the samples that belonged to extinct individuals were collected from natural history museums across the country, and consisted of hair and bone powder (Figure 2).

Figure2. Trophies from Hunting Museum Posada used for sampling (original photo)

2.2 Total DNA isolation and purification

The blood and muscle tissue samples preserved in ethanol were subjected to the extraction protocol: *phenol: chloroform: izoamylalcohol* (25:24:1) (Ausubel et. al, 1995).

The total DNA isolation from fresh blood samples was made according to *DNA-IQ System* protocol (DC6700Promega).

For the blood samples preserved in EDTA the *Thermo Scientific Phusion ® Blood Direct PCR Kit* protocol was used. This method was conceived for amplifying DNA from whole blood and eliminates the necessity of another distinct step of DNA purification before the PCR.

2.3 The amplification of the gene that causes cytochrome b (cyt b), cytochrome oxidase I (cox I) and mitochondrial control region (D-loop) synthesis

The isolated and purified DNA was further subjected to polymerase chain reaction that exponentially multiplied a fragment of informational material. The regions belonging to cytochrome b (*cyt b*), cytochrome oxidase I (*cox I*) and mitochondrial control region (*D-loop*) were amplified.

At *Bison bonasus* species the mitochondrial genome has a length of approximately 16326 base pairs (Figure 3.). The amplification of the gene that codifies the cytochrome b (1070 bp), cytochrome oxidase I (1470 bp) and D-loop (860 bp) using PCR was successful, with a total of roughly 23% length of the mitochondrial genome.

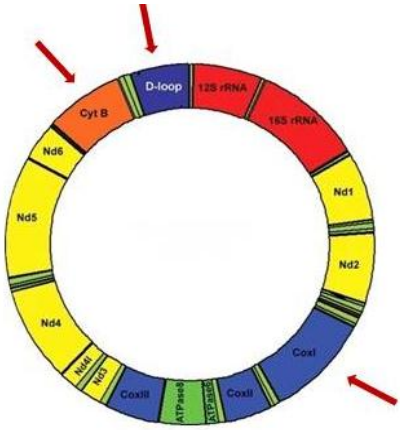

Figura 3. The mitochondrial genome structure of *Bison bonasus*and the analyzed mitochondrial markers

2.4 The amplification of BM2830 and TGLA126 nuclear markers using PCR

To amplify the nuclear DNA, two pairs of primers were used:

- BM2830 (F) (5'-AATGGGCGTATAAACACAGATG-3') and (R) (5'-TGAGTCCTGTCACCATCAGC-3') ;
- TGLA126 (F) (5'-CTAATTTAGAATGAGAGAGGCTTCT-3') and (R) (5'-TTGGTCTCTATTCTCTGAATATTCC-3', primers often used in microsatellites analyses at bovines.

The analysis was performed for 21 individuals belonging to two National Parks from Romania (15 bisons from Vânători Neamț and 6 individuals from Neagra Bucșani). The markers that were used were selected from BOVMAP data base.

2.5 PCR products migration in agarose gel

The obtained PCR products were verified using 1% concentration agarose gel. The results of the amplicons migration were viewed in UV light with a Transiluminator.

2.6 Purifying PCR products

After verifying the PCR products by migrating them in agarose gel, they must be purified. For this Wizard SV Gel and PCR Clean-Up System kit was used.

2.7 Sequencing reaction

For the sequencing reaction it was used Quick Start kit and CEQ 8000 Genetic Analysis System (Beckman Coulter).

CHAPTER3. RESULTS AND DISCUSSIONS

3.1 The identification of interspecific and intrapopulation polymorphism for *Bison bonasus* species

3.1.1 Nuclear microsatellites analysis

*TGLA locus analysis***.** For BM2330 locus, 3 alleles were observed that belong to the bison population from Vânători Neamţ and 3 alleles for the bison population from Neagra Bucşani. Allele 1 was present at 14 individuals from both populations. (Figure4).

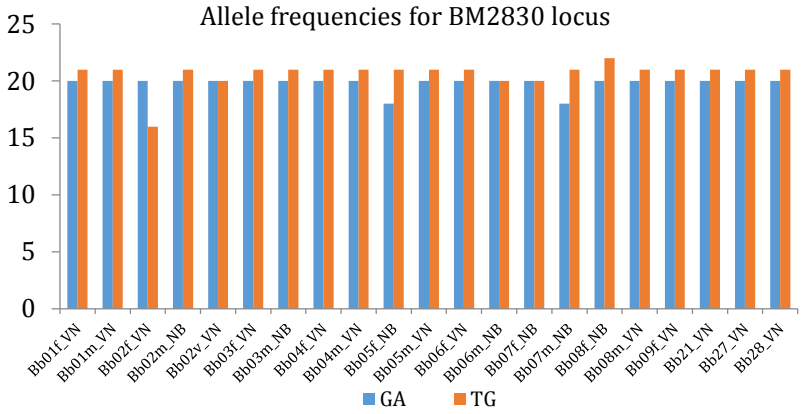

Figure4. The total number estimation of GA and TG tandems for BM2830locus

TGLA locus analysis. For TGLA locus it was observed a total number of 3 alleles from a total number of 21 analyzed sequences (Figure 5). The 3 alleles identified were present for the individuals belonging to both populations. The allele with the highest frequency was allele 1 (A1=66.6%), the tandem TG having 16 repeats in its structure. A1 was identified at 14 individuals (10 from Vânători Neamţ and 4 from Neagra Bucşani).

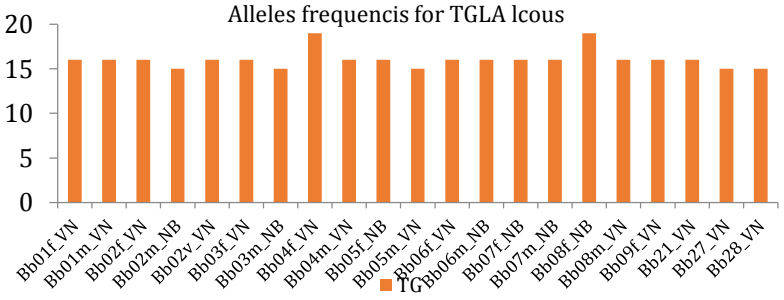

Figure5The total number estimation of TG tandems for TGLA locus

3.1.2Haplotype frequency evaluation by mitochondrial markers analysis

The mitochondrial markers analysis indicated a number of 5 haplotypes (Figure 6). For the Vânători Neamţ population only 1 haplotype was identified (H1), which had a 100% frequency. For the 6 individuals from Neagra Bucşani population, 2 haplotypes were identified: H1 and H2. The 5 sequences obtained from the extinct individuals (hunting trophies or neutralized individuals) belonged to 2 haplotypes (H1 and H3). The frequency of H1 in the extinct individuals was 80% and H3 had a frequency of 20%.

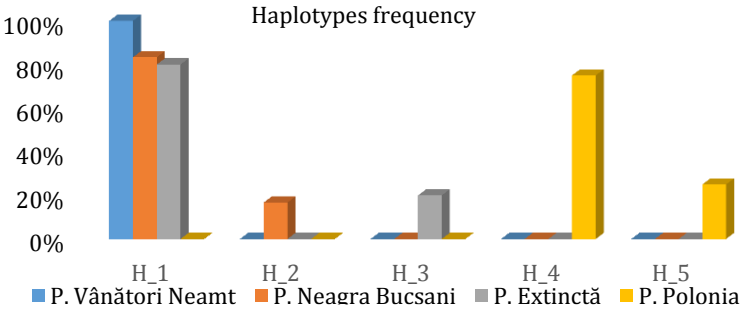

Figure6. Haplotype frequencies in the studied *Bison bonasus* populations

3.1.3 Haplotype network obtained by mitochondrial markers analysis

Analyzing the mitochondrial markers *cyt b*, *cox I* and *D-loop*, the haplotype network was obtained using Network software (Polzin et al., 2003). A number of 5 haplotypes were identified; the evolutionary connections between them are shown in Figure 7.

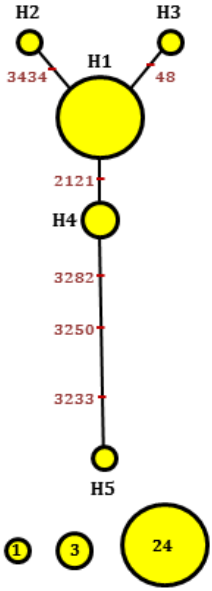

Figure7. Haplotype network in the studied *Bison bonasus*ind ividuals

3.1.4 Haplotype distance matrix obtained using mitochondrial markers

Notable differences can be observed between haplotype pairs H2-H5 and H3-H5 (Figure8). A maximum number of 5 differences were identified between these haplotype pairs. It is known that H2 and H3 are specific haplotypes for the bison population from Romania which belong to Lowland-Caucasius lineage and H5 is the observed haplotype for the bison individuals from Poland which belong to the Lowland lineage. Therefore, it was found that maintaining the two

lineages separately was a success. Analyzing the haplotype distance matrix, a maximum number of 2 differences was identified between the haplotype pairs H1-H2 and H1-H3, haplotypes that were observed for the 2 bison populations from Romania (Vânători Neamț and Neagra Bucșani).

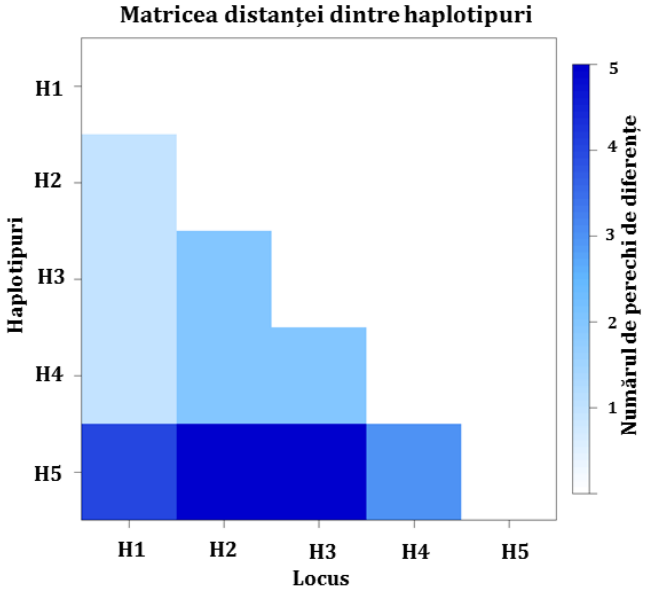

Figure8. Haplotype differences for analyzed populations

3.1.5 Intra- and interpopulational variation

The intra- and interpopulational variation for *Bison bonasus* species was estimated using AMOVA analysis. The variation percentages identified were very similar between the individuals belonging to each population (42.86%) and for the individuals belonging to different populations (57.13%). These genetic differences were also confirmed by the Fst value (0.57136) which suggests a balance between the common allele's frequencies of the 4 populations and the alleles identified in a single population of the 4 compared.

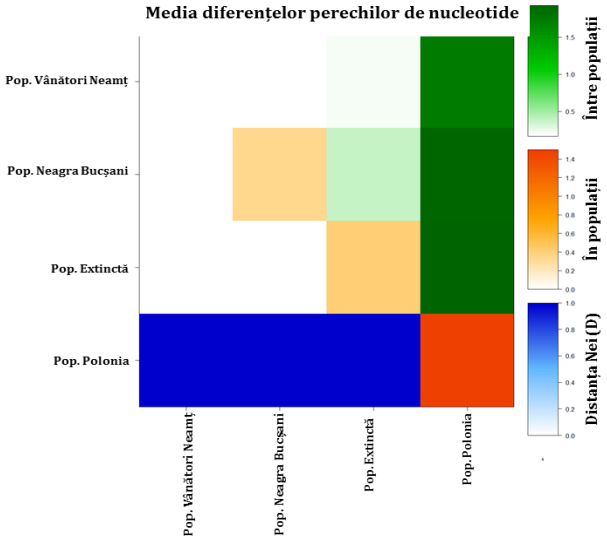

Figure 9. Mean pairwise diffrerences between the 4 studied populations (Vânători Neamț, Neagra Bucșani, Poland and the population formed from extinct specimens)

3.1.6 Genetic structure and gene flow

The total number of clusters for the studied individuals was identified using no admixture model. During the analysis it was observed that the maximum value for ΔK was 3.448759. A K number corresponds to the total number of clusters, which form the populations structure for the maximum value of ΔK. Considering the above, it has been observed the presence of only 2 genetic clusters, with uneven contributions within the analyzed population structure (Figure 10).

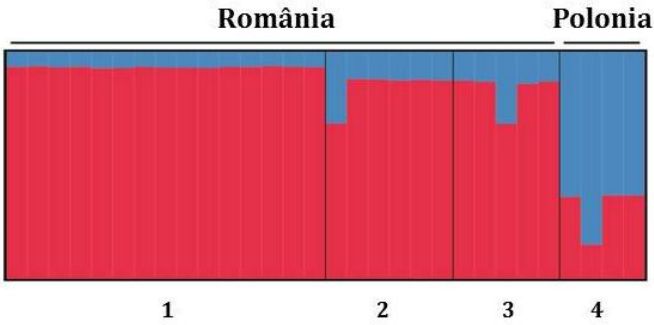

Figure10. Genetic structure and gene flow within the analyzed populations (1. Vânători Neamț; 2. Neagra Bucșani; 3. Extinct specimens; 4. Poland)

3.2 Phylogenetic relationship within Bovine subfamily

3.2.1 Mitochondrial marker with a high topological support

Analyzing the node proportion with PP>0,65 or PP>0,5, lead to a favorable result for *D-loop* (Figure 11). When the mitochondrial control region (*D-loop*) was compared with *cyt b* or *cox* I, the first has shown the highest number of nodes with PP>0,65 (77.52% for *D-loop*, 58,42% for *cyt b*and only 51,68% for *cox I*).

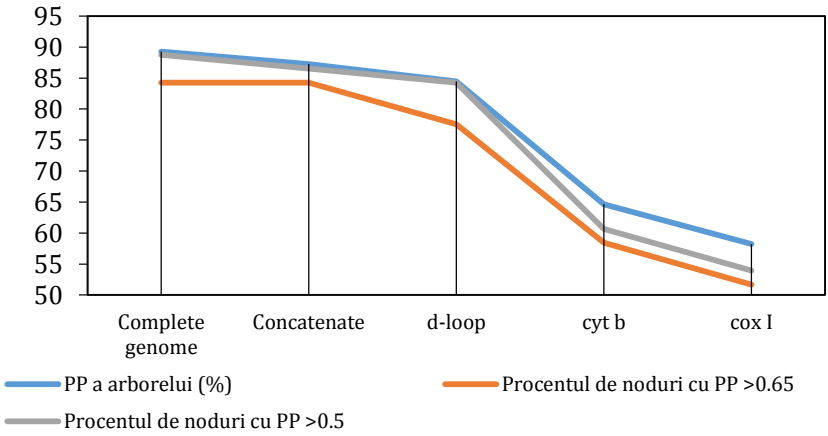

Figure11. Percentage of nodes posterior probability distribution

3.2.2 Different mitochondrial markers impact in divergence time estimation

The best tree model for phylogenetic analysis was obtained using cytochrome b gene. This model had the lowest values for the marginal likelihood (Figure 14).

Once the mitochondrial marker capable to estimate in an accurate way the divergence time for the Bovinae subfamily was identified, the impact of the other markers was investigated on the divergence time for the same group of taxa (Figure 15). In order to achieve this, the divergence time was calculated using Bayesian Inference (for species, tribe and genera level) for the *cyt b, cox I, D-loop*, the 3 concatenated markers and the complete genome.

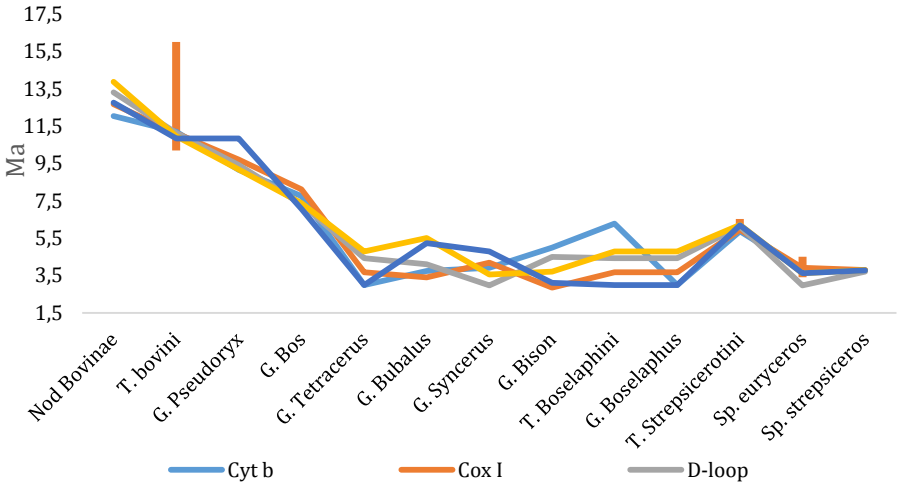

Figure 15. Mitochondrial markers impact on the divergence time estimation within *Bovinae* subfamily (colored red bars mark the variation interval of the calibrated node age)

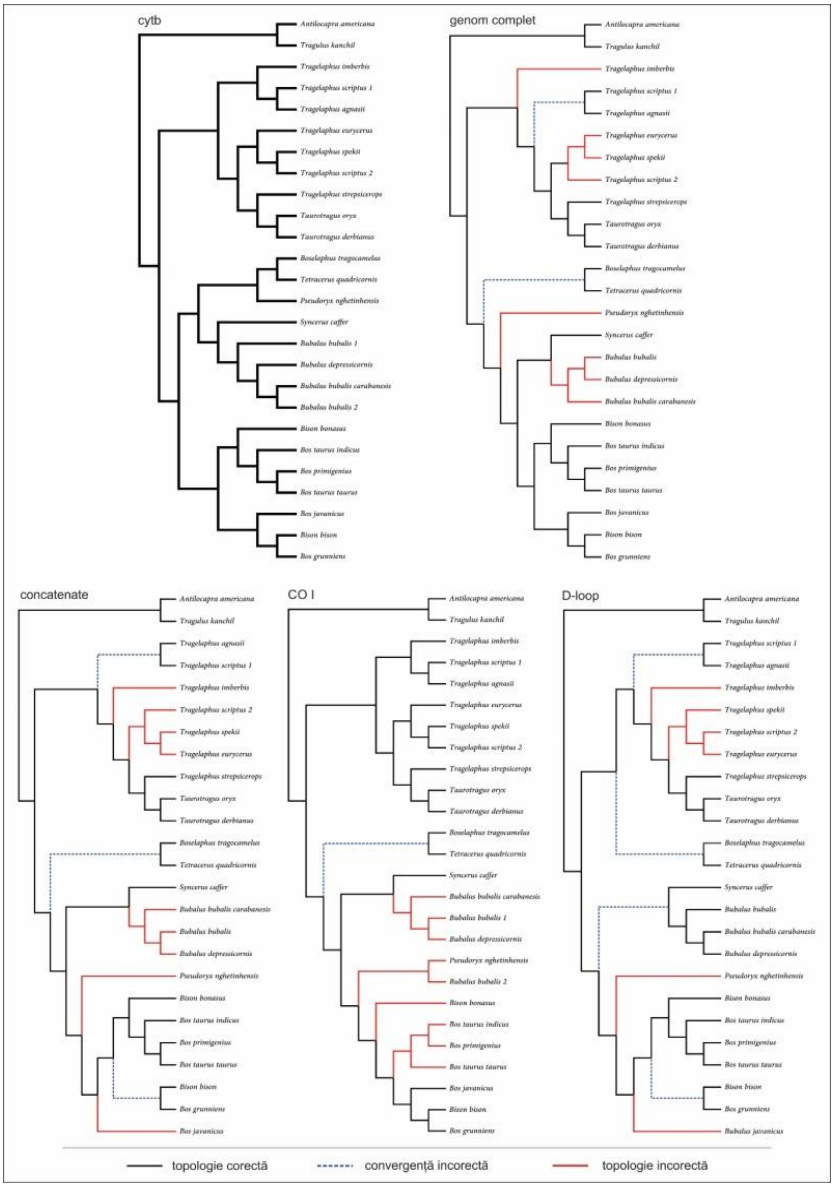

Figure14.The impact of each molecular marker on the MCC tree topology in comparison with the allegedly correct cyt b gene tree

3.2.3 Taxonomical uncertainties resolution within Bovinae subfamily

The first taxonomical uncertainty was represented by the correct position of *Pseudoryx nghetinhensis* (saola) species within the *Bovinae* subfamily. The analyzed data showed without doubt that saola was placed on the major clade of bovine, oxes/buffalo, within the *Bovinae* subfamily. Also, this species evolved from a common ancestor with Boselaphine. Thus, the following classification is proposed: Bovinae subfamily; *Bovini*tribe; *Pseudoryina* subtribe; *Pseudoryx nghetinhensis* species.

The second taxonomical uncertainty consists in the placement of Boselaphine tribe in Bovinae subfamily. According to the obtained topology based on the cyt b gene, it has been observed that the 2 targeted species (*Boselaphus tragocamelus* and *Tetracerus quadricornis*) were placed on the bovine main clade, more accurately on the oxes/buffalo clade.

The last taxonomical uncertainties regarded the 2 bison species (European bison and American bison), their placement within Bovinae subfamily and the relationships between them. Archaeological studies state that the 2 bison species evolved from a common ancestor (*Bison proscus*) and they must be placed on the same clade. The present study showed that based on the topology of the cytochrome b gene, but also for the other mitochondrial markers analyzed, the *Bison* genera is paraphyletic. This fact confirms the hypothesis stated by Prusak et al., in 2004 and reveals that the analyzed species, *Bison bonasus* and *Bison bison*, were grouped on separate clades with individuals belonging to *Bos* genera (Figure 16).

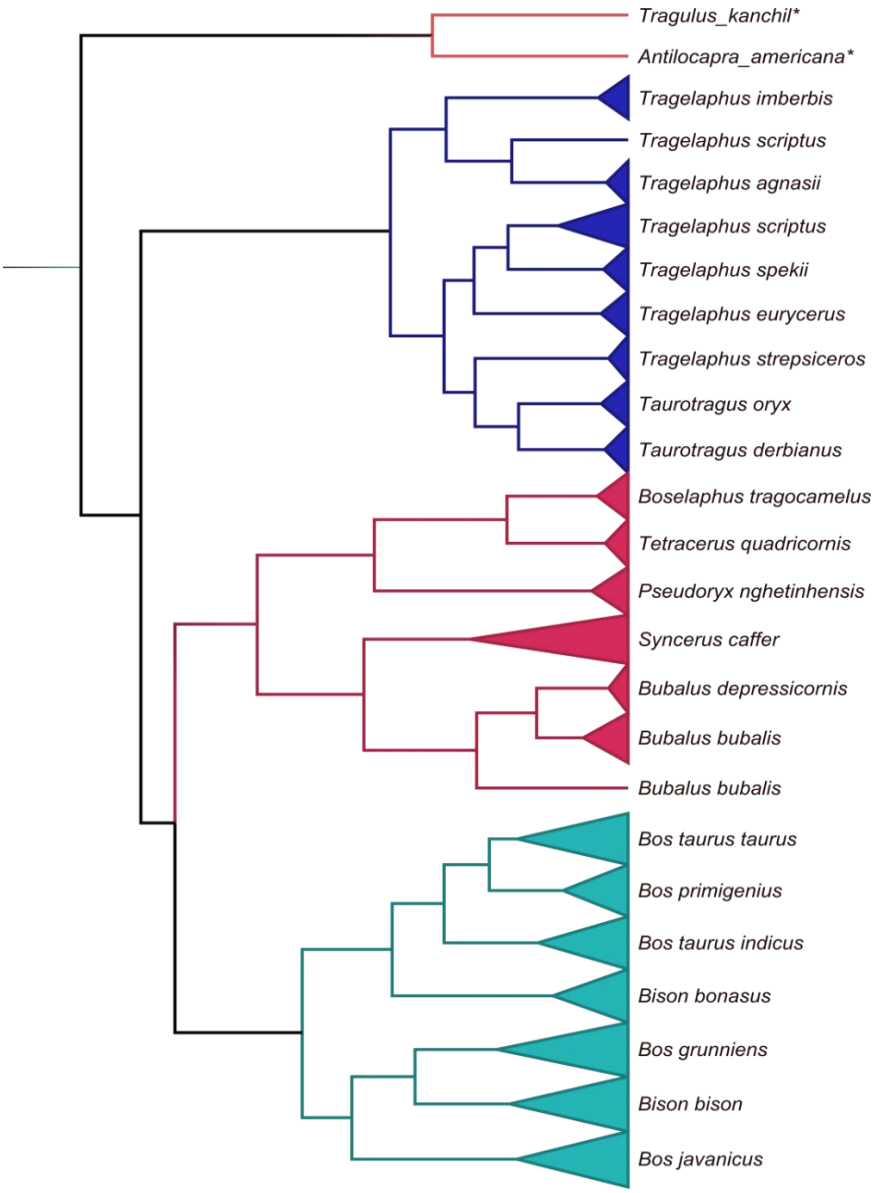

Figure16. Phylogenetic tree obtained by cytochrome b gene analysis, the mitochondrial marker that can accurately describe the phylogenetic relationships within *Bovinae* subfamily

3.2.4 Genetic distance between the European bison (*Bison bonasus*) and the species belonging to Bovinae subfamily

A high level of similarity was observed by estimating the genetic distance between the European bison and other related species. *Bison bonasus* and *Bos indicus* for example are closely related (Figure 17). Likewise, a relatively low number of differences was observed between the European bison and the common cow (*Bos taurus*) (0.069), or with *Bos primigenius* (0.068). Also the European bison has a high level of similarities with species that belong to the *Bos*genera and a high divergence for its alleged relative belonging to *Bison* genera.

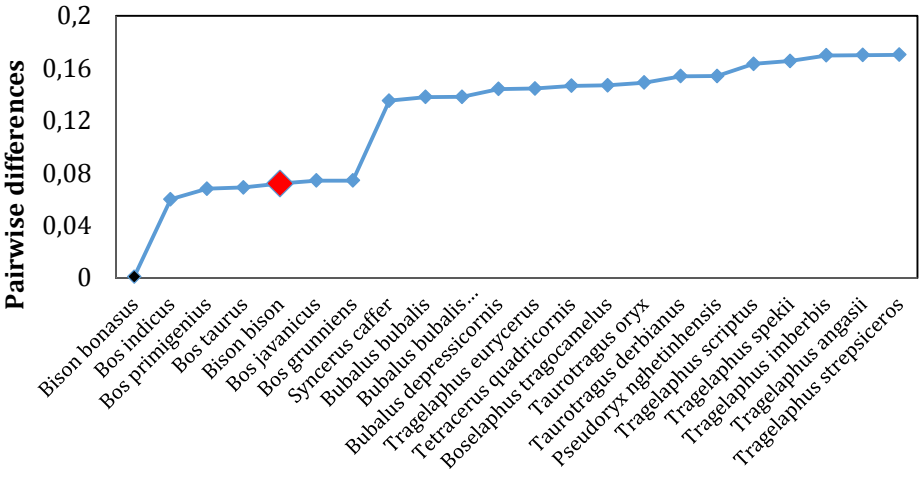

Figure17.Genetic differences between the European bison and its relatives

CONCLUSIONS

The results of our investigations regarding the quantification of the genetic variability of two European bison populations from Romania and the identification of the phylogenetic relationships within Bovinae subfamily lead us to the next important conclusions:

1. Mitochondrial markers (*cyt b, cox I* and *D-loop*) and nuclear markers (BM 2830 and TGLA) are especially important for evidencing the genetic differences between the individuals of *Bison bonasus* species;
2. The mitochondrial DNA sequence analysis for the *Bison bonasus* individuals has shown the presence of 5 haplotypes, 3 local haplotypes (specific for the individuals from Romania) and 2 haplotypes for the specimens from Poland;
3. The high frequency of haplotype H1, for both current and extinct populations, indicates preservation of the pre- and post-"bottleneck" lineage and in the same time a certain degree of uniformity of current populations. Also, the absence of haplotype H3 from the current populations and its presence in only one bone sample suggests that H3 is an extinct haplotype;
4. The haplotype network identified for the studied individuals shows that all current haplotypes are derived from a main haplotype (ancestor), which belongs to the 12 founder specimens. The main haplotype was kept in the founder populations and afterwards was diversified by mutation processes in order to form the other secondary haplotypes;
5. The performed research has shown that the sequences for the mitochondrial marker *cox I* didn't present any polymorphism and it can be considered that the use of only this gene in this type of studies regarding *Bison bonasus* species can lead to inconclusive results when the genetic variability is analyzed.
6. Our results prove that the analyzed individuals belong exclusively to the Lowland-Caucasius lineage, also we can confirm the

existence of the two separate lineages: Lowland-Caucasius in Romania and Lowland in Poland.

7. The molecular analysis regarding the genetic diversity, demographic and spatial expansion of the individuals that belong to the 2 populations from Romania, showed an increase in the heterozygosity, a fact that demonstrates the spatial and temporal sustainability of these populations. This sustainability is also favorable for the reintroduction of this species in the wild.

8. Both Vânători Neamț and Neagra Bucşani bison populations showed a favorable heterozygosity (observed and expected) and a genetic structure well defined. These facts are influenced by the man mediated breeding that carefully selects the specimens which are genetically valuable.

9. Microsatellite loci analysis (BM2830 and TGLA) indicated that most of the allele is common for both studied populations. Still the presence of private alleles (that are specific only for one population) showed a genetic differentiation of the bison from these populations.

10. The molecular investigations performed show that the mitochondrial marker *cyt b* is the best to assess the phylogenetic relationships within Bovinae subfamily. Also it has been shown that the use of inadequate molecular markers can lead to errors regarding time divergence estimation of certain taxa of clades.

11. Phylogenetic analysis within Bovinae subfamily revealed that *D-loop* is the mitochondrial marker with best topological support expressed by the high value of the total posterior probability of the clades. In the same time, this research proves that for a correct quantification of the phylogenetic relationships within this subfamily and a correct estimation of the divergence time, is not enough just the use of a tree with high topological support. This result leads us to the incapacity of estimating correctly the clades age.

12. A Bayesian approach was used in order to understand and clarify the taxonomical uncertainties within Bovinae subfamily, analyzing concatenated and individual mitochondrial markers of the

studied species. The data showed the evolution from a common ancestor of *Boselaphus* tribe and *Pseudoryx nghetinhensis,* the two of them were grouped in a basal clade, positioned in the major clade of cattle-bovines.

13. The phylogeny based on our analysis supports the paraphyletic evolutionary hypothesis of *Bison* genus. This paraphyletic positioning of the two species is also confirmed by the low level of genetic similarities between *B. bonasus* and *B. bison* species.

14. The phylogenetic and phylogeographical studies performed confirmed the hypothesis that Bovinae subfamily was formed in central Africa approximately 12 million years ago and the major radiation of species took place during Pliocene.