ENDANGERED ROMANIAN CATTLE BREEDS–BETWEEN TRADITIONAL BREEDING AND GENETIC CONSERVATION

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Abstract

In Romania, the Grey steppe and Pinzgauer cattle are included in a genetic program of conservation, due to their valuable genetic pool for increased resistance to diseases and high adaptability to severe environmental conditions. Also, these indigenous cattle breeds are part of the history of our country, as different varieties of them represent the same in their countries of formation. Methods applied worldwide in this aim of genetic conservation evolved considerably over the time, and the results reported are interesting on the view of their phylogeny, site of primary formation, and subsequently migration. The present work aims to review the most important aspects related to these two indigenous cattle breeds reared in Romania, the most important genetic markers and techniques which could be applied in further programs of their genetic characterization and preservation.

Key words: indigenous cattle, resistance, conservation

INTRODUCTION

The Grey steppe cattle breed and, also the Pinzgauer, are considered threatened with extinction cattle breeds in Romania, in 2000 being maintained, by Food and Agriculture Organization of the United Nations (FAO), the "endangered-maintained" status for both of them. FAO reports normally draw the attention on the numerical decline of different species, various breeds being ranked, considering the number of individuals for each breed, the female/male ratio, and their including in active programs of preservation or maintenance by companies or research institutions, as one of the following categories (listed in their decreasing order of gravity): extinct, critical, endangered, critical-maintained, endangered-maintained, not at risk [1]. In the case of Grey steppe cattle, the presented FAO data showed a different status for individuals spread all over the Europe, ranging from a "critical" rank (Grey Steppe from Bulgaria, Greece, German cattle population of Hungarian Grey cattle) to and "endangered-maintained" one, such as for Romanian Grey Steppe population (or "Sura de stepă" as is named in our national language) (table 1).

This classification was performed using data reported before 2000 year, but in many cases an appropriate number of animals is still reported in specific researches published up to today ([2], [3], [4], [5], [6], [7], [8]). But not all the Grey steppe varieties presented a continuous decline in their number. For example, the Hungarian variety even increase in its number after 1970 as a result of a good management of these genetic resources in order to serve touristic purposes and not only [9].

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Table 1 Grey Steppe cattle populations size and their status according to FAO ranks

Grey Steppe breed	Country	Population data		Status
		Year	No. of animals	
**according to (1)*				
Iskar Grey *(Bulgarian Grey)*	*Bulgaria*	1994	245, 120♀, 5♂	*critical*
Istrian	*Croatia*	1995	110, 103♀, 7♂	*critical-maintained*
Slavonian Podolian*(Slavonian Syrmian)*	*Croatia*	1995	20, 12♀, 3♂	*critical-maintained*
Katerini	*Greece*	1995	80♀, 5♂	*critical*
Sykia	*Greece*	1995	90♀, 5♂	*critical*
Hungarian Grey Steppe	*Austria*	1994	10♀, 4♂	*critical-maintained*
	Germany	1997	36♀, 5♂	*critical*
Sura de stepă *(Moldavian variety)*	*Romania*	1993	350♀, 12♂	*endangered-maintained*
Ukrainian Grey	*Ukraine*	1990	1500, 684♀, 13♂	*endangered*

In the case of Pinzgauer, a polled variety named Jochberger Hummeln found in Bezirk Kitzbühel, Tirol, received a *"critical-maintained"* status, with 30 females and 4 males reported in 1994. In Germany, the Pinzgauer cattle received an *"endangered"* status, with 286 females registered in the herd book, and 6 males. A similar status was reported for Pinzgauer Fleischnutzung, with 906 females and 42 males registered in the herd book of Germany in 1997. In Romania, the Pinzgauer cattle or *"Pinzgau de Transilvania"* as is reported in Romanian language, included 1092 females registered in the national herd book (table 2), the semen of 23 males being stored at that times of 1993. However, the reported population trend was considered as decreasing [1].

Table 2 Pinzgauer cattle populations size and their status according to FAO ranks

Pinzgauer breed	Country	Population data registered in the herd books		Status
		Year	No. of animals	
**according to (1)*				
Jochberger Hummeln	*Austria*	1994	30♀, 4♂	*critical-maintained*
Pinzgauer	*Germany*	1997	286♀, 6♂	*endangered*
Pinzgauer Fleischnutzung	*Germany*	1997	906♀, 42♂	*endangered*
Pinzgau de Transilvania	*Romania*	1993	1092♀, 7♂	*endangered-maintained*

Both Grey steppe and Pinzgauer cattle are considered, in Romania, autochthonous, the first one – primitive, and the last one – improved. Their origin, aim of breeding, and main phenotypic characteristics were well [9], and presented throughout the time in many scientific papers. In the same way it is worth mentioning the effort of characterization of these Romanian indigenous endangered cattle breeds in terms of various genetic markers ([10], [11], [12], [13], [14], [15], [16], [17], [18], [19]).In this context, the aim of this paper is to briefly present the most important aspects related to Grey steppe and Pinzgauer cattle breeding in Romania, the most important genetic markers for milk and meat production, and molecular techniques which could be applied in further programs of genetic characterization and preservation of these breeds in Romania.

MATERIAL AND METHOD

In order to achieve the assumed objectives of this study, there were consulted

53 references on the chosen topic. The most important aspects are presented in three different sections including: (i) Grey steppe and Pinzgauer cattle breeding, and their morphological and productive evaluation; (ii) genetic markers in the study of threatened with extinction cattle breeds; (iii) techniques used in cattle's DNA or protein analysis. In order to describe phenotypically and genetically the two breeds of cattle that are in genetic conservation in Romania, 4 books from the specialized literature were consulted, respectively 49 scientific articles from different national and international databases. Genetic markers associated with milk production have studied 26 scientific articles, while genetic markers associated with meat production have been highlighted in a number of 14 scientific articles. The techniques used in the analysis of DNA and polymorphism of major milk proteins respectively were studied from a number of 9 bibliographic sources.

Grey steppe and Pinzgauer cattle breeding, and their morphological and productive evaluation. The Grey Steppe breed represents one of the oldest indigenous breeds which belongs to Bos genus, Taurus subgenus, Primigenius species, the horned Taurine subspecies, being known in the popular language as "bour". Till 1850, the national herd book consisted of two indigenous breeds, the Grey steppe and Mocăniţa, the first one being more spread in the steppe and hill areas, and the other one, in the mountain areas. In the formation of these breeds were considered a higher influence of the environmental conditions, and less and even insignificant of man's intervention [14]. The Pinzgauer breed is found out in the Romania's mountain areas at over 1000 m altitude. It originates from Austria, Salzburg, Tyrol, an alpine and subalpine area. The breed was firstly formed between 1690-1740 by the crossing of local red bulls with the Berna type of Switzerland; after 1740, the resulted animals were reproductive used in a true breed [20]. Nowadays, the interest of farmers all over the world is represented by specialized cattle breeds on milk or meat production, which makes financially profitable this activity of animal breeding. Although the Pinzgauer is considered improved indigenous cattle breed in Romania, its recorded productions are still lower than those of highly specialized cattle breeds reared in Romania and not only (2000-2500 kg milk/year, 3.8% fat, 400-500 kg the weight of cows, 650-700 kg the weight of bulls, 800-900 kg the weight of oxen). Considering the Grey steppe breed, the recorded productions are even lower; the milk production of cows raised in households is of 800-900 kg/year, and of 1000-2500 kg for those raised in farms, with a content of 4-6% fat [9]. The main physical characteristics which emphasize some morphological differences between Grey Steppe males and females are shown in table 3, and of Pinzgauer cattle, in table 4.

Table 3 Some morphological differences of varied types of Grey Steppe cattle breed

Grey Steppe cattle breed	Average adult weight (kg)		Average wither height (cm)		Reference
	males	females	males	females	
Iskar Grey *(Bulgarian Grey)*	750	350	140	118	*
Istrian	900	625	148	138	*
Slavonian Podolian*(Slavonian Syrmian)*	600	460	135	128	*
	600-800 (1000 for oxen)	470	135-145	128	**
Katerini	400	285	123	113	*
Sykia	-	-	-	116	*
Hungarian Grey Steppe	900	600	150	140	*
Iugoslav Steppe	800	500	150	135	*
Romanian Steppe *(Moldavian variety)*	780	480	137	129	*
	744	488	-	-	***
Turkish Grey Steppe	470	375.07	126	117.98	****
Ukrainian Grey	780	480	137	129	*

*according to Scherf, 2000; ** according to Keros et al. 2015, *** according to Dascălu et al. 2012; **** according to Soysal and Kök, 2008.

Table 4 Some morphological differences of varied types of Pinzgauer breed

Pinzgauer cattle breed	Average adult weight (kg)		Average wither height (cm)		Reference
	males	females	males	females	
JochbergerHummeln	1200	700	151	139	*
Pinzgauer	-	750	-	140	
PinzgauerFleischnutzung	-	-	-	-	
Pinzgau de Transilvania	900	500	134	127	

* according to Scherf, 2000; "-" unreported data in the studied reference

Excepting a fatty milk, which may be considered by some as an advantage for Grey steppe cattle, its meat is darker and not marbled, being less preferred by human consumers, although one of the original purpose of this breed raising was for meat production beside draught power. The last decades mechanization of agriculture led even this final goal fall. Even so, researchers do not lose their interest in studying these animals, and also those of Pinzgauer breed, at their genetic level being considered a real reservoir of genes linked to various environmental factors resistance, including climatic conditions and various parasites or infectious pathogenic agents.

Genetic markers in the study of threatened with extinction cattle breeds

Techniques of genetic analysis evolved considerably over the time, allowing the genome to be sequenced in various species of interest. The phylogeny of many individuals was studied by researchers since 1980. Another types of genetic research were focused on the study of genetic markers associated to characters of productions, in the case of cattle breeds threatened with extinction this being useful for the appreciation of genetic resource conservation value related to animal origin, the degree of uniformity of breed and, corroborated with many other sensitive molecular markers, their place of formation and domestication.

In Romania, the study of genetic polymorphisms in the major proteins of milk (caseins, lactalbumin, and lactoglobuline) of various native farm species, and the possibility of using them as genetic markers for increasing milk quality or identifying the authenticity / origin of milk and other dairy products, were constantly worked by Ilie D.E. and Bâlteanu A.V. teams. Isoelectric focusing (IEF) and PCR techniques were successfully tested in Romania for the characterization of milk protein polymorphisms. The allele α_{S1}-casein I^{SM} discovered in the Grey Steppe breed was not reported in any other European cattle breed, being an ancestral allele that originates directly from the wild ancestors of the breed, providing the first molecular proof of the phylogenetic position of the breed, which is extremely necessary in the context of its conservation ([10], [21], [22]). Although not in a Romanian population, it seems that Pinzgauer breed is also a carrier of a particular allele. IEF investigated milk samples of Pinzgauer individuals located in Austria and Bavaria, Germany by Erhardt (1996), revealed a new κ-casein variant (κ-casein G) with a frequency of 0.003, allele which was not found in milk samples of Limpurger, another endangered breed which was investigated. However, the family of milk proteins is large, with a significant influence on the milk composition and its physico-chemical properties, which were well documented in the past years for various alleles and genotypes at each locus in part. In an overview of all caseins, in most cases results of various associations are contradictory due to their location in linkage in the structure of the 6^{th} bovine chromosomal pair. When considering caseins, for many scientists and cattle breeders is easier to take into account the genetic structure for κ-casein. Therefore, the κ-casein A allele is well known to be associated with increased milk yield ([23], [24]) and the B allele, with higher κ-casein concentration ([25], [26]), protein and fat yield ([27], [28]), a better reaction with chymosin, a significantly lower clotting time, and a higher rate of curd formation ([20],

[30]). Considering Grey steppe cattle and Pinzgauer indigenous cattle breeds, their characterization on milk protein polymorphism [9] showed a higher frequency of B allele at κ-casein locus in Grey steppe cattle, and of its A allele, in case of Romanian Pinzgauer. This A allele was reported by [31] in the Original Pinzgauer cattle in a unique combination of alleles for milk protein loci located on the 6th chromosome (C-A^2-B-A, encoding the following proteins: α_{S1}-, β-, α_{S2}-, and κ-casein, respectively). In the case of whey proteins, β-lactoglobulin is mostly evaluated due to its polymorphism, the B type and BB genotype being reported in associations with desirable traits for milk industry, such as fat percentage [27], fat and cheese yield, shorter coagulation time and a higher thermal stability of proteins ([27], [32], [33], [34], [35]). In this case, the higher frequency of A allele in Grey steppe cattle was reported over the time, and of B allele, in Romanian Pinzgauer [9].

Beside these, there are many other genes associated with milk production, for example the Pituitary Transcription Factor and the Growth Hormone gene. The polymorphisms of Pituitary Factor 1 (POU1F1 or PIT1) and Growth Hormone Receptor (GHR) genes were investigated on Romanian Grey Steppe by ([36], [12]). The investigation of 60 blood samples showed two alleles at PIT1 locus, the B allele being prevalent to A variant also in Podolica breed [13], although the A allele was found to be desirable to milk production and body conformation, for example in Holstein Friesian, Polish Black and White, Romanian Simmental, and Maramureș Brown [12]. Even so, investigating 352 Holstein cattle for PIT1 gene polymorphism, Bayram et al. (2017) reported the largest frequency of B allele (0.68) as a part of these two identified alleles at this locus. Investigating the polymorphism in exon 6 of PIT1 gene in South Anatolian Red and East Anatolian Red cattle, a low linkage with dairy manufacturing traits was reported by [1]. Considering the GHR gene, the A allele was reported in several cattle breeds to be associated with higher fat and protein percentages; the genotyping of 60 blood samples collected from Romanian Grey Steppe revealed only the including T allele homozygous genotype [36].

In the case of meat production and its quality, it was established an influence of various alleles of some genes, for example Leptin (LEP) and Calpastatin (CAST), on fleshiness, succulence, degree of marinating, carcass quality, and body weight. Bayram et al. (2008) established a higher frequency of A allele of LEP gene in a Holstein Friesian population of 352 cows, considering only two alleles investigated at this locus (A and B). Investigating the polymorphism of the bovine LEP gene at the level of Arg4Cys and Ala59Val amino acids, Komisarek et al. (2005) reported in 187 Black-and-White AI bulls with an average Holstein Friesian genes share of 96.4%, the following frequencies of the genes: for Arg4Cys, 0.55(C) and 0.45(T), and for Ala59Val, 0.73(C) and 0.27(T). The authors of the investigation concluded that the Arg4Cys TT genotype had a positive impact on milk yield growing and protein output, and no significant impact on butterfat yield, even of its greatly reducing. On the other hand, the Ala59Val polymorphism was not reported to affect milk and protein amounts, but was considered by the authors to be responsible for the quantity of obtained butterfat. No significant association was reported by [37] for the leptin receptor gene (LEPR) with reproductive parameters or daily weight gain in three cattle breeds (Brangus Ibagé, Charolais, and Aberdeen Angus). Considering the Calpastatin gene (CAST), in an investigated populations of 71 Turkish Grey Steppe and 61 first-generation crossbreeds of Turkish Grey Steppe and Brown Swiss, two genetic variants (C and G) were reported by [38] for the CAST/Rsal polymorphism, with a higher frequency of C allele in both investigated groups. The aforementioned authors argued that the two single nucleotide polymorphisms found in the CAST gene in intron 5 between exons 5 and 6, which correspond to cross location change between cytosine and guanine, entitle C allele as the most favourable, the genotype CC yielded beef that was more tender than the beef obtained from GG genotype carriers,

the CG genotype being correlate with intermediate beef tenderness.

Another such types of investigations on different types of Grey Steppe breed cover the study of hemoglobin (Hb), transferrin (Tf), and potassium (K) in erythrocytes polymorphisms ([39], [40], [41], [7]). Three Hb variants were reviewed in Grey Steppe cattle by [7], the highest average frecquency being for A type (more than 90%), followed by B and C alleles. On the basis of vertical electrophoresys interpretation using polyacrylamide as a migration support, a single type corresponding to the A type was observed in Romanian Grey Steppe by [40]. Beside the common variants of Hb gene, an unusual hemogloblin polymorphism was recognized in Italian Podolic cattle, involving AY and A^{zebu} variants, which were not detected in other sampled breeds [39]. On the serum transferrin locus, seven alleles were reported in Grey Steppe cattle (A, B, D, D_1, D_2, E, and F), of which A and B variants were reported at a frequency ranged from 0.21 to 0.43, and 0.02 to 0.05 respectively, the average frequencies for other reported allele being in the following decreasing order: $D>D_1=D_2>E>F$. In Romanian Grey Steppe, three alleles were detected at serum transferrin locus, D variant being reported at more than a half of the investigated population; as in previous mentioned report, E allele was found at a lower incidence that A allele. The EE genotype was found to be associated with the highest milk yield, and any of E included allele in genotype seems to be associated with a good milk production, a higher fat percentage, and a higher performance for fat yield. The lowest performances were reported for cows with AD genotype, if there are considered the milk quantity and fat percentage features, and for DD genotype for fat yield. The potassium polymorphism in erythrocytes of Turkish Grey Steppe was investigated by [41], who identified two alleles, K^H and K^L, according to those different red cell potassium concentration types, LK and HK, with a lower and higher potassium concentration than 46 m-equiv l^{-1}, respectively. The reported frequencies in the investigated population of 39 individuals of Grey Steppe breed Turkish variety, showed a higher incidence of K^L allele at a just over ¾ of the individuals, which is in agreement with the highest frequency of K^L allele found by Gonzales and Vallego (1983) in Sayaguesa, Morucha, CordenaAndokza, and Blanca Cocerona breeds [41].

Techniques used in cattle's DNA or protein analysis

In the investigation of the genetic material usually are used several steps of working assuming: (i) the extraction of total genomic DNA from blood samples; (ii) the DNA quantification (purity and concentration determination) by spectrophotometry; (iii) the DNA revealing by agarose gel migration technique; (iv) the amplification of genes of interest by PCR technique (Polymerase Chain Reaction).

(i) The extraction of total genomic DNA from bovine blood samples can be accomplished by several methods, the most well-known of them involving the extraction with Wizard ™ Genomic DNA Purification Kit; the rapid method of extraction of bovine blood DNA; and the automated method of extracting bovine DNA from blood.

(ii) The determination of the concentration and purity of DNA extracted samples can be performed by spectrophotometry by measuring the total absorbance of the extract at wavelengths of 260 nm and 280 nm. The purity of extracted DNA samples is estimated based on A260 / A280 ratio (A=absorbance), and the sample concentration is automatically calculated using a specialized software. DNA integrity is appreciated by a technical migration in agarose gel [42].

(iii) Electrophoresis is a technique of separating molecules according to their molecular mass. Worldwide is a large number of electrophoretic methods applied to the analysis of various polymorphisms in cattle, the most commonly of them including: capillary electrophoresis; polyacrylamide gel electrophoresis under native conditions (NATIVE-PAGE); polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE); isoelectric focusing electrophoresis (IEF technique); agarose gel electrophoresis (AGE). Capillary

electrophoresis combines separation mechanisms of electrophoresis and the automation of chromatography, with an extraordinary separation power, analytical speed, and extreme sensitivity (a single molecule by laser-induced fluorescence detection) [43]. NATIVE-PAGE uses the principle of protein separation based on their electrical charge, molecular mass, and spatial conformation. This technique was successfully applied to the research of major milk protein polymorphisms, to differentiate A, B, C and Dat the locus of β casein, and for the authentication of dairy products. Electrophoresis (SDS-PAGE) is based on the principle of protein separation based on molecular weight. A discontinuous polyacrylamide gel having a supporting medium role and a specific substance sodium dodecylsulfate (SDS) is used to denature proteins. This technique can be applied to both protein separation and separation of DNA and RNA molecules [44]. IEF electrophoresis separates proteins according to their isoelectric point (pI); these molecules are amphoteric ones, which means that they can have positive, negative or zero electrical charge, depending on the pH in the environment. The electrical charge of a protein is given by the sum of positive and negative electric charges, which characterize the amino acids in their constitution. Each amino acid has amino groups and terminal carboxyl groups, and depending on their prevalence in the composition of the protein, the total protein load may be positive (with more amino groups) or negative (with more carboxyl groups) [22]. Agarose gel electrophoresis (AGE) is a standard method of separating, purifying, and identifying DNA molecules, including mixtures that cannot be appropriately separated by other techniques. To verify the integrity of chromosomal DNA as well as to estimate the degree of RNA contamination, the extract is subjected to an agarose gel electrophoresis in a horizontal plate [42].

(iv) Amplification of genes of interest by PCR technique (Polymerase Chain Reaction). This technique was developed by K. Mullis in 1983 and considers the natural process of DNA replication, using the DNA polymerase enzyme in order to synthetize a new complementary strand of DNA using a native template one. As a result, will be formed many copies of DNA sequences (genes) of interest [45]. There are known many variants of the basic PCR technique, the most used of them in the study of various polymorphisms in cattle being the Restriction Fragment Length Polymorphism (RFLP) technique and Amplified Fragment Length Polymorphism (AFLP) technique. RFLP technique is based on the hybridization properties that exist between two DNA fragments presenting a high degree of homology (RFLP-based hybridization) and the PCR technique, highlighting the polymorphisms existing at the restriction enzyme sites. AFLP technique is based on the selective PCR amplification of DNA fragments obtained by a particular restriction using two restriction enzymes and adapters, followed by the analysis of amplified fragments in polyacrylamide gel. It has a great advantage of its not requiring preliminary genome information, and its sensitivity in detecting polymorphism at the whole genome level [13].

Besides classical polymorphisms of genes or of their products represented by proteins or various enzymes, new codominant nuclear markers was found at high density and randomly dispersed across chromosomes, being increasingly used for genetic diversity studies, in particular for breeds that are on the brink of extinction, due to their highly degree of polymorphism and their neutral behavior to selection proceedings ([46], [47]).When these microsatellite's analyses were considered, an increased number of alleles were observed, these loci having a good variability despite of the low populations size and the existed risk of inbreeding. The analysis of phylogenetic relationships established closer or further genetic distances within Grey Steppe breed, or between Grey Steppe and other breeds, being also confirmed some Grey Steppe varieties in Podolian cattle group [9].

CONCLUSIONS

Genetic markers are worldwide used in various programs of cattle's conservation and improvement. In Romania, the Grey steppe

and Pinzgauer cattle breeds are included in a national genetic preservation program in order to maintain their genetic resources. The use and testing of new molecular markers research techniques will further help clarify aspects of their genetic diversity. At the same time, it is possible to establish associations between gene polymorphism and productive, reproductive or adaptation to environmental conditions of the two cattle breeds.

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