

# PHYLOGENETIC RELATIONSHIPS BETWEEN ROMANIAN GREY STEPPE CATTLE AND PODOLIAN CATTLE BASED ON mtDNA SEQUENCES ANALYSIS

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## Abstract

The main purpose of this study was to analyze the phylogenetic relationships between the endangered Grey Steppe cattle and other podolian cattle breeds, including a common ancestor of the wild ox, *Bos taurus primigenius*. The biological material was represented by 32 blood samples collected from the Grey Steppe, from which DNA was extracted to analyze two mitochondrial markers (cytochrome *b* and *d*-loop), relevant for genetic diversity studies, phylogeny, and molecular phylogeography. After PCR amplification of the two molecular markers, sequencing was carried out using the Sanger technique, thus obtaining the nucleotide sequences on the basis of which the phylogenetic tree was built, showing the phylogenetic relationships between the studied breeds. The obtained results demonstrate the belonging of this breed to the *Bos taurus* genus and the close phylogenetic relationships with other podolian cattle. The PQT haplotype, the closest haplotype to the wild ancestor, was predominant among the analyzed individuals. These results are important for efforts to conserve the genetic resources of Grey Steppe cattle.

**Key words:** genetic diversity, Grey Steppe, mtDNA, phylogeny, podolian

## INTRODUCTION

The importance of the protection and conservation of animal genetic resources in all countries of the world resides in the permanent concern of the United Nations Organization for Agriculture and Food - F.A.O. (Food and Agriculture Organization of the United Nations) through annual meetings with nominated specialists from each country and numerous publications presented periodically.

The Grey Steppe cattle breed, that is the subject of this research is an unimproved breed, with direct descent from *Bos taurus primigenius* and according to numerous studies in the specialized literature, it is in very close phylogenetic relationships with the podolian cattle breeds (*Iskar Grey*, *Bulgarian Grey*, *Istrian*, *Slavonian Podolian*, *Katerini*, *Hungarian Grey*,

*Maremmiana*, *Podolica*, *Turkish Grey*, etc), originating from Podolia, a region located in the central-western part of Ukraine [1,2,3]. The breed is closely related to the natural environment, which contributes almost exclusively to the imprinting of special biological qualities of rusticity, adaptability to severe climate and habitat conditions, and increased resistance to diseases [4,5].

In Romania, until the middle of the last century, the breed was predominant, and later, its share recorded a pronounced numerical decline. Currently, it is found in the form of crossbreds with a different degree of absorption, sporadically in the Danube Delta and within the Research and Development Station for Cattle Breeding, Dancu-Iasi (S.C.D.C.B., Dancu-Iasi), there is a nucleus of taurines, located in a national genetic conservation program. Several

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varieties are known, which were named after the name of the area of formation: *Moldavian*, *Transylvanian*, *Ialomita* and *Dobrogean variety*. The Transylvanian variety is characterized by large body development, followed by the Moldavian variety, which is located between medium-sized bulls, with the largest spread in the Moldova area in the past [6,7].

The revised bibliographic references [1,8,9,10,11,12], regarding the podolian cattle breeds, confirm that they are carriers of a reservoir of valuable genes that must be conserved by applying various modern reproduction biotechnologies such as embryo transfer or in vitro fertilization.

The national genetic conservation programs of the Grey Steppe cattle breed require concrete information regarding the genetic diversity, the phylogenetic relationships with the podolian cattle breeds, and evidence of the breed belonging to the origin of the wild ancestor *Bos taurus primigenius*.

Therefore, the main purpose of this study was to analyze the phylogenetic relationships between endangered Grey Steppe cattle and other podolian cattle breeds, including a common ancestor of the wild ox, in order to support the efforts of genetic conservation.

The results of this research demonstrate the belonging of this breed to the *Bos taurus* genus and its close phylogenetic relationship with other podolian cattle. The PQT haplotype, the haplotype closest to the wild ancestor, was predominant among the analyzed individuals.

The authors of this study can recommend the use of these results in order to develop some strategies regarding the genetic conservation of the Grey Steppe breed.

## MATERIAL AND METHOD

As part of this research, a series of genetic analyses was carried out to evaluate the genetic diversity of the Grey Steppe cattle. One of the main objectives was to

analyze two mitochondrial markers, cytochrome b and d-loop, based on which clear conclusions were drawn regarding the phylogeny.

Phylogenetic analysis of the breed was carried out by comparing the rate of evolution of the two mitochondrial markers with the rate of evolution of the complete mitochondrial genome, both for the breeds, species, and genus. The gene sequences resulting from the sequencing of the two mitochondrial markers in the Grey Steppe were also statistically processed and the results were compared with the gene sequences specific to the Podolian cattle (*Iskar Grey*, *Bulgarian Grey*, *Istrian*, *Slavonian Podolian*, *Katerini*, *Hungarian Gray*, *Maremmana*, *Podolica*, *Turkish Grey*). Based on the obtained results, a phylogenetic tree was constructed to show the phylogenetic relationships between these breeds and estimate the divergence time.

The biological material was represented by 32 blood samples collected from the Grey Steppe cattle, age range between 22-288 months, with an average of 134.5 months, within S.C.D.C.B., Dancu-Iasi (Figure 1).



Fig. 1 Grey Steppe cattle breed

The 32 analyzed samples were marked with SS1...SS32 (SS being the romanian abbreviation from the title of the breed-Sura Stepă).

To quantify the genetic diversity, a series of analyses were carried out in the Laboratory of Molecular Genetics, as follows: DNA isolation from blood samples using the

Maxwell™ 16 and 16 MDx instruments method, total DNA quantification using spectrophotometry with a NanoDrop Spectrophotometer, amplification of mitochondrial markers by Polymerase Chain Reaction (PCR), analysis of amplicons by electrophoresis in agarose gel, sequencing of the two markers by the Sanger method, statistical analysis of nucleotide sequences, and phylogeographic reconstruction of the studied breed in relation to the podolian cattle breeds, using statistical programs such as Mega X, SeaView, and DnaSP [13].

## RESULTS

According to the interpretation of spectrophotometric analysis, most biological substances have a characteristic absorption rate in the field of ultraviolet radiation. In the case of nucleic acids, it corresponds to an absorption rate with a wavelength  $\lambda=260$  nm, and in the case of proteins,  $\lambda=280$  nm [14,15].

The amounts of DNA extracted from the analyzed individuals varied in the value range 7.0 - 28.60 ng/ $\mu$ l (Figure 2).

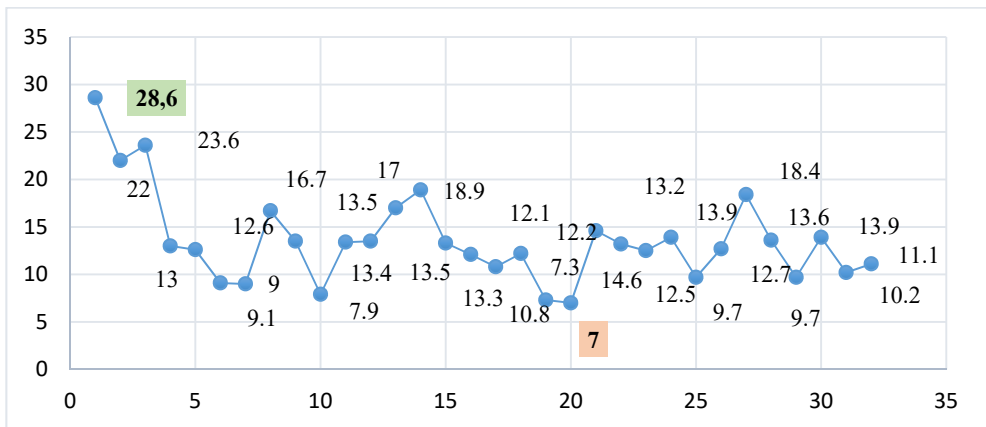


Fig. 2 DNA concentration values, measured with the Nanodrop spectrophotometer –Gray Steppe breed (ng/ $\mu$ l)

The ratio of the two absorption rates, A260/A280 being between 1.03 and 1.72. The average concentration of the DNA samples was 19.85 ng/ $\mu$ l, thus demonstrating the effectiveness of the applied extraction method as well as the purity of the samples.

Following the amplification of mitochondrial markers using the PCR method, PCR amplicons were obtained that were checked in a 1% agarose gel, performing migration at 70 volts for 45 min (Figure 3). The molecular weight marker used was 100 bp.

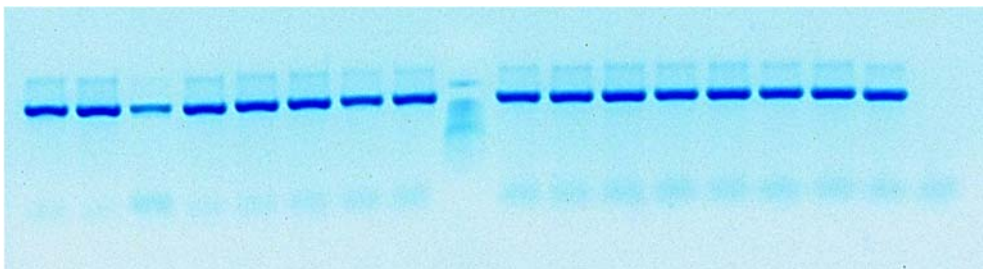
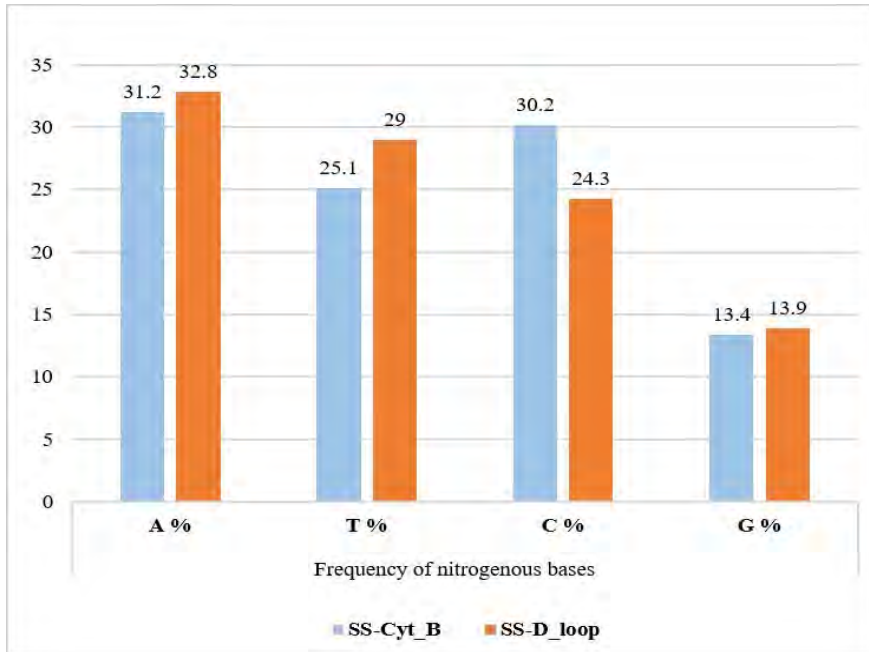


Fig. 3 Agarose gel electrophoresis of PCR amplicons

As can be seen from the figures above, non-specific products or other contaminants were not identified in the 32 samples analyzed. The alignment of the 32 nucleotide sequences of individuals from the Grey Steppe breed was carried out using

the MEGA X program and based on the SeaView program, the proportions of the 4 nitrogenous bases (A,T,C,G) were calculated for each individual and respectively for each sequence (Figure 4).



**\*Note:** Adenine-A; Thymine-T; Cytosine-C; Guanine-G

Fig. 4 Graphical representation of the frequency of nitrogenous bases

In the case of cytochrome b sequence analysis, the percentages of nitrogenous bases were of 31.2% for A, 25.1% for T, 30.2% for C, and 13.4% for G. Also, after sequence analysis using statistical programs for mitochondrial control region d-loop, the frequency of nitrogenous bases was 32.8% for A, 29.0% for T, 24.3% for C and 13.9%

for G. In order to estimate the relationships within the cattle population, based on the analysis of the nucleotide sequences of cytochrome b and the mitochondrial control region d-loop, the phylogenetic trees were made, with the help of the SeaView program, represented in figure 5.a for cytochrome b and 5.b for d-loop.

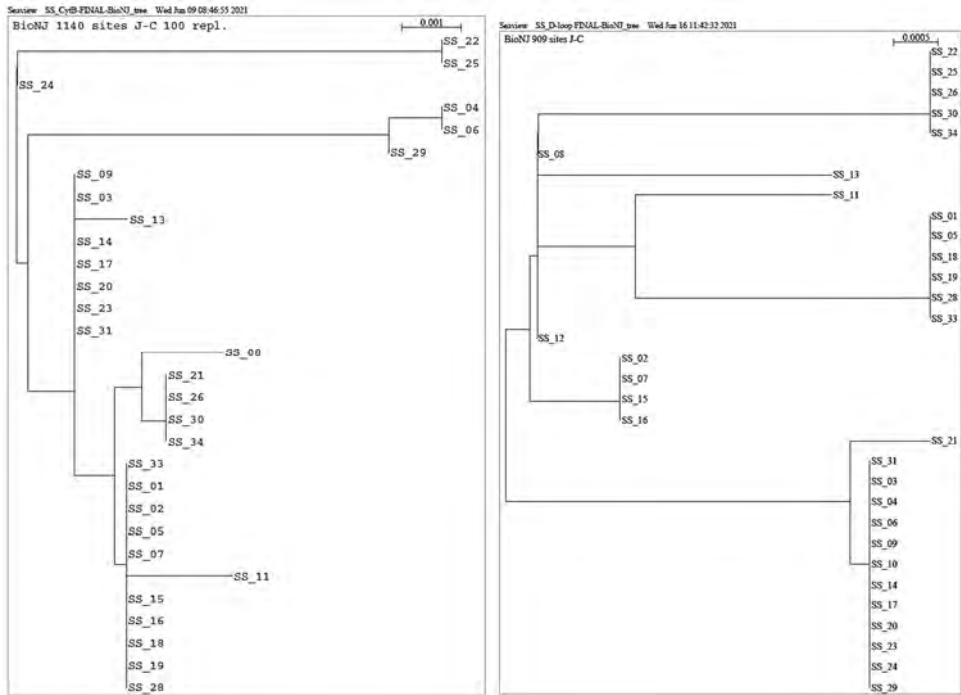


Fig. 5 a. Phylogenetic tree based on cytochrome b gene sequence analysis for the Grey Steppe; b. Phylogenetic tree based on d-loop gene sequence analysis for the Grey Steppe population

In addition, to estimate the divergence time within the Grey Steppe cattle breed population, a haplotype network was constructed. The distribution of haplotypes can be seen in table 1.

Table 1 Identified haplotypes and representative individuals for each haplotype

Haplotypes identified	Representative individuals	Total individuals/haplotype
T3/T4	SS_02; SS_03; SS_04; SS_06; SS_07; SS_08; SS_09; SS_10; SS_12; SS_13; SS_14; SS_15; SS_16; SS_17; SS_20; SS_21; SS_23; SS_24; SS_28; SS_30	20
T2	SS_01; SS_05; SS_18; SS_19; SS_27; SS_31	6
T1	SS_11	1
P'QT	SS_22; SS_25; SS_26; SS_29; SS_32	5

**DISCUSSIONS**

Recent studies have revealed that nearly all taurines are members of macro-haplogroup T and that the estimated time of

divergence was approximately 16 thousand years. This suggests that the evolutionary history of *Bos taurus primigenius* has a brief bottleneck. Macro-haplogroup T was



separated into two sister subclades (T5 and T1/T2/T3), with T1/T2/T3 serving as the most common. T4 was incorporated into T3 over time [16,17,18].

By examining the d-loop mitochondrial regulatory area of the northern and central European taurines, another haplogroup-P was discovered [19].

In this research, through the analysis of the distribution of the four haplotypes, it was found that the T3 haplotype had the highest weight, being identified for 20 individuals out of the total 32 that were analyzed.

According to a study by Achilli et al. (2009) on the origin of taurines based on mitochondrial genome analysis, not all taurines in Europe are members of haplogroup T. For 26 European cattle breeds (22 from Italy and 4 from other parts of Europe), the author examined the mtDNA gene sequences. Haplogroup T and its subclades comprised the majority of breeds. Of the examined breeds, 1.4 belonged to haplogroups P and Q, which are unique to ancient cattle from northern and central Europe and share a common ancestor with *Bos taurus primigenius* [20]. Following examination of the nucleotide sequences of the Grey Steppe cattle breed, the P'QT haplogroup was discovered. This suggests that this haplotype is ancestral and is unique to *Bos taurus primigenius*, from which these cattle breeds arose.

The findings of this study can help improve breeding programs and the state of knowledge on population genetic diversity.

## CONCLUSIONS

Sequencing of the cytochrome b gene and the d-loop mitochondrial control region revealed that they are particularly relevant in highlighting genetic differences and establishing phylogeny between individuals of a species.

The identification of the P'QT haplogroup following the analysis of the nucleotide sequences of the Grey Steppe cattle breed, indicates that this haplogroup

is of an ancestral type, being specific to the wild ox (*Bos taurus primigenius*), from which this breed evolved. Within this haplogroup, 16% of the total samples were analyzed, which could be used as the basis for the conservation and genetic consolidation of the breed in a pure state.

In order to actively conserve the Grey Steppe breed, the authors recommend the creation of a gene bank as well as the application of modern reproductive biotechniques within the population, such as embryo transfer and in vitro fertilization.

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