

## Genetic diversity among Romanian cattle breeds with a special focus on the Romanian Grey Steppe Breed

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### Abstract

The genetic diversity found in domestic breeds allows farmers to develop new characteristics in response to changes in the environment, the appearance of diseases, or of modifications in market conditions. Indigenous breeds often possess gene combinations and special adaptations (such as disease resistance, adaptation to harsh conditions or poor-quality feeds) not found in other breeds. Five Romanian cattle breeds (Romanian Spotted, Romanian Black Spotted, Romanian Brown, Montbeliarde and Romanian Grey Steppe) were investigated in order to characterize their genetic structure and to study their phylogenetic origin. Romanian Grey Steppe is an indigenous cattle breed, part of the Grey Steppe/Podolian group originating in the steppe of Ukraine. The observed heterozygosity per breed ranged from 0.580 (Montbeliarde) to 0.690 (Romanian Brown) and the expected heterozygosity from 0.711 (Romanian Spotted) to 0.778 (Romanian Black Spotted). Hardy-Weinberg equilibrium was tested for all breed-combinations and the exact P-value over all loci and breeds was not significant. Only 7% of the total genetic variability could be attributed to differences among breeds ( $F_{ST}=0.07$ ,  $p<0.01$ ). The Romanian Grey Steppe was clearly segregated from the other breeds in the NJ tree.

Keywords: Romanian Grey Steppe, microsatellite, genetic diversity, clustering.

### Introduction

Livestock breeds have been formed by centuries of natural and human selection. Breeds have been selected to fit a wide range of environmental conditions and human needs but the selection of a few highly productive breeds caused the decline of others breeds. The Food and Agriculture Organization of the United Nations (FAO) indicated that approximately 30% of the world's farm animal breeds were at risk of extinction.

In Romania, the last local cattle breed is the Grey Steppe which originates from the wild ancestor *Bos taurus primigenius* and is included in the Grey breed group encountered in various European countries (Ukrainian Grey, Hungarian Grey and Italian Grey, Yugoslavian Grey Steppe, Greek Grey Steppe, etc.). This breed was formed over the centuries, under the exclusive influence of natural conditions and of usage systems and specificity.

The number of individuals in the Grey Steppe breed in our country was prevalent until the middle of the 19th century, but during the last century a marked decline was registered. The breed used to be wide-spread through all the regions of our country, with the exception of the mountainous areas, including numerous ecological varieties (Moldavian, Transylvanian, the Ialomita variety and the Dobrudja variety). The Grey Steppe breed is on the brink of extinction in its pure form, thus the genetic fund of this breed must be conserved.

Microsatellites are the best markers for population genetic studies because they offer advantages which are particularly appropriate for conservation projects: they are widely available and exhibit a high degree of polymorphism. In addition, it is assumed they are neutral to selection, since the observed genetic diversity is constituted as the consequence of two forces: genetic drift and mutation.

Studies of genetic relationships between cattle breeds using microsatellites provide useful information on the evolution of breeds, gene pool development and the magnitude of genetic differentiation (MacHUGH & al. 1998 [1], DEL BO & al. 2001 [2], MATEUS & al. 2004 [3]).

The current study was carried out in order to characterize genetic diversity and phylogenetic relationships among five cattle populations: Romanian Grey Steppe, Romanian Spotted, Romanian Black Spotted (Romanian Holstein-Friesian), Romanian Brown and Montbeliarde, based on allelic frequencies for 11 microsatellite loci. This analysis included the distribution within and between breeds of the observed genetic variation, and a phylogenetic analysis of breed assignment from microsatellite allele frequencies.

## Materials and Methods

### Sampling and DNA extraction

Fresh blood was collected from 190 individuals in the following bovine populations: Romanian Spotted (RS), Romanian Black Spotted (RBS), Romanian Brown (RB), Montbeliarde (M) and Romanian Grey Steppe (RGS). The individuals were chosen at random and we avoided closely related animals (Table 1). The isolation of genomic DNA from fresh blood was performed with Wizard Genomic DNA Extraction Kit (Promega).

**Table 1.** Population size sampled.

Breed	Number of animals sampled		
	F	M	Total
Romanian Spotted (RS)	30	10	40
Romanian Black Spotted (RBS)	30	10	40
Romanian Brown (RB)	32	8	40
Montbeliarde (M)	32	8	40
Romanian Grey Steppe	22	8	30

### Microsatellite analyses

Amplification of the microsatellite loci was realized by multiplex PCR using StockMarks® for Cattle Bovine Genotyping Kit (AppliedBiosystems) and it was carried out using a GeneAmp 9700 System (AppliedBiosystems). PCR products were detected by capillary electrophoresis using an ABI Prism 310 DNA Genetic Analyzer (AppliedBiosystems). The size of alleles was determined by using GeneScan-500 ROX Size Standard and the results were processed with the GeneScan®3.1.2 and Genotyper®2.5.2 Software (AppliedBiosystems). As a reference we used the DNA control from the kit.

The 11 microsatellite markers included in our study and their sizes for each breed are shown in Table 2.

### Statistical analysis

We estimated the allelic frequencies, and the observed and expected heterozygosities ( $H_o$  and  $H_e$ ) using the CERVUS 2.0 program (MARSHALL & al. 1998 [4]). An exact test was used to determine deviations for the Hardy-Weinberg equilibrium (HWE) using the computer program GENEPOP 4.0 (ROUSSET & al. 2007 [5]). Exact P-values were

calculated using the complete enumeration method (LOUIS & al. 1987 [6]) for loci with fewer than five alleles or using the Markov-Chain algorithm for loci with more than five alleles (1000 dememorization steps for 100 batches and 5000 iterations per batch). In addition, we tested the significance of difference between breeds, on the basis of genic and genotypic differentiation, and also the significance of genic and genotypic differentiations for all pairs of populations.

Wright's F coefficients ( $F_{IT}$ ,  $F_{IS}$  and  $F_{ST}$ ) were calculated using FSTAT software (GOUDET & al. 1995 [7]). The neighbor-joining tree topology was obtained with the PHYLIP 3.5 statistical package (FELSENSTEIN & al. 1989 [8]) using the Reynolds ( $D_R$ ) genetic distance. Bootstrap values were computed over 1,000 replicates.

**Table 2.** Cattle specific loci and their sizes.

Microsatellite loci	Size obtained (bp)				
	RS	RBS	RB	M	RGS
TGLA227	72-100	76-104	68-98	76-100	76-100
BM2113	118-140	116-140	116-140	128-136	116-136
TGLA53	150-184	148-182	154-180	150-190	150-182
ETH10	210-220	200-222	214-220	210-218	198-222
SPS115	242-264	242-254	242-254	242-250	240-262
TGLA126	114-126	114-124	116-126	114-120	112-126
TGLA122	138-160	134-164	138-164	138-162	138-170
INRA23	198-212	198-212	198-216	196-216	198-214
ETH3	114-126	114-128	106-128	114-126	114-126
ETH225	134-146	134-146	134-144	134-144	134-144
BM1824	178-192	178-200	178-190	176-190	178-188

## Results and Discussions

### Level of variation and HWE

A total of 125 distinct alleles were detected across the 11 loci analyzed (Table 3). All the loci were polymorphic and the number of alleles varied between four (ETH10, SPS115, TGLA126) and 12 (TGLA122, TGLA53).

**Table 3.** The number of alleles per locus in each population.

Locus	RS	RBS	RB	M	RGS	Total
TGLA227	12	13	10	7	5	16
BM2113	9	11	11	5	8	12
TGLA53	11	11	8	12	8	16
ETH10	4	8	4	4	6	9
SPS115	8	5	5	4	6	12
TGLA126	7	6	5	4	7	8
TGLA122	8	12	9	8	5	15
INRA23	7	7	6	8	8	11
ETH3	7	8	9	7	7	10
ETH225	6	5	5	6	5	7
BM1824	7	7	6	7	5	9

Observed and expected heterozygosities per breed ranged from 0.580 (M) and 0.711(RS) to 0.690 (RB) and 0.778 (RBS), respectively (Table 4). HWE was tested for all

breed combinations. When results were pooled across breeds, two microsatellites offered a deviation, BM2113 ( $p < 0.05$ ) and TGLA53 ( $p < 0.01$ ); and when pooled across loci no population gave a significant deviation ( $p > 0.05$ ).

The results obtained by the Hardy-Weinberg test demonstrate that all the five bovine populations are in equilibrium, without any digressions from it.

**Table 4.** Observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities over 11 microsatellites in five bovine populations.

Population	$H_O$	$H_E$	MNA
Romanian Spotted	0.593±0.163	0.711±0.127	7.818±2.227
Romanian Black Spotted	0.641±0.151	0.778±0.083	8.454±2.841
Romanian Brown	0.690±0.140	0.746±0.088	7.181±2.358
Montbeliarde	0.580±0.239	0.725±0.159	6.636±2.419
Romanian Grey Steppe	0.687±0.216	0.763±0.054	6.363±1.286

#### F-statistics

The  $F_{ST}$  values indicate that around 7% of the total genetic variation could be explained by the breeds' differences and the remaining 93% may correspond to differences among individuals (Table 5). The  $F_{ST}$  values for single loci varies from 0.02 (TGLA227) to 0.208 (ETH10). On average, each of the four breeds had a 15% deficit of heterozygotes, whereas the entirety of individuals had a 21% deficit of heterozygotes.

The genetic variation (7%) observed between the breeds studied is comparable with the differentiation observed between the European beef cattle breeds (7%) (CANON & al. 2001 [9]), but is lower than that observed in Portuguese cattle breeds (9%) (MATEUS & al. 2004 [3]), and in north-eastern Asian cattle (10.9%) (KIM & al. 2002 [10]).

In comparison with other animal species, this difference is lower than that observed in some horse breed: the Norwegian horse breed, 12% (BJØRNSTAD & al. 2000 [11]), German draught horse breeds 11.6% (ABERLE & al. 2004 [12]), Spanish Celtic horse breeds 8% (CANÓN & al. 2000 [13]), Romanian horse breeds 12.3% (GEORGESCU & al. 2008 [14]); in dogs (9.9%) (JORDANA & al. 1992 [15]) and goats (17%) (SAITBEKOVA & al. 1999 [16]), but is greater than that found among Spanish donkey breeds (3.6%) (ARANGUREN-MENDEZ & al. 2001 [17]).

**Table 5.** F statistical estimates and their significances by locus.

Locus	$F_{ST}$	$F_{IS}$	$F_{IT}$
TGLA227	0.02	0.085	0.103
BM2113	0.068	0.322	0.368
TGLA53	0.075	0.593	0.623
ETH10	0.208	0	0.208
SPS115	0.047	0.042	0.087
TGLA126	0.036	0.291	0.317
TGLA122	0.046	0.092	0.134
INRA23	0.123	0.148	0.253
ETH3	0.053	-0.004	0.05
ETH225	0.052	-0.04	0.013
BM1824	0.031	0.045	0.075
<b>Mean</b>	<b>0.07</b>	<b>0.151</b>	<b>0.21</b>

The global population differentiation tests (genic and genotypic) showed highly significant ( $p < 0.01$ ) results for all 11 loci. Highly significant ( $p < 0.01$ ) genetic differences for

all breeds combinations were shown through pair-wise tests. The  $F_{ST}$  values ranged from 3.56% for the RS-M pair to 1.13% for the RB-RS pair (Table 6).

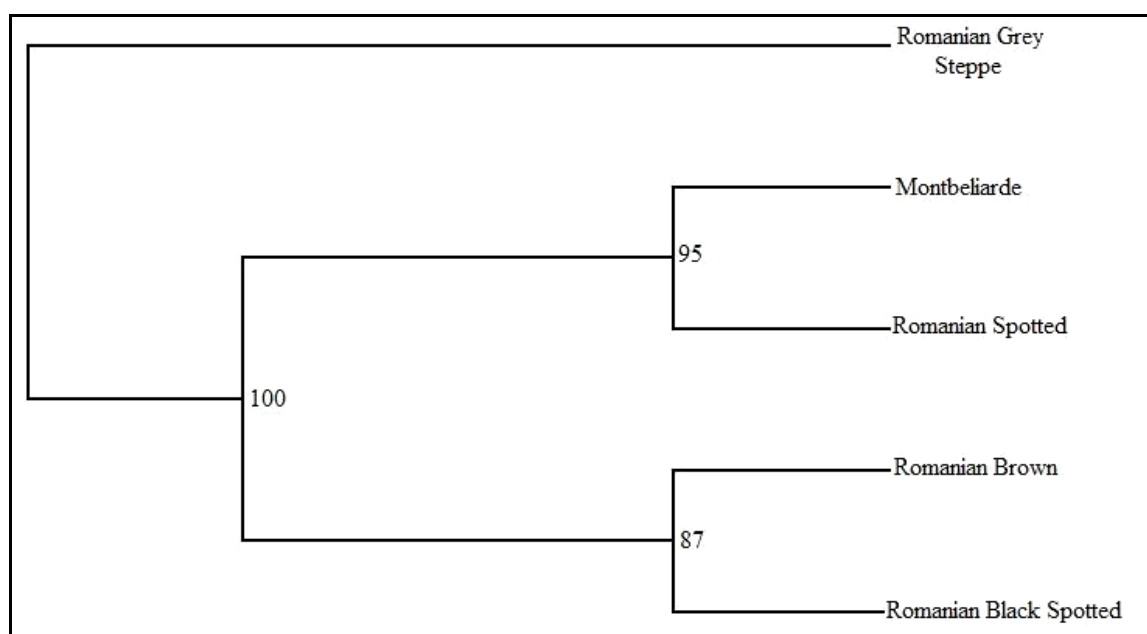
**Table 6.** Fst estimates compared in pairs (above diagonal) and Reynolds's genetic distance (below diagonal).

	RS	RBS	RB	M	RGS
RS		0.0662	<b>0.1132</b>	0.0356	0.0660
RBS	0.083546		0.0522	0.0675	0.0400
RB	<b>0.131480</b>	0.071088		0.1054	0.0696
M	0.056757	0.087499	0.126578		0.0753
RGS	0.090666	0.065676	0.095277	0.102635	

#### Breed relationships

The  $D_R$  distance ranged from 0.056757 to 0.131480. The two smallest distances for the pair M-RS and the largest distance between RB and RS were calculated (Table 5).

A NJ tree based on Reynolds's genetic distance relating the five cattle populations is presented in Figure 1. The numbers of the nodes are bootstrapping values for 1000 replicates of the 11 loci genotyped.



**Figure 1.** Neighbour-joining dendrogram of genetic relationships among five cattle breeds using  $D_R$  genetic distances based on 11 microsatellite loci. The numbers on the nodes are percentage bootstrap values in 1000 replications.

The phylogenetic tree obtained using the NJ method based on Reynolds' genetic distances shows that the Romanian Grey Steppe breed is clearly distinct from the other four cattle populations, which form two distinct clusters. Romanian Grey Steppe is part of the Grey Steppe/Podolian group which originates in the Steppe of Ukraine from where it moved westward and immigrated into Italy in ancient times, where it formed the Podolian cattle group.

The two clusters are in agreement with the expected relationships between breeds. Thus the Montbeliarde breed, also known as the French Dairy Simmental, belongs to the Simmental breed group, just as the Romanian Spotted and they form the first cluster. This group includes a variety of breeds such as Fleckvieh from Austria, Abondance from France,

Pezzata Rossa from Italy, Masny Simmental from the Czech Republic, etc. In addition, the Romanian Brown and Romanian Black Spotted breeds are included in a separate cluster. This conclusion is logical however, since the Romanian Brown breed was formed by absorption cross-breeding between the Schwyz breed in Switzerland and the local cattle breeds of the Grey Steppe and Mocanitsa type. Subsequently, this breed participated in the formation of the Romanian Black Spotted breed together with other imported breeds (Holstein) and local breeds (Dobrudja Red, Brown and Romanian Spotted).

## Conclusions

Assessment of genetic variability in domestic animals is an important issue for the preservation of genetic resources and the maintenance of future breeding options, in order to satisfy the demands of changeable markets. Conservation policies of native breeds will depend to a large extent on our knowledge of historic and genetic relationships among breeds, as well as on economic and cultural factors.

The data obtained from this study clearly demonstrate the ancient origin of this local breed. Taking into account the fact that currently this breed is endangered in its pure form, taking steps in order to save it is clearly required. Since this breed could be considered a 'biological treasure', the genetic fund of the breed should be preserved through measures taken for the inventory of the genetic material, and by centralizing the individuals in breeding centers using specific exploitation technologies, as well as structuring the individuals into lines and adopting well-balanced breeding programs and limiting to a minimum the selection and in-breeding.

These data confirm that the Romanian Grey Steppe breed is an ancient one which has undergone a divergence from the common branch before the other breeds. Our results are going to be completed through the mtDNA analysis and through construction of phylogenetic trees based on mtDNA sequences.

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