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Genetic Polymorphism of Main Lactoproteins of Romanian Grey Steppe Breed in Preservation

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Abstract**—**The paper presents a part of the results obtained in a complex research project on Romanian Grey Steppe breed, owner of some remarkable qualities such as hardiness, longevity, adaptability, special resistance to ban weather and diseases and included in the genetic fund (G.D. no. 822/2008.) from Romania.

Following the researches effectuated, we identified alleles of six loci, codifying the six types of major milk proteins: alpha-casein S_1 (α S1-cz); beta-casein (β-cz); kappa-casein (K-cz); beta-lactoglobulin (β-lg); alpha-lactalbumin (α-la) and alpha-casein S_2 (α S2-cz). In system αS1-cz allele α_{s1}-Cn B has the highest frequency (0.700), in system β-cz allele β-Cn A_2 (0.550), in system K-cz allele k-CnA_2 (0.583) and heterozygote genotype AB (0.416) and BB (0.375), in system β-lg allele β-lgA_1 has the highest frequency (0.542) and heterozygote genotype AB (0.500), in system α-la there is monomorphism for allele α-la B and similarly in system αS2-cz for allele α_{s2}-Cn A.

The milk analysis by the isoelectric focalization technique (I.E.F.) allowed the identification of a new allele for locus α_{S1}-casein, for two of the individuals under analysis, namely allele called αS1-casein I^{RV}. When experiments were repeated, we noticed that this is not a proteolysis band and it really was a new allele that has not been registered in the specialized literature so far. We identified two heterozygote individuals, carriers of this allele, namely: BI^{RV} and CI^{RV}. This discovery is extremely important if focus is laid on the national genetic patrimony.

Keywords**—**allele, breed, genetic preservation, lactoproteins, Romanian Grey Steppe

I. INTRODUCTION

IN the foreign specialized literature there are many researches related to the chemical composition of milk, the features of the main bovine lactoproteins, genetic parameters for a series of milk components as well as of the non-genetic factors influencing the protein content of milk. When presenting the bovine lactoproteins, access is logically laid on the primary, secondary and tertiary structure of kappa-casein and the main protean fractions: β-casein, β-lactoglobulin, αS_1 casein and αS_2 casein [1, 2, 3, 4, 5].

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We have knowledge of the latest researches that have managed to establish the three dimensional structure of kappa-casein thus clarifying a series of properties of this protean fraction of milk having such an important role in milk coagulation with a direct influence on the output and quality of cheese. Many quantitative and qualitative genetics researches focused on the structure of the genes responsible for the synthesis of the main bovine lactoproteins and the punctiform mutations suffered by these within evolution, a thing that allowed the presence of this accentuated polymorphism of lactoproteins [6, 7].

In a first stage of our researches, we established the average value and the variability of the milk yield indices and lactoprotein indices under study. On this occasion, we made a full analysis of locus of kappa-casein (K-cz), establishing the gene frequency, genotype frequency, the standard error of the gene frequency and the state of genetic balance according to Hardy-Weinberg law.

II. Material and Method

Researches were effectuated on 30 Romanian Grey Steppe cows raised semi-intensively, tied-up stalling, at the Research-Development Station for Bovine Growing Dancu, Iaşi (S.C.D.C.B. Dancu, Iaşi). Due to the strictly genetic determinism of lactoproteins, what makes genotype be identical to phenotype, lactoprotein frequency is very different from one breed to another. Hence, the need to run these researches that might establish the genotypic and allelic frequencies of lactoproteins for Romanian Grey Steppe breed, the Moldavian variety from the North-Eastern part of Romania. The study of polymorphism of milk proteins was made by PCR-RFLP technique, and for the study of polymorphism of all bovine lactoproteins we also used the isoelectric focalization technique (I.E.F.) [8, 9].

The milk samples were collected individually in 15 ml Falcon tubes, transported at 4^0 C and then frozen at -20^0C until tests were run. Defrosting occurred slowly at room temperature and subsequently, samples were centrifuged at 8.000 rotations/minute, for 5 minutes for milk separation. They were stored for 30 minutes at 4 degrees for fat solidification and then it was removed from each tube by means of a spatula.

For an optimal protein concentration, samples were diluted with a urea and β-mercaptoethanol solution. Samples were migrated in a polyacrylamide gel with 4% concentration. After migration, the gel was immersed in a solution 10% of trichloroacetic acid. Colouring occurred for 2 hours by means of a solution 0.025% Coomassie Brilliant Blue R-250 in 40% ethanol and 7% glacial acetic acid.

III. Results and Discussions

We suspected the presence of some ancestral alleles undiscovered so far for the loci codifying the milk proteins due to the lack of an improvement programme, what made Romanian Grey Steppe breed keep a high variability for a long time. Unfortunately, the drastic reduction of the number of individuals and the replacement of this breed with other more productive breeds has led to the loss of this variability and this is why the breed has been introduced in a preservation programme of the animal genetic resources from Romania.

In figure 1 we may see the alleles identifies for the six loci codifying the six types of major proteins of milk (α S1-cz; β-cz; K-cz; β-lg; α-la; α S2-cz).

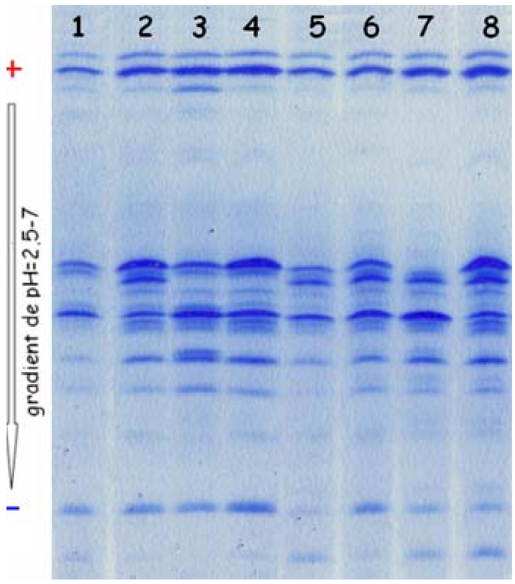

Fig. 1 IEF Profile belonging to some individuals from Romanian Grey Steppe breed highlighting alleles of milk major proteins

The genetic structure for polymorph systems of milk proteins: alpha-casein S_1 (αS1-cz), beta-casein (β-cz), kappa-casein (K-cz), beta-lactoglobulin (β-lg), alpha-lactalbumin (α-la) and alpha-casein S_2 (αS2-cz) is presented in table 1.

As the specialized literature mentions [10, 11], in our case too α_{s1}-Cn B is more frequently encountered with a higher frequency than 0.7 and allele α_{s1}-Cn C had the frequency of 0.2 for the animals under study.

Casein α_{s2} is monomorphous for Romanian Grey Steppe nucleus under study, for allele α_{s2}-Cn A., as it appears at all bovine breeds studied so far.

Casein β has the two universal variants β-Cn A_1 and β-Cn A_2 found out at bovines and. In our case, allele β-Cn A_2 (0.550) is the most frequently met and β-Cn A_1 has a frequency of 0.45, variants B, C and A_3 being absent. Variant β-Cn A_1 has a higher frequency for the breeds originating in North-West Europe and the breeds of improved members of the Bovidae family [12].

The higher frequency of allele A_2 has a special significance since this allele is the ancestral one from which all the others derived phylogenetically.

TABLE I
GENETIC POLYMORPHISM OF MILK PROTEINS FOR ROMANIAN GREY STEPPE BREED

Registration no.	α S1-cz	β-cz	K-cz	β-lg	α-la	α S2-cz
9991	BI^{RV}	A_1A_2	AB	AB	BB	AA
9993	BB	A_1A_1	BB	AB	BB	AA
9983	BB	A_1A_2	AB	AB	BB	AA
9988	BB	A_1A_2	BB	AA	BB	AA
0004	CI^{RV}	A_2A_2	AA	AB	BB	AA
9985	BC	A_1A_2	AB	AB	BB	AA
9990	BB	A_1A_2	BB	AA	BB	AA
9998	BC	A_2A_2	AB	AB	BB	AA
9723	BB	A_1A_1	BB	AB	BB	AA
9986	BC	A_2A_2	AB	AB	BB	AA
Genotype frequency	BB = 0.5 BC = 0.3 CI^{RV} = 0.1 BI^{RV} = 0.1	A_1A_1 = 0.2 A_1A_2 = 0.5 A_2A_2 = 0.3	AA = 0.209 AB = 0.41 BB = 0.375	AA = 0.292 AB = 0.50 BB = 0.208	BB = 1	AA = 1
Allele frequency	$p_B = 0.7$ $q_C = 0.2$ $r_{IRV} = 0.1$	p_{A1} = 0.45 q_{A2} = 0.55	p_{A1} = 0.417 q_{A2} = 0.583	p_{A1} = 0.542 q_{A2} = 0.458	P_B = 1	$p_A = 1$

All researches have undoubtedly showed the favorable influence of variant k-Cn B on milk quality, cheese output and quality. Consequently, in the study of bovine lactoproteins, most researches focused on the determination of the frequency of kappa-casein alleles at different breeds and the possibility of "limited" promotion by selection of kappa casein B.

Variants k-Cn A and k-Cn B are universally discovered at bovines and zebu. In recent years, 3 more variants have been identified: k-Cn C, k-Cn D and k-Cn E, all having frequencies lower than 0.1 and being identified only in some local breeds.

For Romanian Grey Steppe breed from S.C.D.C.B. Dancu Iaşi, K-cz system has a high frequency for allele k-CnA_2 (0.583) and the heterozygote genotype AB (0.416) and BB (0.375). As we already mentioned, ancestral allele B is associated to diverse breeds and a better quality of milk.

Variant k-Cn B has a higher frequency in the breeds from Brună group, of different origins raging between 0.4 and 0.6. The failure to promote k-Cn B by selection triggers in time a reduction of its frequency. In the crossbreeds of different breeds, the frequency of k-Cn B is intermediate between the frequencies of pure breeds showing the strong influence of crossbreeding in the transmission of the wanted type of kappa-casein.

In system β-lactoglobulin, variants β-Lg A and β-Lg B are universally encountered in bovines and zebu. The distribution of the two variants in most breeds is quite balanced. In our case, β-lgA$_1$ has the highest frequency (0.542) and the heterozygote genotype AB (0.500).

For α-lactalbumin, variants α-La A and α-La B apparently exist in most zebu populations. In almost all breeds of bovines we encounter only variant α-La B. α-La A, encountered in zebu, is less rare in the countries from Central and Meridional Europe being discovered in 11 Italian breeds and some Russian and Romanian local ones. In our researches too on Romanian Grey Steppe nucleus, we have encountered a monomorphism for allele α-la B.

The milk analysis by IEF and PCR-RFLP allowed the identification of a new allele for locus α_{S1}-casein, for two of the individuals analysed from S.C.D.C.B. Dancu, Iaşi, namely allele called αS1-casein I^{RV} [8, 9]. It appears under the shape of a band with isoelectric point situated between alleles B and C, closer to that of allele C as one may see in figures 2, 3, 4, 5, 6 and 7

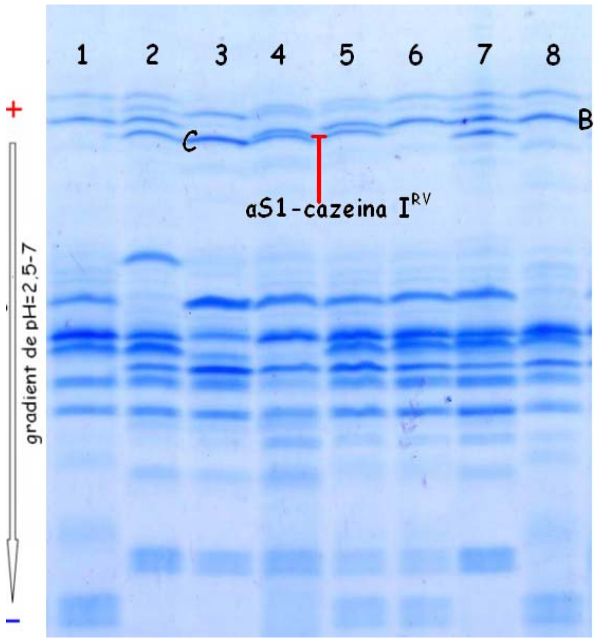

Fig. 2 IEF profile for bovine lactoproteins of Romanian Grey Steppe (lanes 1, 4, 5, 6, 8), as against Bălţata Românească breed (lanes 2, 3, 7) for locus of αS1 casein.
Genotypes of αS1 casein are:
1- BB; 2- BC; 3- CC; 4- CI^{RV}; 5- BI^{RV}; 6- BB; 7- BC; 8- BB.

When repeating the experiments, we noticed that this is not a proteolysis band but it really is a new allele that has not been registered so fat in the specialized literature: BI^{RV} and CI^{RV} [8, 9].

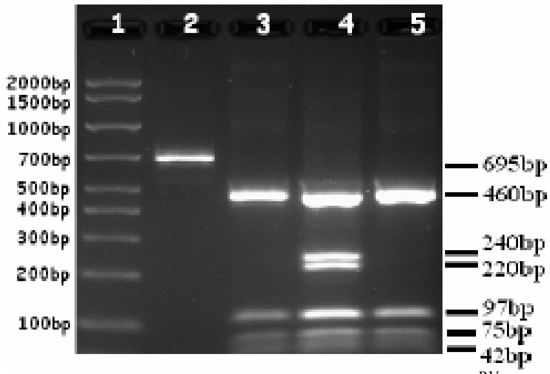

Fig. 3 Comparison between αS_1-CN B, C and I^{RV} cDNA's restriction map with BseGI enzyme. Note the presence of 240 and 220 by fragments specific to I^{RV} allele and 460 bp product specific to B and C alleles respectively. Lane 1: Ampli Size^{TM} Molecular Ruler, BioRad, Lane 2: Uncut cDNA; Lane 3 and 5: αS_1-CN BB and CC samples, respectively; Lane 4: BI^{RV}

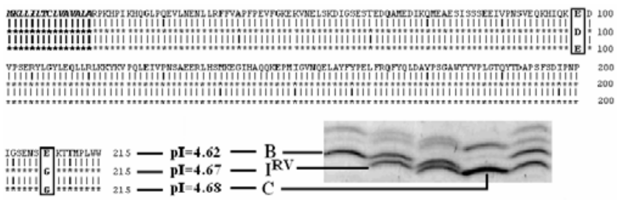

Fig. 4 Comparison between protein sequences features of B, C and I^{RV} variants (Romanian Grey Steppe breed).
The mutations, which are making the difference between this 3 protein variants, are marked with rectangles. Note the differences between isoelectric points (**pI**) of the 3 proteins, which explain the observed IEF profiles (gel image from the right side). Signal peptide is highlighted with bold italic letters.

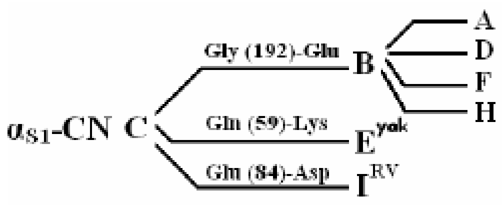

Fig. 5 Phylogenetic origin of alpha S$_1$ I^{RV} allele

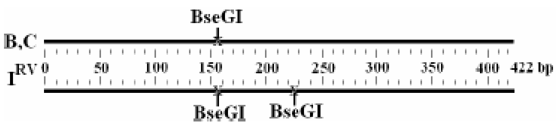

Fig. 6 Comparison between B, C and I^{RV} cDNA theoretical restriction maps of BseGI enzyme. Note 1 restriction sites for BseG in B and C allele and 2 in I^{RV}

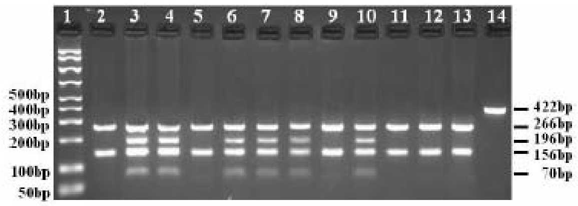

Fig. 7 Identification of αS_1-CN I^{RV} allele by PCR-RFLP.
The reference samples αS_1-CN BB (Lane 2), CC (Lane 5) and BC (Lane 9) revealed the same BseGI digestion pattern: 266 bp and 156 bp fragments. Digestion of the 422 bp fragments amplified from reference samples BI^{RV} (Lane 3) and CI^{RV} (Lane 4) revealed 2 additional fragments specific to I^{RV} allele (196 and 70 bp). Lanes 6, 7, 8, 10 – samples belonging to 4 bulls carriers of I^{RV} allele. Lanes 11, 12, 13, - samples which not carry this new variant. Lane 1: Ampli Size™ Molecular Ruler, BioRad.

This discovery is extremely important if they lay focus on the preservation of the national genetic patrimony. It has not been noticed in other European breeds of members of the Bovidae family or the breeds from the Podolic Family where Romanian Grey Steppe belongs to. Despite all these, a similar IEF profile was noticed for Kuri breed from Africa and Nepalese Bos taurus [13, 14]. Unfortunately, they have no conducted studies regarding the frequency of this possibly new allele, which is why it has not been registered in the specialized literature.

If this should ever be done and one should confirm that IEF profile noticed by these authors and us belongs to an allele identical for this locus, we may draw the conclusion that there are common phylogenetic relations between Romanian Grey Steppe and the primitive members of the Bovidae family from the two continents.

The presence of this ancestral allele specific to Romanian Grey Steppe breed demonstrates it seniority and represents a premiere for the Podolic Family. Consequently, this new casein may represent an important genetic marker of bred origin. It might represent an alarm signal for the intensification of efforts to keep Romanian Grey Steppe breed, unique among the primitive bulls' descendants.

Romanian Grey Steppe breed is one of the breeds that may offer many peasant surprises in terms of understanding the mechanisms of resistance to diseases and turning to good use of the low nutritive food, characters that have been diminished or lost in the improved cows.

Allele αS1-casein I^{RV} was fully sequentialized in 2008. Following sequentialization, they identified the following substitutions as compared to the common alleles B and C: position 297 (exon 11): gaA - Glu (B, C) gaT- Asp (I^{RV}); position 620 (exon 17): gAa – Glu(B) gGa - Gly (C, I^{RV}). Based on substitution A-T from exon 11, they elaborated a protocol PCR-RFLP for the identification of allele I^{RV}.

The sequentialized allele was downloaded in GenBank (access number GenBank EU908730.1) and in the prestigious data base of NCBI - National Center for Biotechnology Information, U.S.A.

IV. Conclusion

1. For Romanian Grey Steppe breed, we identified alleles for the six loci codifying the six major proteins of milk (α S1-cz; β-cz; K-cz; β-lg; α-la; α S2-cz). In system K-cz, we noticed a high frequency for allele k-CnA_2 (0.583) and the heterozygote genotype AB (0.416) and BB (0.375).
2. We have identified new alleles for locus α_{S1}-casein, namely the allele called αS1-casein I^{RV}. Two of all individuals under study are heterozygote carriers of this allele, namely BI^{RV} and CI^{RV}. This discovery is extremely important if access is laid on the preservation of the national genetic patrimony.
3. The breeding bulls of Romanian Grey Steppe breed should be checked in terms of milk quality by genomic testing and use of genetic markers as modern methods recently introduced in the genetic improvement of the members of the Bovidae family.

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