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Review

Milk Protein Polymorphism Characterization: A Modern Tool for Sustainable Conservation of Endangered Romanian Cattle Breeds in the Context of Traditional Breeding

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Abstract: The paper aims to review literature data with respect to Romanian native cattle breeds which are considered at risk of extinction. In the last decades, the number of individuals of Romanian indigenous cows decreased significantly, as a consequence of the intensification and specialization of animal productions and agriculture modernization. Some of the native cattle breeds are already lost, due to their crossing with improved breeds. However, after the accession of Romania to the European Union, various preservation programs were initiated, and most of them included biochemical research and studies of molecular or quantitative genetics. All these, associated with the application of reproduction biotechnologies, give a chance to these animals, which are extremely valuable in terms of their genetic resistance to diseases and environmental factors. The reviewed literature on Romanian indigenous endangered cattle breeds confirms that these animals are carriers of a valuable gene pool, which can be kept and bred while applying different reproductive biotechnologies. Consequently, this paper raises awareness on two issues: the decrease of genetic diversity in two Romanian native cow breeds threatened with extinction (Grey Steppe and Romanian Pinzgauer); and the benefits of genetic diversity of the two breeds.

Keywords: indigenous cattle; endangered breeds; genetic resistance; sustainable conservation

1. Introduction

Modern and intensive agricultural systems have been criticized as often being detrimental and nonviable, when considered from the ecological and social point of view [1,2]. These issues led experts to reflect on intensive agricultural systems, and find alternatives in traditional agricultural systems that have been developed and maintained by local farmers for centuries with ingenious practices that often result in both community food safety and the conservation of ecosystem services [2–4]. Traditional animal husbandry involves, on the one side, traditional practices and, on the other side, rustic livestock.

Genetic diversity and conservation of local animal breeds are valuable assets for countries where agriculture is still an important economic sector. Moreover, the diversity, in general, represents an important cultural heritage [5], and preservation of domestic animal breeds represents, among others, an essential tool towards maintaining rural traditions. To a large extent, the decrease of animal breed diversity appears to have been accelerated by the expansion of market systems and associated processes of globalization [6]. The benefits of genetic diversity in animals include high tolerance for extremely harsh environmental climatic conditions, resistance to disease and external parasites, and quick recovery after illness.

The aim of this paper is to present literature data with respect to endangered Romanian native cattle breeds. In this work, we are focusing on two breeds: Grey Steppe, as the last autochthonous primitive cattle breed; and Romanian Pinzgauer, an autochthonous improved cattle breed, which is now considered to have an endangered-maintained status [7]. The relevant research focused on their origin and spreading, on milk protein polymorphism as a part of genetic conservation programs, and, nonetheless, on the conservation perspectives through the use of genetic markers of great finesse, such as microsatellites. A vast majority of discussions pored over the Grey Steppe cattle—considered to be a maternal breed and a gene reservoir for most of the autochthonous improved cattle breeds. The relevance of this review is given by the scrutiny of indigenous breeds characterized, on the one hand, by low productivity, and on the other hand, by a set of qualities which ensure their survival in natural conditions.

From ancient times, mankind was primarily preoccupied with cattle breeding, at first for meat and draught power (in agriculture, transportation, and construction), and then for milk production. In some civilizations, such as Minoan Crete (from 2000 to 1500 BC), longhorned and spotted bulls were used as an entertaining activity in the sport of bull-leaping [8,9].

Back, in remote history, it seemed that there were several domestication centers, such as those from Asia, North Africa, and Europe, some hypotheses suggesting a different place and time arrangement models of domestic cattle origin and spreading [10]. The idea of a single site of domestication is supported both by Epstein [11] and by Reed [12], who consider either Sumeria or the Southern Balkans as the first domestication center for all cattle. Other authors are talking about domestication as a non-localized process. This hypothesis seems to be sustained by some proofs of using cattle for plowing in the lower Danube Valley, England, Poland, or in Southern Spain [8]. A series of stone paintings also demonstrated the presence of domesticated cattle in the Northern Sahara, Egypt, and Crete [8].

A large number of cattle breeds diverged from the primitive animals as a consequence of their adaptation to local environmental conditions, and of multiple selections for health, longevity, fertility, and production traits. Nowadays, people's interest in highly productive cattle breeds is well-known, and has led to the decrease in the number of indigenous breeds [13,14].

2. Indigenous Cattle Breeds: Origin and Phylogenetic Relationships

The Romanian indigenous cattle breeds are classified into primitive and improved. The autochthonous primitive cattle breeds are the Grey Steppe (in Romanian: Sură de Stepă) and the Mountain Breed (in Romanian: Mocănița), and autochthonous improved ones are the Romanian Spotted (in Romanian: Bălțată Românească), Brown (in Romanian: Brună), Pinzgauer (in Romanian: Pinzgau de Transilvania), and Red of Dobrogea (in Romanian: Roșie Dobrogeană) [9,15].

The Grey Steppe breed is considered a descendant of *Bos primigenius*. In fact, all domesticated cattle originated from *Bos primigenius* or *Bos urus*, which moved west and south from the Ukrainian steppe in ancient times (8000 years Before Present) to form different varieties of Grey cattle, such as: Iskar Grey and Bulgarian Grey (or Bulgarian Steppe) in Bulgaria; Istrian, and Slavonian Podolian (Syrmian cattle) in Croatia; Katerini, and Sykia in Greece; Hungarian Grey in Hungary; Cinisara, Maremmana, and Podolica in Italy; Romanian Steppe in Romania; Turkish Grey (Boz Steppe or Plevne) in Turkey [16–18]. Other Grey cattle varieties are Tyrolean Grey and Rhaetian Grey. Tyrolean Grey is a typical alpine cattle breed from Tyrol in Austria and South Tyrol in Italy [19]. We also know that a lighter type of this breed (known as the "Rätisches Grauvieh" or Rhaetian Grey) can be found in Switzerland [20].

Although initially considered a subspecies of *Bos primigenius*, *Bos brachyceros* appears to be a result of *Bos primigenius* selection in flocks of domesticated animals. In Romania, *Bos brachyceros* became extinct before *Bos primigenius*, contributing to Mountain Breed formation [15,21]. The Mountain Breed existed until the second half of the 19th century, and after that, it became the subject of crossbreeding with Pinzgauer and Brown cattle breeds [9,15].

The autochthonous improved breeds were formed by crossings between local primitive breeds and improved breeds imported from other countries. For example, the Romanian Spotted was formed by crossings between Grey Steppe cows and imported Simmental bulls, the Brown breed by crossings between Grey Steppe or Mountain Breed cows and Schwyz bulls, the Romanian Pinzgauer by crossings between Grey Steppe cows and Pinzgauer bulls, and the Red of Dobrogea breed by crosses between Grey Steppe cows and red bulls of Angler, Red Steppe, Red Polish, Red Estonian, Red Danish, or Red-Brown Latvian breeds [15]. The Romanian Spotted cattle accounted for 36% of the Romanian livestock [22] and, over the last 25 years, it has been the subject of improvement with Simmental bulls. The Brown cattle (of Maramureș) accounted for 33% of the Romanian livestock, as it was well-adapted to hill and mountain conditions. The Red of Dobrogea was also included in improvement programs with Friesian breeds; therefore, currently, it can no longer be found as a pure breed [9,15].

The Grey Steppe and Romanian Pinzgauer—Indigenous Breeds Threatened with Extinction

In 2000, the Food and Agriculture Organization of the United Nations (FAO) included the Grey Steppe and the Romanian Pinzgauer cattle breeds in a list of domestic animals threatened with extinction, with an endangered-maintained status. At that time, 60 females and the storage of 5 males' semen were reported for the Grey Steppe cattle, and for the Romanian Pinzgauer, 1092 females were registered in herd book, and the semen of 23 males was stored [7].

The Grey Steppe breed is important because it represents a direct descendant of *Bos primigenius*. Although until 1897 its population counted slightly more than two million animals, nowadays, recent surveys have showed that their number could be less than 150 [23–25]. For most of the Grey Steppe breeds, the decreasing number was visible, especially after the World War II. For example, just 130 animals were reported for the Yugoslav Steppe located in Serbia and Montenegro [26], 500 individuals in Turkey [17], 60 Estonian Grey Steppe cattle [27], 24,000 of Italian Podolian animals [28], and 13 bulls and 171 cows of Croatian Grey Steppe [29]. This decreasing number is related to several factors. Thus, these animals have a narrowed milk production, mainly used for calves feeding; they have a medium slaughter yield, and the bulls get to the slaughter weight at an older age, compared to bulls of early maturing breeds (Angus, for instance); their meat is darker and not particularly marbled, since most of the suet forms subcutaneously, and intestinal fat deposits with lower arrangement of fat at intramuscular level. Furthermore, during the last century, the agricultural sector experienced modern technologies, which allowed a lower use of these animals for draught power [17,25,29–32].

An interesting fact is that not all reports present a continuous decline of the Grey Steppe cattle number. For example, the Hungarian Grey Steppe number of cattle was in decline until the year 1960, but after 1970, the stock has continuously increased from 500 cows and 19 bulls to 2500 cows and 100 bulls in the year 1998 [33]. The same trend is expected in many countries, since the interest in the preservation of threatened cattle breeds has considerably increased over the last decades, and various preservation programs were initiated in order to reduce the decrease of genetic diversity [16,34].

The scientific literature presents various advantages and drawbacks of Grey Steppe cattle breeding. As a benefit for the farmer, keeping such animals in shelters is cheap, due to their natural ability to be raised in wild and semi-wild conditions, by considering that the breeds' natural habitats are the forests, wetlands, marshy places, or rough grounds. In addition to high tolerance for extremely harsh environmental climatic conditions, these breeds are very resistant to disease and external parasites, having a quick recovery after illness. Moreover, these animals are very resistant to feed change, due to their very adaptive digestive tract; their grazing ability and the possibility to use a very poor diet consisting mainly in straw and stalks in cold winters are well known [17,29–32,35–37].

The Grey Steppe cows usually calve easily and have a good maternal instinct, but the average age at first calving is more belated compared to other specialized breeds (3.82 years vs. 2.23–2.99 years) [38]. These cows are ill-tempered and have fewer abilities to be milked, their udder being small, divided into fore udder and rear udder with a reduced space between teats, in some cases pseudo-teats being visible. Most of the Grey Steppe types found in Europe were not bred for milk production, but rather for meat or draught power, considering their hard and robust hoof, solid skeleton, and pronounced joints (Figure 1a,b). Milk productions of 800–900 kg per year for cows raised in households, of 1000–2500 kg for those raised in farms, with a content of 4–6% fat, and medium slaughter yield of 50–51% for oxen, and 47–49% for cows were reported [17,25,29–31]. As a part of sexual dimorphism, bulls have stronger forequarters, larger dewlaps and are more robust than cows [17]. The color of hair generally varies from light silver to dark ash, while the muzzle, eyelashes, and hooves are black. Bulls generally have a darker color than cows, and calves are reddish in color, but after 2–3 months of life, the phenomenon of molting appears, and they gradually reach the characteristic color of the breed [25]. The Grey Steppe cattle have long horns disposed in lyra shape, bulls having more robust horns than cows; oxen are more gracile than bulls, and have longer horns [39]. The length of the horn grows by 10 mm annually, and the circuit of the horn core base grows by 4.22 mm annually [40].

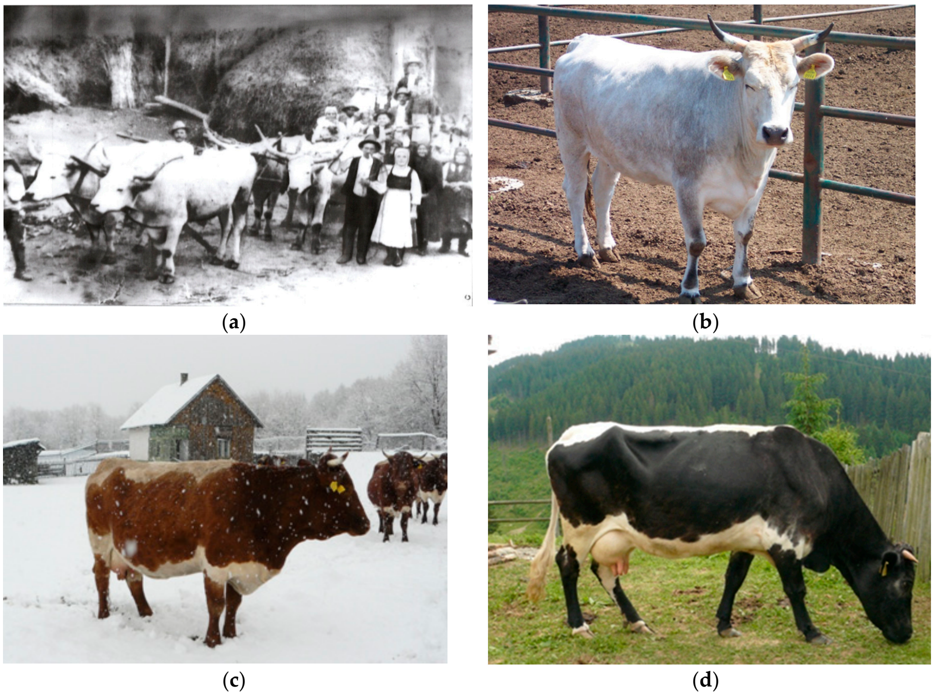

Figure 1. Grey Steppe and Romanian Pinzgauer: (**a**) Hungarian Grey Steppe (originating from Transylvania; picture from 1920s in Cluj County); (**b**) Romanian Grey Steppe (originating from Moldova); (**c**) Red variety of Pinzgauer; (**d**) Black variety of Pinzgauer (known also as Dorna variety; it is endemic to Dorna region, Romania). Original photos by Vasile Cighi (**a**), and Valentin A. Bâlteanu (**b**–**d**).

Among autochthonous improved cattle breeds, the Romanian Pinzgauer is included in the list of endangered animals, as it is still found as a pure breed (Figure 1c,d). The other improved native breeds are present in number of individuals high enough not to be considered in danger of extinction. Therefore, they will not be discussed in this paper. The Romanian Pinzgauer cattle were characterized in 1964 by a milk–meat type conformation, their milk production ranging between 2000 and 2500 kg per year (with a content of 3.8% fat), with a maximum reported of 6520 kg. The weight of cows was reported to be between 400 and 500 kg, and of bulls between 650 and 750 kg. At the end of fattening, the oxen weight ranged between 800 and 900 kg with a slaughter yield up to 54% [15].

3. The Characterization of Romanian Indigenous Endangered Cattle Breeds in Terms of Their Milk Protein Polymorphism

The genetic conservation efficiency depends both on modern biotechnologies and on genetic marker usage. Semen preservation, multiple-ovulation, egg conservation, and embryo transfer were reported as being successful in small herds' preservation. The biochemical and molecular markers proved to be equally valuable, in particular, due to their accurate information provided by (in)direct DNA analysis.

The use of isoelectric focusing technique (IEF)—as an efficient tool for screening populations at the milk protein phenotypic level—is recommended, due to the low costs and to the possibility to have six gene polymorphism in one picture, including four caseins (α_{S1}-, β-, α_{S2}-, and κ-CN) and two whey proteins: alpha-lactalbumin (α-LA) and beta-lactoglobulin (β-LG). The lack of milk in some past situations restricted the use of electrophoresis for milk protein genotyping, but nowadays, with newly developed techniques based on DNA analysis (restriction fragment length polymorphism—RFLP, and single strand conformation polymorphism—SSCP), genotype identification regardless of sex or physiological stage became possible [41–44] (Table 1).

Over the last three decades, many investigations have been developed in relation to different breeds on the certain milk protein genetic variants and yield traits, composition, and technological milk properties' associations [45–52]. Considering the Grey Steppe and Pinzgauer cattle breeds, milk protein polymorphism has been studied to establish the gene, genotype, and haplotype frequencies [23,30,41,53–59], to identify and characterize possible new alleles [23,60,61], and to argue that the Grey Steppe breed belongs to some originating groups associated to centers of domestication [53,62]. The results obtained so far in the Grey Steppe cattle showed that four of the milk proteins exhibited polymorphism, and the α_{S2}-CN and α-LA proteins showed monomorphism in all reviewed studies.

The prevalence of B allele at the α_{S1}-CN locus has been reported in Hungarian, Romanian, and Turkish varieties of Grey Steppe cattle [23,30,54,55,58], which is in agreement with its predominance in most of common European cattle breeds [42,46,63–66]. Instead, the α_{S1}-CN C allele was reported at a higher frequency than B allele in the Bulgarian Grey population by Neov et al. [53], the predominance of C allele being characteristic in zebu (*Bos indicus*) and yak (*Bos grunniens*), with about 90% of frequency in the former, and about 63% in the latter [67]. Although Chianese et al. (1988) and Rando et al. (1992, 1993) (cited by Formaggioni et al. [67]) reported the D and G allele, respectively, in the Podolian cattle group, neither of these variants has been found in the reviewed Hungarian, Romanian, or Turkish Grey Steppe cattle populations. Instead, a possible new protein variant (α_{S1} I^{SM}) was reported in the Romanian Grey Steppe cattle Moldavian variety, at a higher frequency when 30 milk samples from pure breed individuals were analyzed by PCR-RFLP and IEF technique [30,58], compared to the results obtained from the IEF analyses of 13 milk samples from pure breed, and 11 milk samples from individuals of uncertain origin [23]. This I^{SM} new allele was identified only in pure breed individuals, the pedigree analyses showing that the carrying cows in heterozygous form (BI^{SM}, CI^{SM}) had a common ancestor [35]. After a three times genotyping experiment iteration, Bâlteanu et al. [23] concluded that the IEF profile was the same every time, the isoelectric point of this new protein being situated between the B and C alleles, closer to C. The α_{S1} I^{SM} allele was fully characterized by cDNA sequencing, and this was different from the B variant by two substitutions, and from C variant by one substitution [61].

The β-CN locus is known as particularly polymorphic, A^1 and A^2 alleles being found both in *Bos taurus* and *Bos indicus* breeds. The A^2 allele was reported at a frequency of more than 50% in Romanian and Hungarian Grey Steppe, with a higher incidence in Hungarian variety [23,30,54,58].

The A^2 allele was reported at a higher frequency in the Romanian Grey Steppe population, which included both pure breed and individuals of uncertain origin, in contrast with the A^1 higher reported frequency when 30 milk samples from pure breed individuals were considered [30,58]. The highest rate of ancestral A^2 allele was confirmed in Holstein Friesian reared in Italy, Romania, and Estonia, and in other cattle populations, located from Southern to Northern Europe, such as different Italian breeds, Czech Fleckvieh, Romanian Spotted, Romanian Black and White (formed by crossing with Holstein Friesian cattle), Brown, Black Pinzgauer, and Montbéliarde cattle populations reared in Romania, and Estonian Native cows [42,55,63,66,68–70].

The A^1 allele was reported at a higher frequency in improved cattle breeds from the Northwestern Europe [30]. Mishra et al. (2009) (cited by Mir et al. [71]) reported the absence of the A^1 allele in most of the Indian cattle breeds. The β-CN B and C variants were revealed at low frequencies in the Romanian Grey population, which also included the individuals of uncertain origins. These allelic variants were unreported in milk sasmples analyses collected from Grey Steppe pure individuals, although the applied method was the same (IEF). In the Hungarian Grey population investigated by Baranyi et al. [54], the B allele reported frequency was the lowest, while the C allele was unreported in those 101 analyzed milk samples. The B and C alleles were also reported in a Romanian Spotted cattle population by Grădinaru et al. [42], at close frequencies to those reported in the Romanian Grey Steppe by Bâlteanu [23]. The Romanian Spotted is a multipurpose breed formed by crosses between Romanian Grey Steppe, as a maternal breed, and imported Simmental bulls. In yak and zebu, the C variant has never been detected [67].

Although most of the genetic analyses allow the identification of α_{S2}-CN locus as monomorphic with A allele in the most of cattle breeds, other alleles, such as B and C, were reported in Nepalese *Bos taurus* and *Bos indicus* populations (reviewed by Formaggioni et al. [67]). According to Formaggioni et al. [67], the D variant was found by Grosclaude et al. (1976; 1978) in two French bovine breeds, Montbéliarde and Vosgienne, respectively. The B allele was reported first in Podolian cattle by Chianese et al. (1988) (cited by Formaggioni et al. [67]), but it was absent in the Romanian Grey Steppe cattle population investigated by Bâlteanu et al. [23].

The κ-CN was the last of the main milk proteins for which polymorphism has been detected [67]; the A and B alleles are universally spread in bovine and zebu. The predominance of the κ-CN A allele in Hungarian Grey cattle [54] was also reported in Holstein Friesian populations located in Estonia, Romania, Iran, or China, and in several other breeds reared in Europe and Iran [42,65,66,68–70,72–77]. In fact, this is not a selection response, since the B allele is associated with higher κ-casein concentration [48,49], protein, fat, and milk yield [46,50], a better reaction with chymosin, a significantly lower clotting time, and a higher rate of curd formation [78,79]. This could be explained by the linkage present among the α_{S1}-, β-, and κ-CN loci, with higher rates of possible combinations, including A allele at the κ-CN locus. The A allele was also more frequent than B allele in *Bos taurus* and *Bos indicus*, considering the research reviewed by Mir et al. [71], and in contradiction with the findings of Ceriotti et al. [80]. Investigations carried out on Bulgarian and Romanian Grey Steppe cattle populations showed a higher frequency of the B allele compared to A allele [23,30,41,53,58,59]. Baranyi et al. [54] argued the gradually decreasing frequency of κ-CN A allele proceeding from North-West of Europe to South-East of Europe, but the B allele frequency in the Romanian Grey Steppe investigated population was higher than that reported in the Bulgarian Grey Steppe-investigated population.

A study which took into account the linkage of bovine casein locus (α_{S1}-, β-, α_{S2}- and κ-CN), proved that the distribution of the casein haplotypes clearly depended on the geographic origin of breeds. Therefore, considering a haplotype structure of α_{S1}-CN prom, α_{S1}-CN, β-CN, α_{S2}-CN, and κ-CN, the haplotypes BBA^2AA and BBA^1AA were found predominantly in cattle breeds from Central and Northwestern Europe, the BBA^1AB and BBA^2AB are predominant in taurine breeds from Southern Europe and Africa, while in *Bos indicus*, the haplotypes BCA^2AA_i, CCA^2AA, BCA^2AH, and CCA^2AH occurred as specific haplotypes or at a high frequency [62].

The α-LA B allele is the most reported in all *Bos taurus* western breeds, the α-LA A allele being found in *Bos indicus* populations and in some European cattle breeds, particularly in those imported from South Africa [67]. The A allele was reported in the Podolian cattle reared in the South of Italy, which may suggest a common origin of such breeds to *Bos indicus*. This A allele was absent when it was studied in the Romanian Grey Steppe population [23].

The A and B variants of β-LG are diffused both in bovines and in zebu, the ancestral B allele prevailing in *Bos taurus*, as well in *Bos indicus* breeds [71], in Hungarian Grey Steppe [54], and in 55 individuals of Romanian Grey Steppe genotyped using the PCR-RFLP technique [41,59]. However, the investigations performed on Romanian Grey Steppe cattle using IEF technique revealed a higher frequency of the A allele at this locus [23,30,58].

Table 1. Milk protein alleles and frequencies found by different authors in the Grey Steppe and Romanian Pinzgauer.

Locus	Breed	Alleles	Frequency (Reference)		Frequency (Reference)			
α_{S1}-CN	Grey Steppe	B	0.770	[23] 1	0.7			[58] 2
		C	0.189	[23] 1	0.2			[58] 2
		I^{SM}	0.041	[23] 1	0.1			[58] 2
	Romanian Red Pinzgauer	B	0.867	[66] 4				
		C	0.133	[66] 4				
	Romanian Black Pinzgauer	B	0.692	[66] 4				
		C	0.308	[66] 4				
α_{S2}-CN	Grey Steppe	A	1	[23] 1, [58] 2				
	Romanian Red and Black Pinzgauer	A	1	[66] 4				
β-CN	Grey Steppe	A^1	0.354	[23] 1	0.45			[58] 2
		A^2	0.563	[23] 1	0.55			[58] 2
		B	0.063	[23] 1				
		C	0.020	[23] 1				
	Romanian Red Pinzgauer	A^1	0.483	[66] 4				
		A^2	0.400	[66] 4				
		B	0.117	[66] 4				
	Romanian Black Pinzgauer	A^1	0.115	[66] 4				
		A^2	0.750	[66] 4				
		B	0.097	[66] 4				
		C	0.038	[66] 4				
κ-CN	Grey Steppe	A	0.417	[23] 1	0.417	[58] 2	0.464	[41] 3, [59] 3
		B	0.583	[23] 1	0.583	[58] 2	0.536	[41] 3, [59] 3
	Romanian Red Pinzgauer	A	0.534	[66] 4				
		B	0.466	[66] 4				
	Romanian Black Pinzgauer	A	0.674	[66] 4				
		B	0.326	[66] 4				
α-LA	Grey Steppe	B	1	[23] 1, [58] 2				
	Romanian Red and Black Pinzgauer	B	1	[66] 4				
β-LG	Grey Steppe	A	0.542	[23] 1, [58] 2			0.409	[41] 3, [59] 3
		B	0.458	[23] 1, [58] 2			0.591	[41] 3, [59] 3
	Romanian Red Pinzgauer	A	0.267	[66] 4				
		B	0.650	[66] 4				
		C	0.083	[66] 4				
	Romanian Black Pinzgauer	A	0.269	[66] 4				
		B	0.731	[66] 4				

1 Isoelectric focusing (IEF), 24 individuals; 2 IEF and PCR-RFLP, 30 individuals (differences in frequency can be the result of the different investigation techniques used); 3 PCR-RFLP, 55 genotyped individuals; 4 IEF, 26 Black and 30 Red Romanian Pinzgauer (differences between the two varieties can be due to the small number of individuals investigated; Romanian Pinzgauer have a different origin from other Pinzgauer populations, so that results cannot be extrapolated to all the Pinzgauer varieties in the world).

Concerning the Romanian Pinzgauer cattle breed, research carried by Bâlteanu et al. [66] on red and black varieties showed the α_{S2}-CN and α-LA as monomorphic loci. The α_{S1}-CN, β-CN, κ-CN and β-LG were reported as polymorphic loci. For some of them, a different number of alleles among the two Romanian Pinzgauer varieties were reported. For example, the C allele of β-CN was reported only in the black variety, and the C allele of β-LG, only in the red variety (both at the lowest frequencies). At β-CN locus, the A^2 allele was reported at the highest frequency in the black variety, and the A^1 was reported at the highest frequency in the red variety. At α_{S1}-CN, κ-CN, and β-LG loci, the B, A, and B alleles, respectively, were reported at the highest frequencies in both types of Romanian Pinzgauer [66].

In the Slovak Pinzgauer cattle, at α_{S1}-CN locus, the A allele was reported at a higher frequency than B allele; their identification was carried out using the PCR-RFLP technique by MaeIII on 3% agarose gel [57]. At the β-CN locus, Miluchová et al. [56] reported A^1 and A^2 alleles, with the A^1 allele showing the highest frequency (using PCR-RFLP technique for CSN_2 gene by DdeI on 3% agarose gel).

4. Trends in Genetic Conservation Studies Using Microsatellites

Microsatellites are a new type of genetic marker found at high density and randomly dispersed across chromosomes, increasingly used for genetic diversity studies, in particular, for breeds that are on the brink of extinction, due to their high degree of polymorphism and their neutral behavior to selection [16,43].

Considering the Grey Steppe cattle, various investigations on different numbers of microsatellite loci from those recommended for genetic diversity studies in cattle [81] were already performed [16,29,34,43,82]. Considering the allelic variability reported in the studied varieties of Grey Steppe, the highest allelic polymorphism (16 different alleles) was identified at TGLA227 locus in the Syrmian Podolian cattle [29]. A rough similarity of allelic polymorphism was reported at BM2113 locus for the Syrmian Podolian cattle by Keros et al. [29], TGLA53 locus for the Podolica cattle breed by Moioli et al. [82], and TGLA122 locus for the Romanian Grey Steppe by Ilie et al. [34], with 15 different alleles at each locus and for each breed. If one considered the investigated Romanian [34,43], Croatian [29], and Bulgarian Grey Steppe [16] populations where similar microsatellites were taken into account, all the loci were polymorphic, with the highest average number of alleles at TGLA227 locus (11.5 alleles), followed by TGLA53 (10.75 alleles), TGLA122 (10 alleles), BM2113 (9.5 alleles), INRA023 (9.25 alleles), ETH3, and TGLA126 (8.25 alleles each), SPS115 (8 alleles), ETH 10 (7.25 alleles), BM1824 (6.75 alleles), and ETH225 (6 alleles) loci. The highest average number of alleles per locus in those four Grey Steppe investigated populations was 11.73 in the Syrmian Podolian cattle [29], followed by 9.09 alleles in 32 individuals of the Romanian Grey Steppe [34], 7.55 alleles in the Bulgarian Grey Steppe [16], and 6.36 alleles in 30 individuals of the Romanian Grey Steppe [43].

Considering the results obtained on microsatellite analyses, phylogenetic relationships inside of Grey Steppe varieties or between Grey Steppe cattle and other breeds were established by Moioli et al. [82], Georgescu et al. [43], Pariset et al. [18], Xuan et al. [83], D'Andrea et al. [28], and Ilie et al. [34]. Looking at the Reynolds' genetic distances, the Romanian Grey Steppe breed represents a clearly distinct group from those of Montbéliarde and Romanian Spotted (both belonging to the Simmental group), or Romanian Brown and Romanian Black Spotted [43]. A cluster of Romanian Grey Steppe separate from those of Romanian Black and White and Romanian Brown, and Romanian Spotted and German Spotted cattle breeds, was also demonstrated by Ilie et al. [34]. Part of these results was confirmed by mtDNA D-loop region sequences analysis performed by Xuan et al. [83], and the three Romanian cattle breeds (Romanian Black Spotted, Romanian Brown, and Romanian Grey Steppe) were divided in two distinct clusters, one comprising Romanian Black Spotted breed and Romanian Brown, and the second including Romanian Grey Steppe.

Considering the Nei's standard genetic distance when three Podolitic breeds were deemed, the Maremmana is more distant from the Hungarian Grey Steppe than from the Turkish Grey Steppe, and the Turkish Grey Steppe is more distant from the Hungarian Grey Steppe than from Maremmana [18]. The shortest distance between the Maremmana and Podolica breeds was reviewed by Moioli et al. [82], which is in line with their common historical origin. Another confirmation of Podolian cattle breeds' group came from the results reported by D'Andrea et al. [28],where three clusters included the following breeds: Piemontese, Holstein Friesian, Simmental, and Pezzata Rossa Italiana (cluster 1), Limousine, Grigio Alpina, Rendena, Cabannina, and Swiss Brown (cluster 2), and Istrian Podolian, Italian Podolian, Romagnola, and Chianina (cluster 3).

5. Conclusions

The reviewed literature on Romanian indigenous endangered cattle breeds confirms that these animals are carriers of a valuable gene pool which can be kept and bred by applying different reproductive biotechnologies.

The Grey Steppe cattle group is considered a direct descendant of the ancestor *Bos primigenius*, and a maternal breed for many improved cattle breeds formed in Romania. Its genetic heritage is found in numerous other cattle breeds, as it is demonstrated by the variety of studies performed using different genetic markers. Some of the reported results may be contradictory, due to the different types of Grey Steppe cattle, natural selection rather than the artificial selection, or due to an explanation based on geographical considerations. Furthermore, many alleles spreading suggest either a phylogenetic relationship of taurine (*Bos taurus*) and zebu (*Bos indicus*), or the introgression of *Bos indicus* in Southern and Eastern European cattle breeds.

From a practical perspective, given the low number of livestock (the situation also called population bottleneck), the sustainable conservation of old breeds, such as the Grey Steppe cattle, must be made based on consistent molecular evidence; otherwise, the genetic drift becomes very probable, the variability decreases, while some alleles are lost. In this regard, the most unrelated genitors from a numerically reduced population can be identified using molecular markers, and mated, in order to avoid excessive inbreeding and to maintain the diversity within one rustic breed. It is known that in Hungary, but also in other countries, this type of selection is already practiced.

Identification of the rare genotypes at individual level, which are close to the ancestral gene pool for each breed, is the key to sustainable conservation of many cattle breeds. Rustic cattle breeds are carriers of valuable genes, and these genes can be sustainably conserved only together with their reservoir, i.e., the breed as a whole.

The more conserved the rustic breeds, the higher the diversity of ancestral genes. Ancestral genes can offer in the future, on the one hand, solutions to improve the animal livestock and heal some diseases and, on the other hand, answers to questions of animal evolution.

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