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Genetic Characterization of Romanian Local Breeds Using Microsatellite Markers

Georgescu Sergiu Emil and Costache Marieta
University of Bucharest, Faculty of Biology
Department of Biochemistry and Molecular Biology, Splaiul Independentei, Bucharest
Romania

1. Introduction

The rapid evolution of civilization led to a loss in the genetic diversity of domestic animals. Due to the need to exploit the highly productive breeds, with production characteristics features constantly improved in time, the less productive local breeds have been neglected, changed by crossbreeding or even replaced. This practice has become extremely dangerous because it endangers the possibility of future improvement and development of domestic animals. In the future, such practice may affect the durability of genetic resources conservation and improvement because, together with the populations' disappearance, the species genetic pool is critically reduced.

During the last years, the issue of conserving the genetic diversity as a component of the conservation of the environment has been raised at an international level. In this respect, one of the main aspects of scientific research activities is conserving the biodiversity of local breeds, especially those of economic interest. FAO (Food and Agriculture Organization) included the issue of conservation, evaluation and use of animal genetic resources in its fields of interest four decades ago. In this context, a list of domestic breeds threatened with extinction has been published (World Watch List for Domestic Animal Diversity).

Some of the most important molecular markers used to study genomes are VNTR (Variable Number Tandem Repeat) and SNP (Single Nucleotide Polymorphism). They can be highlighted through various techniques such as: RFLP (Restriction Fragment Length Polymorphism) analysis, SSCP (Single-Strand Conformation Polymorphism), genotyping fluorescent labeled fragments or sequencing. Microsatellites are useful 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.

Worldwide, there is a special concern regarding the evaluation and genetic characterization of local populations, the so-called rare animal breeds. Most of the studies performed refer to the breeds' genetic characterization and the identification of the phylogenetic relations among them. Due their characteristics (high polymorphism, a higher power of discrimination comparative to other genetic markers, codominant Mendelian inheritance,

easily amplified by PCR), microsatellites prove themselves very useful for genetic characterization of farm animals. During recent years, a whole series of studies was performed regarding horses (Canon et al., 2000, Tozaki et al., 2003, Aberle et al., 2004), bovines (Del Bo et al., 2001, Kim et al., 2002, Mateus et al., 2004), swine (Li et al., 2000, Fabuel et al., 2004) and sheep (Arranz et al., 2001, Baumung et al., 2006).

Microsatellites are tandem repeats present in all the genomes of vertebrates. They are short repetitive sequences, of 2-9bp, dispersed throughout the entire genome. Their frequencies are increased in non-coding regions. It was noted that certain categories of repetitions are encountered with a much higher frequency, in which case repetition of the type (CA)n is the most popular. This class of repetitive elements shows a high polymorphism and is species-specific. Microsatellite is the term usually used when the length of the repeating unit is bellow 10bp, and the term of minisatellite is used when the repeating unit is between 10 and 100bp.

Microsatellites have the highest variability among DNA sequences originating in the genome, since their polymorphisms are derived from fragment length and not from primary sequence. The cause of variation between individuals of the same species at the microsatellites or minisatellites level consists in the different number of the repetitive unit. The rate of mutations in the minisatellite/microsatellite sequences is very high (about 10^{-4} per kb) and the frequency of exchange for each locus is assumed to be proportional to the minisatellite/microsatellite length. The high variability makes them especially useful for genomic mapping, because there is a high probability of individual variation in their alleles for each locus.

2. Study of microsatellite markers

2.1 Microsatellite analysis

The study of these markers implies the analysis of sets comprising over 10 microsatellites, each set being species-specific. Their analysis is done by individual genetic profile, determining the interrelations between different individuals and evaluating their allele frequency in populations. Microsatellite analysis involves the following steps: i) sample collection; ii) DNA extraction, purification and evaluation; iii) multiplex PCR amplification; iv) evaluation of amplified fragments by electrophoresis in polyacrylamide gels or by automatic genotyping using capillary electrophoresis and amplicons fluorescence detection.

The biological materials used consist in blood samples collected on anticoagulant or hairs. DNA extraction from biological samples can be done using specific kits or conventional processes.

The study of microsatellite markers will involve analysis of sets comprising 10 to 17 microsatellites, each set being species-specific. Their analysis is done by individual genetic profiling (genetic fingerprinting), determining the interrelations between different individuals and evaluating their allele frequency in populations. Microsatellite sets will be selected based on of relevance in terms of population analyses from the international databases. A microsatellite analysis test consists in extracting DNA, multiplex PCR amplification, capillary electrophoresis and fluorescence detection in the fragments amplified. In the case of the genotyping technique, first a multiplex PCR reaction is done

using genomic DNA, followed by capillary electrophoresis and fluorescence detection in the resulting product. For the multiplex PCR reaction we use a combination of several primers with specific sequences of fragments of interest, resulting in simultaneous amplification of these areas. The method for amplification should use primers with hybridization temperatures similar to the DNA sequences of interest. The size of amplified fragments is identified using an automated genetic analyzer system using high-resolution separation by capillary electrophoresis. For each DNA marker alleles are detected and then genotyped by comparison with international standardized sets of markers. One of the primers of each microsatellite is marked with an available fluorescent dye to enable the multiplex analysis of markers in a single reaction. The resulting products are compared with molecular weight standards that ensure the accuracy and precision of determinations.

2.2 Statistical analysis

For a neutral marker, the degree of polymorphism is proportional to the mutational rate. Rates of mutations and their effects are important factors in the calculation of genetic distance based on data obtained from microsatellite analysis. Researchers can determine the period of time passed since the separation of two populations or measure the degree of alleles transfer between them by applying theoretical models of empirical data obtained. Thus, they are able to establish mathematical models that enable them to assess genetic diversity and phylogenetic relationships between different populations.

Genetic diversity is given by multiple alleles and genotypes established for a study group (population, species or species group). According to the Hardy-Weinberg equilibrium principle, allele frequencies and genotypes in a population remain constant - meaning that they are in equilibrium - from generation to generation, except in the case of outside influences. These influences could be controlled by mating, mutations, small populations, genetic drift and gene exchange. Hardy-Weinberg equilibrium is extremely important in conservation studies and genetic evolution. It provides basic information to identify random mating, mutations occurrence or inbreeding effects. Deviations from expected values for the Hardy-Weinberg equilibrium can have several causes, such as low population size, inbreeding, or presence of null alleles among populations that can lead to an excess of false homozygosity. Analysis of a larger number of loci can provide an accurate picture of genetic diversity because each locus will contain an independent history of the population which depends on the proportion of mutations, the genetic drift or migration.

Heterozygosity is one of the most important parameters that can give us information about diversity and even the history of a population. Values vary from 0 (absence of heterozygosity) and 1 (where a large number of alleles have the same frequency). Higher values of average heterozygosity are equal to high levels of genetic variation. Conversely, if the average heterozygosity is reduced, genetic diversity is also reduced.

Over time, as a result of human intervention, many populations of animals around the world have been fragmented. The impact of fragmentation on genetic diversity, inbreeding and extinction risk of these populations depends largely on the gene exchange between different occurring sub-populations. Subsequent to the fragmentation of a population, there are gradual differences between these sub-populations. The degree of differentiation between different sub-populations is directly correlated with inbreeding coefficients in both populations and interpopulations.

The inbreeding coefficient of the whole population (F_{IT} = Factor of Inbreeding in the Total population) can be divided into: i) inbreeding coefficient of individuals in relation to a sub-population which includes individuals (F_{IS} = Factor of Inbreeding relative to sub-population); ii) inbreeding coefficient due to differences between sub-populations, to the whole population at baseline (F_{ST} = Factor of Inbreeding Relative to Total Population). F_{ST} is decreased when the exchange of genes between subpopulations is large. If the exchange rate drops, F_{ST} grows and subpopulations are separated and distinguished from one another (Weir & Cockerham, 1984). Regarding F_{IS}, a positive value shows a deficit of heterozygosity. The deficit is even greater as the value obtained is higher, which means a high level of inbreeding. In general, the values obtained for F_{IS} vary between -1 (no inbreeding) to 1 (complete identity).

2.3 Construction of phylogenetic trees based on microsatellite frequencies

The analysis of phylogenetic relationships is based on the definition of that sequence of steps (algorithms) which can build the best phylogenetic tree. A phylogenetic tree is the graphic representation of the phylogeny of a group of organisms. To obtain phylogenetic trees the following algorithms must be followed: obtaining microsatellites data, comparing the data, selecting optimal phylogenetic methods, constructing and evaluating the trees.

To study the phylogenetic relationships between closely related species or populations microsatellites are the markers to be used. Based on their frequency, genetic distances are calculated and are subsequently used to build phylogenetic trees. Some of the genetic distances used are the following: the Nei standard distance (Nei, 1972), the Cavalli-Sforza distance (Cavalli-Sforza and Edwards, 1967) and the Reynolds genetic distance (Reynolds et al., 1983). The calculation of these three distances is based on similar assumptions that admit that the differences between populations are largely due to genetic drift.

Thus, the evaluation of Nei standard distance (DS) is performed based on two assumptions: i) all loci have the same neutral mutation rate and genetic variability is due, in equal proportions, to both mutations and genetic drift, and ii) population size remains constant in time. In this context, distance is expected to increase proportionally with time. The other two genetic distances are evaluated without considering the mutational process and based on the assumption that allele frequency differences are due to genetic drift only in a population whose size is not kept constant over time. Thus, distance does not increase proportionally with time, but with the ratio 1/N (where N is the population size).

After calculating the genetic distance, a phylogenetic tree can be built using several methods, including Neighbor-Joining or UPGMA (Unweighted Pair Group Method with Arithmetic Mean).

3. Genetic characterization of some Romanian local breeds using microsatellites

The rapid evolution of civilization has led to a loss of genetic diversity in farm animals. Apart from the exploitation of very productive breeds, with constantly improved production features, there is an increase tendency to overlook or to simply replace the less productive indigenous species. This situation endangers the possibilities for any future improvement and development of farm animals. In the future, such practice may affect the

durability of the conservation and improvement of genetic resources because, together with the populations' disappearance, the species genetic pool is seriously reduced.

The assessment of genetic variability in domestic animals is an important issue for the preservation of genetic resources and the maintenance of protection of future breeding options, in order to satisfy the demands of changing market needs. Conservation policies of native breeds will depend on an increase of our knowledge about historic and genetic relationships among breeds, as well as on cultural factors.

During recent years, the issue of conserving the biodiversity as a component of the conservation of the environment has been raised at an international level. In this respect, one of the main aspects of scientific research activities is conserving the biodiversity of local breeds, especially those of economic interest. FAO included the issue of conservation, evaluation and use of animal genetic resources in its fields of interest four decades ago. In this respect, a list of domestic breeds threatened with extinction has been published (World Watch List for Domestic Animal Diversity). In Romania it was determined several domestic populations and breeds are threatened with extinction, needing urgent rescue and conservation measures. Such populations are the following: Grey Steppe (cattle), Tsurcana and Karabash (sheep), Mangalitsa and Bazna (swine) and Hucul (horses). These are the only local breeds for which steps have been taken in order to be saved until now, but they are still considered to be endangered.

The new genetics and molecular biology methodologies applied via the identification and genetic characterization of indigenous breeds will provide new possibilities to reclaim natural resources by genetically improving animal populations and by preserving biodiversity.

3.1 Genetic structure of Romanian Hucul horse inferred from microsatellite data

The equine genome is distributed on 31 pairs of autosomes and the X/Y sex chromosomes. What they have in common with other mammals is the fact that the majority of horse DNA is made up of repeated sequences, which do not encode proteins. The repeated sequences comprise many different types such as the minisatellite, microsatellite, SINE, LINE and telomeric amongst others (Bowling & Ruvinsky, 2000).

The horse microsatellites were characterized first by Ellegren et al., 1992 a,b, who isolated microsatellite sets with repetitive motifs CA/GT and showed that they are polymorphic, so that they could be used for paternity tests, genetic linkage studies and genetic diversity analysis. Subsequently, many studies have described the isolation of equine microsatellites and their use in studies of paternity and genetic linkage maps of horse population composition. In recent years, microsatellites were used for the analyses of different horse breeds around the world, including primitive horse breeds like the German draught horse (Aberle et al., 2004), the Spanish Celtic breeds (Canon et al., 2000), or the Norwegian breeds (Bjørnstad et al., 2000).

The Hucul breed is an indigenous equine race, a true descendant of the wild horses in Romania. The rusticity and adaptability to the environment traits are unique in the equine breeds in our country, and constitute a valuable genetic inheritance. The Hucul represents the only local horse breed and its origin is controversial. The Hucul could be considered a direct descendant of the Tarpan horse in the Northern segment of the Carpathians and it

was consolidated as a breed starting with 1872, together with the founding of the Rădăuți Stud. Five bloodlines were established and named after the foundation stallions: Goral, Hroby, Ouşor, Pietrosu and Prislop. Currently the Hucul horse is bred in stud farms in Slovakia, Romania, Hungary and Poland.

The name of these horses originates from the ethnic group, Hutsul, which originally bred them. Acclimatized in an area with low temperatures, in mountain areas, the Hucul is considered to be a component of the mountain ecosystems, a cultural landscape formed in hundreds of years by human interaction with nature.

The morphological characteristics of the Hucul horses make them similar to the Tarpan. It is a small horse, perfectly adjusted to mountainous areas, possessing great endurance in the wilderness, characterized by the ease with which it finds its food and extremely resistant to disease. As an adaptation trait, to the mountainous areas, these horses have very resistant hooves and do not require horseshoes.

In order to analyze the genetic diversity of the Hucul breed, a comparative study was initiated between four breeds of horses from Romania (Hucul, Arabian, Romanian Sport Horse and Thoroughbred), using a set of 12 microsatellite markers (Georgescu et al., 2008). This study included the analysis of heterozygosity, inbreeding, the Hardy-Weinberg equilibrium (HWE) test and breeds relationship. An UPGMA tree based on the Reynolds's genetic distance relating the four horse populations was built using the PHYLIP 3.5 software (Felsenstein, 1989).

We collected fresh blood samples from 240 randomly chosen individuals (60 from each breed) and the isolation of genomic DNA was performed with the Wizard Genomic DNA Extraction Kit (Promega). The amplification of the microsatellite loci (VHL20, HTG4, AHT4, HMS7, HTG6, HMS6, HTG7, HMS3, AHT5, ASB2, HTG10, and HMS2) was performed using StockMarks® for Horses Equine Genotyping Kit (AppliedBiosystems). PCR products were detected using an ABI Prism 310 DNA Genetic Analyzer (AppliedBiosystems) and the allele sizes were determined using GeneScan-500 LIZ Size Standard (AppliedBiosystems).

A total of 119 different alleles were detected for all the four analyzed species and the entire group of 12 microsatellites was polymorphic (Table 1).

Microsatellite	Thoroughbred	Arabian	Romanian Sport Horse	Hucul
VHL20	6	8	8	10
HTG4	7	3	4	5
AHT4	6	8	7	7
HMS7	5	5	6	6
HTG6	9	6	6	7
AHT5	8	7	5	7
HMS6	6	7	5	5
ASB2	11	8	8	7
HTG10	12	11	7	9
HTG7	7	7	3	5
HMS3	10	8	7	7
HMS2	5	9	8	5

Table 1. The numbers of alleles per locus for each breed (Georgescu et al., 2008).

Observed and expected heterozygosities per breed (Table 2) ranged from 0.662 and 0.676 (Hucul) to 0.759 (Thoroughbred) and 0.741 (Romanian Sport Horse), respectively (Georgescu et al., 2008).

Breed	**H_O**	**H_E**
Thoroughbred	0.759±0.09	0.720±0.108
Arabian	0.691±0.12	0.738±0.101
Romanian Sport Horse	0.709±0.064	0.741±0.07
Hucul	0.662±0.135	0.676±0.122

Table 2. Observed (H_O) and expected (H_E) heterozygosities of 12 microsatellites in four horse breeds from Romania (Georgescu et al., 2008).

	Thoroughbred	**Arabian**	**Romanian Sport Horse**	**Hucul**
Thoroughbred		0.0997	*0.0878*	0.1501
Arabian	0.108410		0.1116	**0.1877**
Romanian Sport Horse	*0.096567*	0.119528		0.0979
Hucul	0.157910	**0.194924**	0.105775	

Table 3. Fst estimates compared in pairs - above diagonal - and Reynolds's genetic distance - below diagonal- (Georgescu et al., 2008).

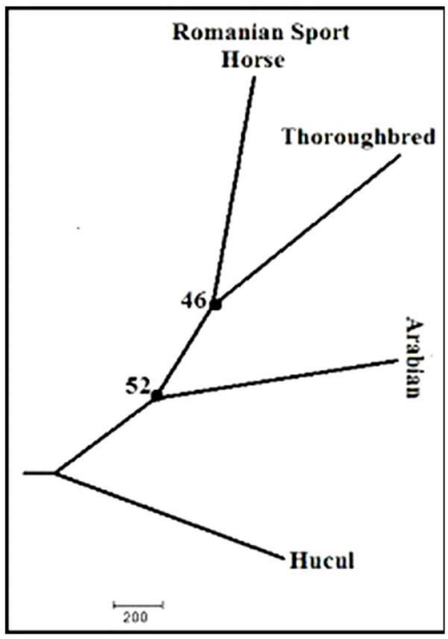

Fig. 1. Phylogenetic tree constructed based on Reynolds' genetic distance by UPGMA method. The numbers at the nodes are values for 1000 bootstrap replications (Georgescu et al., 2008).

The F_{ST} values ranged from 9.7% for the Romanian Sport Horse-Hucul pair to 1.8% for the Arabian-Hucul pair. The Reynold's genetic distance ranged from 0.096 to 0.194 (Table 3).

The results obtained by the Hardy-Weinberg test demonstrate that all the four horse populations are in equilibrium, without any digressions from it (Georgescu et al., 2008).

The phylogenetic tree obtained by the UPGMA method shows an early and clear divergence of the Hucul breed in comparison to the other three breeds analyzed (Figure 1). These data confirm the clear-cut divergence of the Hucul from the common branch (Georgescu et al., 2008). This was the first microsatellite-based genetic diversity study performed on the Hucul population in Romania.

3.2 Genetic diversity of Romanian cattle breeds based on microsatellites

The cattle genome consists of 29 pairs of autosomes and one pair of sex chromosomes. The total amount of DNA per cell is $6x10^{-12}$g, similar to other mammals. As in the case of all vertebrate genomes, the cattle genome is interspersed with numerous repetitive sequences with different structures and origins, such as microsatellites.

In the last decade, useful microsatellites studies have been published on the subject of European cattle breeds (MacHugh, 1998; Martin-Burriel *et al.,* 1999, 2007; Del Bo et al., 2001, Canon et al., 2001, Mateus *et al.,* 2004), African cattle breeds (Ibeagha-Awemu & Erhardt, 2005), and Asian cattle (Kim *et al.,* 2002; Mao *et al.,* 2007, 2008).

The last local cattle breed from Romania 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, Yugoslavian Grey Steppe, Greek Grey Steppe). Grey Steppe was spread on Romanian territory, except for the mountain areas, and had various ecologic species: the Moldavian, Transylvanian, Ialomița and Dobrudja varieties (Georgescu et al., 2009). The number of specimens registered a serious decline after the 1st World War. Grey Steppe has the following characteristics: robust-rough constitution, lively temper, strong tardiness and longevity, good fertility. It has very good qualities of rusticity, health, resistance to bad weather and diseases and has universal uses (traction, milk, meat). Currently, the Grey Steppe breed is on the brink of extinction, thus the genetic fund must be conserved.

The first study regarding genetic diversity and phylogenetic relationships of cattle breeds from Romania was carried out focusing on five populations: Grey Steppe, Romanian Spotted, Romanian Black Spotted, Romanian Brown and Montbeliarde, and was based on allelic frequencies of 11 microsatellite loci (Georgescu et al., 2009). This study included analyses of heterozygosity, inbreeding, the Hardy-Weinberg equilibrium test and breeds relationship. Also, a Neighbor-Joining (NJ) tree based on Reynolds's genetic distance relating the five cattle populations was built using the PHYLIP 3.5 software (Felsenstein, 1989).

We collected fresh blood samples from 190 randomly chosen individuals and the isolation of genomic DNA was performed with the Wizard Genomic DNA Extraction Kit (Promega). The amplification of the microsatellite loci (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, and BM1824) was carried out using StockMarks® for Cattle Bovine Genotyping Kit (AppliedBiosystems). PCR products were detected using an ABI Prism 310 DNA Genetic Analyzer (AppliedBiosystems) and the size of alleles was determined using GeneScan-500 ROX Size Standard (AppliedBiosystems).

A total number of 125 distinct alleles were detected across the 11 microsatellites analyzed in the five cattle breeds (Table 4) and the number of alleles varied between four and 12 (Georgescu et al., 2009).

Locus	Romanian Spotted	Romanian Black Spotted	Romanian Brown	Montbeliarde	Grey Steppe
TGLA227	12	13	10	7	5
BM2113	9	11	11	5	8
TGLA53	11	11	8	12	8
ETH10	4	8	4	4	6
SPS115	8	5	5	4	6
TGLA126	7	6	5	4	7
TGLA122	8	12	9	8	5
INRA23	7	7	6	8	8
ETH3	7	8	9	7	7
ETH225	6	5	5	6	5
BM1824	7	7	6	7	5

Table 4. The number of alleles per locus in each population (Georgescu et al., 2009).

Observed and expected heterozygosities per breed (Table 5) ranged from 0.580 (Montbeliarde) and 0.711 (Romanian Spotted) to 0.690 (Romanian Brown) and 0.778 (Romanian Black Spotted), respectively. The Hardy-Weinberg equilibrium was tested for all breed combinations and the results obtained demonstrated that all the five bovine populations are in equilibrium (Georgescu et al., 2009).

Breed	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
Grey Steppe	0.687±0.216	0.763±0.054	6.363±1.286

Table 5. Observed (H_O) and expected (H_E) heterozygosities of 11 microsatellites in five Romanian cattle populations (Georgescu et al., 2009).

The F_{ST} values indicate that 7% of the total genetic variation could be explained by the breeds' differences and the remaining 93% correspond to differences among individuals. In Table 6 the Reynold's genetic distance was calculated ranging from 0.056757 to 0.131480, with the smallest distances for the pair Montbeliarde - Romanian Spotted and the largest distance between Romanian Brown and Romanian Spotted (Georgescu et al., 2009). The phylogenetic tree obtained using the Neighbor-Joining (NJ) method based on Reynolds' genetic distances (Figure 2) shows that the Grey Steppe breed is clearly distinct from the other four cattle populations, which form two distinct clusters (Georgescu et al., 2009).

The two clusters presented in Figure 2 are in agreement with the expected relationships between breeds. The data obtained from this study represent a starting point in the genetic characterization of the Grey Steppe breed and can be useful for the preservation of these natural resources on the brink of extinction.

	Romanian Spotted	Romanian Black Spotted	Romanian Brown	Montbeliarde	Grey Steppe
Romanian Spotted		0.0662	**0.1132**	*0.0356*	0.0660
Romanian Black Spotted	0.083546		0.0522	0.0675	0.0400
Romanian Brown	**0.131480**	0.071088		0.1054	0.0696
Montbeliarde	*0.056757*	0.087499	0.126578		0.0753
Grey Steppe	0.090666	0.065676	0.095277	0.102635	

Table 6. Fst estimates compared in pairs -above diagonal- and Reynolds's genetic distance - below diagonal- (Georgescu et al., 2009).

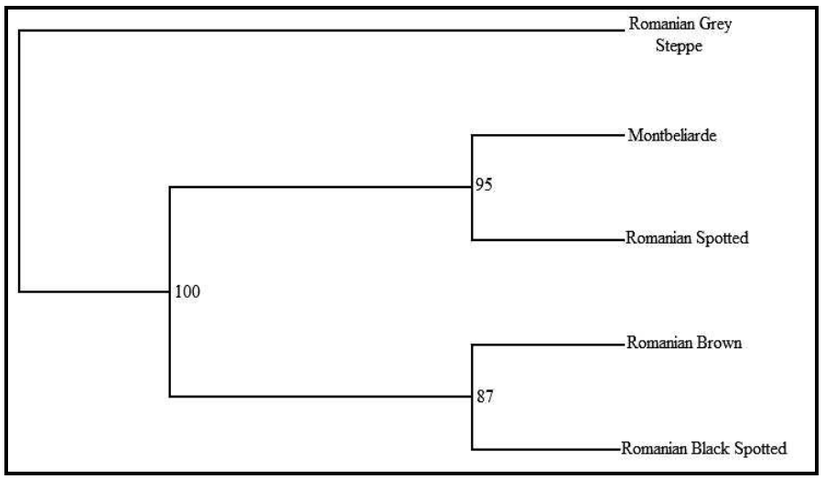

Fig. 2. NJ dendrogram of genetic relationships between the five Romanian cattle breeds. The numbers on the nodes are percentage bootstrap values in 1000 replications (Georgescu et al., 2009).

3.3 Genetic diversity of Mangalitsa pig population from Romania based on 10 microsatellites

In the last century, swine were included in improvement programs. Thus, over the years these programs have obtained breeds that satisfy the increasingly commercial requirements. But the selection methods used have involved a reduction of the numbers of animals in the population selected for breeding. Therefore, the consequence of selection for price efficiency is a reduction of the genetic variability of populations.

Several studies based on microsatellites focused on studying genetic diversity of pig breeds from Europe (Laval et al. 2000; Fabuel et al., 2004) and China (Li et al. 2000; Zhang et al., 2003), but only one such study was carried out in the case of pig breeds from Romania (Manea et al., 2009).

The Mangalitsa breed, although not originally from Romania, is still considered a local breed, since it has been bred in Romania for more than a hundred years. This breed is known for the remarkable quality of the meat, often used in traditional dishes.

Today, Mangalitsa is one of the swine breeds from Romania included on the FAO list of endangered species. The Mangalitsa breed is the only swine breed in our country dedicated to lard production. The prolificacy of the breed is good with the average of 5-6 piglets per farrowing. Subcutaneous fat (lard) and intramuscular fat obtained from the Mangalitsa breed have a lower content in "noxious" cholesterol than plant margarines.

One of the first study regarding phylogenetic relationships and genetic diversity of Romanian swine breeds based on microsatellite markers was carried out on seven populations: Synthetic Line-345 Peris (LS-345), Synthetic Line LSP-2000 (LSP-2000), Pietrain, Large White, Landrace, Mangalitsa and Wild Boar. This study included analysis of inbreeding and heterozygosity, breed relationships and Hardy-Weinberg equilibrium tests. Phylogenetic analysis between swine populations were performed using PHYLIP v3.5 software and a dendrogram was constructed using the Neighbor-Joining method (Manea et al., 2009).

Fresh blood samples from swine individuals, chosen at random, were collected and the isolation of genomic DNA was performed with Wizard Genomic DNA Extraction Kit (Promega). The amplification of the microsatellite loci (SW936, SO228, SO155, SW911, SO355, SW240, SW857, SO101, SO386, and SO005) was performed in two multiplex polymerase chain reactions using dye labeled primers (Manea et al., 2009). The combined PCR products were detected by capillary electrophoresis using an ABI Prism 310 DNA Genetic Analyzer (AppliedBiosystems) and the size of alleles was determined using GeneScan-500 LIZ Size Standard (AppliedBiosystems).

A total of 112 different alleles were identified for 10 microsatellites in all seven breeds analyzed (Table 7). The most polymorphic marker was SO005 with a total of 21 alleles, while microsatellite SO101 was the least polymorphic, showing only 7 alleles (Manea et al, 2009).

Locus	LS-345 Peris	LSP-2000	Pietrain	Large White	Landrace	Mangalitsa	Wild Boar	Total
SW936	8	9	7	8	6	7	4	12
SO155	7	7	6	6	5	4	4	8
SO228	6	5	6	6	5	6	4	9
SW911	4	4	6	5	3	5	3	9
SO355	6	6	6	7	6	6	1	9
SW240	8	7	6	8	7	9	5	14
SW857	8	9	7	7	7	7	4	10
SO101	4	4	3	5	4	4	5	7
SO386	9	7	3	8	5	7	8	13
SO005	11	13	6	14	8	8	8	21

Table 7. The numbers of alleles per locus in each breed (Manea et al., 2009).

As shown in Tabel 8, the range of heterozygosity of the analyzed markers in the seven evaluated pig breeds was between 0.5 and 0.699. Observed and expected heterozygosities ranged from 0.5 and 0.591 (Wild Boar) to 0.699 (Large White) and 0.765 (Pietrain). HWE was tested for all breed-combinations and the results demonstrated that all seven populations are in equilibrium (Manea et al., 2009).

Population	H_O	H_E	MNA
Synthetic Line-345 Peris	0.68±0.115	0.723±0.115	7.2±2.25
Synthetic Line LSP-2000	0.674±0.122	0.728±0.122	7.1±2.726
Pietrain	0.65±0.283	0.765±0.121	5.6±1.43
Large White	0.699±0.141	0.746±0.083	7.5±2.592
Landrace	0.626±0.159	0.706±0.083	5.7±1.337
Mangalitsa	0.651±0.138	0.616±0.138	6.3±1.636
Wild Boar	0.5±0.286	0.591±0.267	4.6±2.117

Table 8. Observed (H_O) and expected (H_E) heterozygosities and the mean number of alleles (MNA) of 10 microsatellites in seven Romanian swine populations (Manea et al., 2009).

Fst estimates compared in pairs and the Cavalli-Sforza's chord distance (Cavali-Sforza and Edwards, 1967) have been presented in Table 9. The Cavalli-Sforza genetic distance (D_C) ranged from 0.034, for LS-345-LSP-2000 pair, to 0.219, for Pietrain-Wild Boar pair. Also, a Neighbor-Joining tree of the seven pig populations (Figure 3) was constructed using Cavalli-Sforza's chord distances, based on the 10 microsatellite loci data (Manea et al., 2009). This dendrogram presents the phylogenetic relationships among the analyzed Romanian swine populations.

	LS-345	LSP-2000	Pietrain	Large White	Landrace	Mangalitsa	Wild Boar
LS-345		*0.018*	0.058	0.056	0.057	0.094	0.192
LSP-2000	*0.034*		0.025	0.057	0.055	0.113	0.169
Pietrain	0.067	0.046		0.067	0.067	0.133	0.203
Large White	0.056	0.056	0.084		0.036	0.105	0.176
Landrace	0.083	0.085	0.097	0.069		0.123	**0.203**
Mangalitsa	0.107	0.111	0.133	0.094	0.130		0.155
Wild Boar	0.198	0.173	**0.219**	0.170	0.199	0.146	

Table 9. Fst estimates compared in pairs -above diagonal- and Cavalli-Sforza's chord distances -below diagonal- (Manea et al., 2009).

As shown in Figure 3, with a bootstrap value of 100% Mangalitsa is the closest breed to the Wild Boar. At the same time, LS-345 and LSP-2000 together with the Pietrain breed, form a distinct cluster, which is normal because three different swine breeds participated in the formation of the LS-345 (Belgian Landrace, Duroc and Hampshire), while LSP-2000 was formed by crossing swine from the Synthetic Line-345 with the Pietrain breed (Manea et al., 2009).

This study was the first based on microsatellite markers for the genetic characterization of swine populations from Romania and the data obtained confirmed the ancient origin of the Mangalitsa. Also, this study contributed to an improved knowledge of the genetic diversity and phylogenetic relationships of pig breeds.

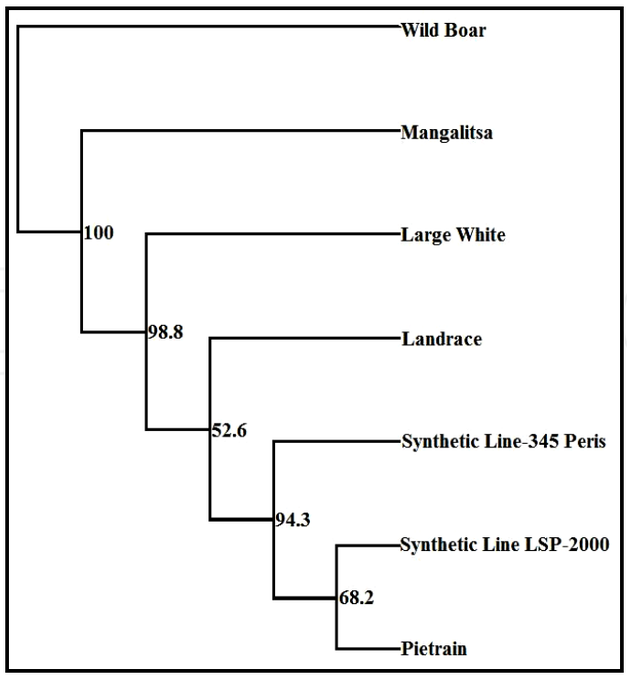

Fig. 3. Neighbor-Joining dendrogram of genetic relationships among seven Romanian swine breeds. The numbers on the nodes are percentage bootstrap values in 1000 replications (Manea et al., 2009).

3.4 Genetic diversity and phylogenetic relationships of four Romanian sheep breeds based on microsatellites

Today, the need to preserve local genetic resources is a global high priority and many measures to support primitive breeds have been established. Regarding sheep breeds, the maintenance of genetic diversity in livestock requires the adequate implementation of conservation measures, which should be based on complete information concerning the genetic characterization of the populations.

In the last decade, several studies based on microsatellites were performed on sheep breeds from Europe (Arranz et al. 2001; Stahlberger-Saitbekova et al., 2001, Rendo et al., 2004, Baumung et al., 2006) and Asia (Mukesh et al., 2006), but in Romania the first study was conducted in 2010 (Kevorkian et al., 2010).

The Karabash breed is an endangered race and it was saved by individual breeders, not through an organized effort. The Karabash together with Tsurcana represent the last two local sheep breeds from Romania. This particular sheep breed was observed due to some remarkable external characteristics, but mainly because of the high production and lamb earliness. The Karabash was mentioned for the first time in 1912 and it is considered that the two local sheep breeds, Tsigai and Tsurcana, contributed to its formation.

The aim of the first study, based on molecular markers, was to analyze the genetic diversity, variability and the phylogenetic relationships of four Romanian sheep breeds (Botoşani Karakul, Karabash, Palas Milk Line and Palas Meat Line), using 11 microsatellites. Blood samples from a total of 161 individuals from the four different sheep breeds were collected

and the isolation of total DNA was performed with Wizard Genomic DNA Extraction Kit (Promega). Two PCR multiplex reactions were performed to amplify 11 microsatellites: 8-Plex reaction for OarCP20, OarCP34, MAF70, MAF214, MAF65, BM143, McM42, HSC and 3-Plex reaction for OarFC11, OarFCB20 and MAF33. The forward primers were labeled with a fluorescent compound. The combined PCR products were detected with ABI Prism 310 automated sequencer (Applied Biosystems), using the GeneScan-500 LIZ Size Standard (Kevorkian et al., 2010).

Table 10 presents the total number of allele identified in for the four breeds analyzed. All the 11 microsatellites were successfully amplified. MAF70 marker showed the highest number of alleles while OarCP20 was the least polymorphic (Kevorkian et al., 2010).

Locus	Milk Line Palas	Meat Line Palas	Karabash	Botoşani Karakul	Total
OarFCB 11	8	12	6	13	18
Oar FCB 20	10	9	7	13	20
Oar CP34	10	8	9	11	14
MAF 70	11	16	6	18	30
Oar CP20	6	7	3	9	11
MAF214	7	4	7	7	12
BM143	12	11	6	9	16
MAF33	7	3	7	15	17
McM42	5	4	10	10	17
MAF65	9	7	10	9	15
HSC	15	12	15	17	27

Table 10. Number of alleles per locus for each analyzed breed (Kevorkian et al., 2010).

Table 11 presents the results concerning the observed and expected heterozygosities for the four Romanian sheep breeds; these ranged from 0.580, respectively 0.670 (Karabash) to 0.720 (Meat Line Palas) and 0.790 (Botosani Karakul). HWE was tested for all breed-combinations and the results demonstrated that all four investigated populations are in equilibrium (Kevorkian et al., 2010).

Breed	H_o	H_e	MNA
Karabasch	0.580	0.670	7.8
Meat Line Palas	0.720	0.770	8.5
Milk Line Palas	0.590	0.740	9.2
Botoşani Karakul	0.670	0.790	11.6
Mean	0.640	0.740	9.275

Table 11. Mean number of alleles (MNA), mean observed heterozygosity (Ho) and mean expected heterozygosity (He) across 11 loci (Kevorkian et al., 2010).

The matrix of Nei's standard genetic distances and estimates of pairwise distances between breeds are presented in Table 12. The genetic distances between breeds ranged from 0.263

for Milk Line Palas and Meat Line Palas to 0.606 for Karabash and Meat Line Palas. The correspondent phylogenetic tree is presented in Figure 4 (Kevorkian et al., 2010).

	Botoşani Karakul	Karabash	Milk Line Palas	Meat Line Palas
Botoşani Karakul	-	0.0855	0.0803	0.0811
Karabash	0.381176	-	0.1063	0.1256
Milk Line Palas	0.412315	0.459566	-	0.0493
Meat Line Palas	0.448552	0.606219	0.263112	-

Table 12. Estimates of pairwise F_{ST} distances between the analyzed breeds (above diagonal) and Nei's standard genetic distances (below diagonal).

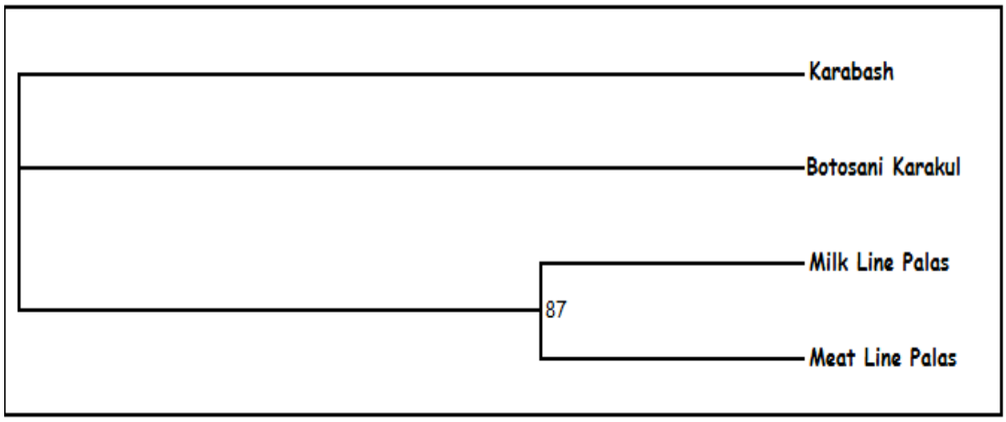

Fig. 4. Neighbor-Joining phylogenetic tree based on Nei's standard genetic distances for four Romanian sheep breeds (Kevorkian et al., 2010).

The resulting tree revealed that the most closely related breeds were the two synthetic lines. Although for the Karabash breed several clear difference are noticed (Kevorkian et al., 2010). This study was the first which has led to results regarding the characterization of genetic variability based on microsatellite markers of Romanian sheep breeds.

4. Conclusions

The need for characterizing the genetic variability of local breeds is one of the global priorities of scientific research and it is dictated both by the revaluation of practices in livestock breeding and by the conservation of genetic resources. The local breeds, perfectly adapted to the environment conditions in their foundation area, are preserved as gene pools for the next generations.

The originality of our studies is based on the fact that local breeds from Romania have never been studied and characterized using microsatellite markers. In addition these breeds were never analyzed in terms of phylogenetic relationships in comparison with other local breeds all over the world and their origins had not been clearly established. The research activities could open new horizons in clarifying issues concerning the origins of the Romanian local breeds and their kinship with other breeds.

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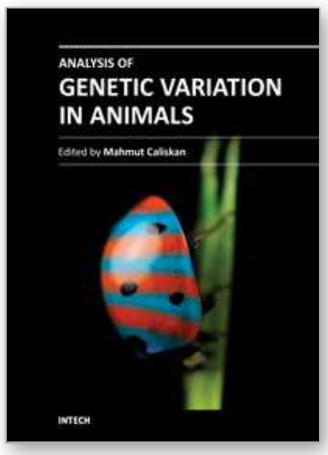

Analysis of Genetic Variation in Animals
Edited by Prof. Mahmut Caliskan

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Analysis of Genetic Variation in Animals includes chapters revealing the magnitude of genetic variation existing in animal populations. The genetic diversity between and within populations displayed by molecular markers receive extensive interest due to the usefulness of this information in breeding and conservation programs. In this concept molecular markers give valuable information. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in animals and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation in animals by presenting the thoughts of scientists who are engaged in the generation of new idea and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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