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# Application of Milk Proteins Genetic Polymorphism for Selection and Breeding of Dairy Cows in Bulgaria

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Additional information is available at the end of the chapter

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## 1. Introduction

Main goals of the dairy cattle breeding are the search of an economically efficient way of the improvement of milk production and the qualitative milk traits. Selection and breeding of animals with desirable genotypes is of crucial importance for the genetic improvement of dairy cows.

Milk protein genetic polymorphisms of the genus *Bos* provoke a significant scientific interest, mainly associated with their evolution, population structure, breeding and hybridization. Over the last decades, studies have been concentrated on the influence of the genetic variants of the major milk proteins on the quantitative and qualitative milk traits and their technological properties (Di Stasio & Mariani, 2000; Martin et al., 2002).

Cow milk contains two classes of specific proteins, i.e. the group of caseins and the group of whey proteins. This division has been based on the milk proteins behaviour at pH 4.6. The former class, which is about 80% of the protein content in bovine milk, is precipitated at pH 4.6 (isoelectric pH). It contains four caseins, i.e.  $\alpha$ s1- (CSN1S1),  $\alpha$ s2-,  $\beta$ - and  $\kappa$ -casein (CSN3). The latter class, which constitutes about 20% of milk proteins, is soluble under those conditions. It contains two main whey proteins, i.e.  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin (LGB) as well as some other proteins presented by insignificant concentrations (Fox & McSweeney, 1998). Each of the above mentioned proteins is presented by at least two genetic variants. Genetic variants of milk proteins differ from each other by one or more aminoacid residues in the polypeptide chains, which is due to various types of mutations in the genes encoding them. There are several methods for genotyping milk protein polymorphisms; however, the most frequently applied one is the PCR-RFLP assay. The alleles of a particular gene can be identified through their restriction profile.

Most of the studies have been focused on CSN1S1 and CSN3 of the group of caseins and LGB of the whey proteins. These proteins have a great effect on milk production and milk constituents (Erhardt, 1996). The genetic variants of the CSN3 gene have a dominant role due to the protein influence of this protein on the formation, structure and stabilization of the casein micelles with respect to milk technological properties and cheese production (Farrell et al., 1996). In bovine milk, the CSN3 protein is calcium-insensitive because it contains only one phospho-seryl residue and prevents the precipitation of the other three caseins. Some milk proteins are potential allergens (especially CSN1S1) because they are missing in human milk (EFSA, 2004; Crittenden & Bennett, 2005). LGB is the major whey protein of ruminant species. Its biological functions are not well studied but there are data that it may have a role for the phosphate metabolism in the mammary gland (Hill et al., 1997). LGB also have a role for the transport of retinol and fatty acids in the gut because it seems to be resistant to gastric digestion *in vivo* and remains intact after it passes through the stomach (Yvon et al., 1984). There are numerous reports on the polymorphism of the milk protein genes from various areas of the world. However, data for Bulgarian cattle populations are scarce. Such studies have been initiated by our team (Zlatarev et al., 2008). Subsequently, several cow breeds were examined (Hristov et al., 2011a, b). Our recent investigations have been focused on the Bulgarian Rhodopean Cattle Breed (BRC), which is of high significance due to its long-term exploitation and the relative independence from environmental conditions. It is incomparable with any other breeds in Bulgaria with respect to the milk yield, fat and protein contents, viability, use continuance and fertility. Because of all these features, the BRC breed is a valuable gene fund for the country and the region and its preservation and improvement are of significant interest.

The selection and breeding of the BRC breed (and all the other breeds in Bulgaria) with respect to the milk production and milk quality have been accomplished mainly on the basis of phenotypic features. The goal of the analysis of the polymorphisms of the milk protein genes is to obtain data applicable as additional criteria in the marker-assisted selection and breeding. This is a reliable approach, which may facilitate and accelerate the selection process, increasing the qualitative and quantitative characters of the farm production and the competitiveness of the milk industry. This strategy will also increase the economic efficiency of the dairy cattle farming and genetic improvement of dairy cattle populations.

## **2. Genotyping of Bulgarian Rhodopean Cattle Breed and Shorthorn Rhodopean Cattle Breed**

The Shorthorn Rhodopean Cattle Breed (SRC) is one of the two native cow breeds in Bulgaria. Its population is threatened by extinction. It is considered as one of the last forms of the prehistoric European cattle breeds together with Albanian, Illyric Dwarf and Montenegro cattle. Due to the fact that the gene fund of highly productive dairy cows is getting narrower in the course of years of selection, this breed is a genetic resource for the enrichment of other breeds used in the region. On the other hand, it is of great importance to preserve its own population structure as a native cattle breed for Bulgaria. The SRC breed is also a basic breed for creation of the BRC, officially declared as a new race in 1989 (Nickolov,

1999). Therefore, the application of highly efficient molecule markers for genotyping, genetic identification and marker-assisted selection is very important for the evaluation and preservation of the biodiversity of these native Bulgarian cow breeds. Consequently, studies of milk protein genes polymorphism and their application for the identification of individuals with desirable characteristics as well as for the selection and breeding programs is the main focus of the recent work of our team.

## 2.1. Genotypes and allelic forms identification

Each of the studied genes is presented by at least two variants, which are genetically determined by autosomal and codominant alleles. The absence of genetic dominance is useful because the homozygous individuals give only one variant for each protein in the electrophoregram, while heterozygous ones give both variants. Thus, the estimation of the gene frequencies for a population is easy. The variants can be detected by both protein electrophoresis, isoelectric focusing (IEF) and analysis of DNA. At DNA level, polymorphisms are due either to single nucleotide substitutions or to DNA re-arrangement phenomena. The differences between genotypes could be identified using specific restriction enzymes. Particular alleles could be evidenced on the electrophoresis gel with different band length (PCR-RFLP assay).

### 2.1.1. *CSN1S1* gene

The four casein genes are localized in a 250 kb cluster (Ferretti et al., 1990; Threadgill & Womack, 1990) situated in the Chromosome 6 (Hayes et al., 1993a; Popescu et al., 1996). Their order is *CSN1S1*, *CSN2*, *CSN1S2* and *CSN3*. This gene cluster is also referred to as the casein locus (Martin et al., 2002) or as the super locus (Freyer et al., 1999). The genomic DNA encoding the *CSN1S1* milk protein is about 17.5 kb. A recent review of the milk protein nomenclature (Caroli et al., 2009) indicates nine genetic variants of the *CSN1S1* gene (A, B, C, D, E, F, G, H and I) in the genus *Bos* (Table 1). For this gene, the most common allele is B followed by C. These allele forms can be found in all cattle breeds.

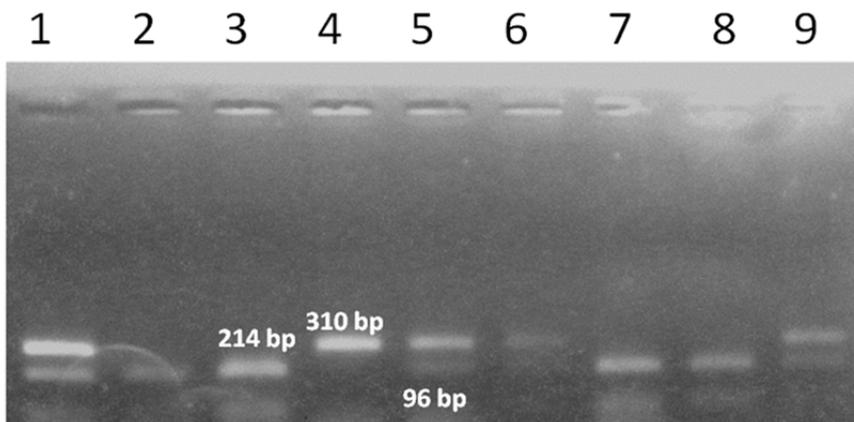
The genotype identification in our recent study was performed using PCR-RFLP assay. Total DNA for PCR reaction was extracted from blood samples by the application of GeneJet™ Genomic DNA Purification Kit (Fermentas). For the amplification of the polymorphic region of the *CSN1S1* gene, primers described by Koczan et al. (1993) were used. They covered parts of the 5'-flanking region and exon 1 (in total 310 bp fragment). The restriction profiles after digestion with *Tsp45I* specific endonuclease showed particular genotyping differences.

Totally, 87 animals of the BRC breed were examined for variants of the *CSN1S1* gene. Three genotypes were obtained, two homozygous (BB and CC) and one heterozygous (BC). About 71% of the animals (62 cows) were heterozygous and their RFLP profiles showed three electrophoretic bands (310bp, 214 bp and 96 bp). Only 26% (23 cows) were homozygous BB animals and two electrophoretic bands were characteristic for them (214 bp and 96 bp). The homozygous CC genotype was presented by the lowest frequency (2%), which could be

pointed out as an insignificant presence. There were only two cows found with that genotype expressed, with one unrestricted fragment on the electrophoregram (310 bp). The RFLP profile of the CSN1S1 gene is shown on Figure 1.

Gene	CSN1S1 variants								
Protein	B	A	C	D	E	F	G	H	I
14891–14929									
14–26		Del.							
17383	<b>GCC</b>			<b>ACC</b>					
53	Ala			<b>ThrP</b>					
17377–17400									
51–58								Del.	
18901	CAA				<b>AAA</b>				
59	Gln				Lys				
18923	TCG					TTG			
66	SerP					Leu			
19836	GAA								GAT
84	Glu								Asp
26181	GAA		GGA		GGA				
192	Glu		Gly		Gly				

**Table 1.** Position within the gene and mature protein of the CSN1S1 genetic variants in the genus *Bos* (Caroli et al., 2009). Del. – deletion. In bold – non-synonymous mutations.



**Figure 1.** PCR-RFLP assay (2% agarose gel electrophoresis) of CSN1S1 gene of BRC breed after restriction of the polymorphic region with *Tsp45I* restrictase. 1, 5, 6, 9 – BC genotype; 2, 3, 7, 8 – BB genotype; 4 – CC genotype. The size of the restriction fragments is shown (white letters).

Genotype frequencies were estimated after a direct count. On the other hand, allelic frequencies were calculated from the observed genotype frequencies.

The genotypic and allelic frequencies for the CSN1S1 gene are shown on Table 2. For the CSN1S1 gene in BRC breed, it is obvious that the B allele frequency is predominant in comparison with the C allele. This finding is in agreement with previous studies, which have defined the B allele as being the most frequent in many cattle breeds (Beja-Pereira et al., 2003).

The validity of Hardy-Weinberg equilibrium for the population was evaluated using  $\chi^2$  test (Preacher, 2001). The observed and the expected genotype frequencies were of similar values, thus confirming the validity of Hardy-Weinberg equilibrium for the BRC population. The prevailing frequency of the B allele and the heterozygous BC genotype for the CSN1S1 gene allowed the assumption that animals possessing the BB and/or the BC genotypes have been used during the selection and reproduction of the BRC breed. The extremely low frequency of the homozygous CC individuals corroborated with the above-mentioned assumption.

Gene	Genotype	Genotype frequencies		Allele frequencies	$\chi^2$	p-value
		Observed	Expected			
CSN1S1	BB	0.264	0.385	B – 0.621 C – 0.379	0.26 NS	0.88
	CC	0.023	0.144			
	BC	0.713	0.471			
CSN3	AA	0.318	0.345	A – 0.587 B – 0.413	0.01 NS	0.99
	BB	0.143	0.170			
	AB	0.540	0.485			
LGB	AA	0.395	0.439	A – 0.686 B – 0.314	0.13 NS	0.94
	BB	0.023	0.099			
	AB	0.581	0.416			

**Table 2.** Genotype and allele frequencies for the CSN1S1, CSN3 and LGB genes in the Bulgarian Rhodopean Cattle. NS - non-significant differences.

The SRC was genotyped as well (Table 3). A total of 38 animals were studied for the polymorphisms of the CSN1S1 gene and almost half of them (20 cows) were homozygous by the B allele (about 53%). This contrasted with the BRC breed where the dominant genotype was heterozygous. For the examined native breed, low and almost insignificant frequency (13%) of the CC genotype was estimated, similarly to the BRC breed. The allelic frequencies of the BRC and the SRC were similar but the B allele was slightly prevailing in the BRC. This was mostly due to the fact that Jersey cow, for which the B allele is highly frequent (Miciński et al., 2007), has been used as the basic cattle breed for selection and improvement of the BRC breed during the past 50 years.

Gene	Genotype	Genotype frequencies		Allele frequencies	$\chi^2$	p-value
		Observed	Expected			
CSN1S1	BB	0.526	0.486	B - 0.697 C - 0.303	0.04 NS	0.98
	CC	0.132	0.091			
	BC	0.342	0.422			
CSN3	AA	0.237	0.213	A - 0.461 B - 0.539	0.01 NS	0.99
	BB	0.316	0.291			
	AB	0.447	0.497			
LGB	AA	0.063	0.250	A - 0.500 B - 0.500	0.56 NS	0.75
	BB	0.063	0.250			
	AB	0.875	0.500			

**Table 3.** Genotype and allele frequencies for the CSN1S1, CSN3 and LGB genes of Shorthorn Rhodopean Cattle. NS - non-significant differences.

The observed the expected genotype frequencies were with similar values, thus confirming the validity of Hardy-Weinberg equilibrium for the SRC population.

### 2.1.2. CSN3 gene

The genomic DNA encoding the CSN3 milk protein is about 13 kb. Recently, 14 genetic variants have been identified (A, A<sup>1</sup>, B, B<sup>2</sup>, C, D, E, F<sup>1</sup>, F<sup>2</sup>, G<sup>1</sup>, G<sup>2</sup>, H, I and J) (Caroli et al., 2009) for the gene encoding the CSN3 protein. Their characteristic nucleotide and aminoacid substitutions are shown on Table 4. The A and the B alleles are the most frequent among all the species of the genus *Bos* (Neelin, 1964; Woychik, 1964).

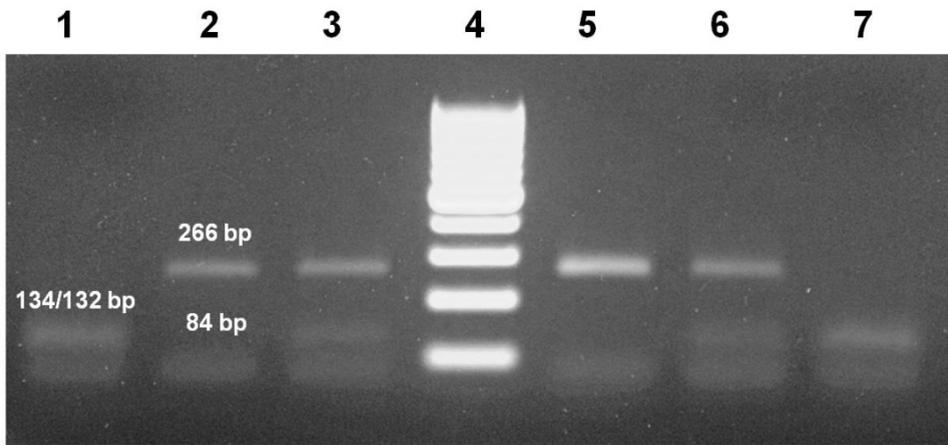
The total DNA extraction and the PCR amplification of the CSN3 gene were performed in the same way and under the same conditions as described in section 2.1.1. For amplification of the polymorphic region of the CSN3 gene (located between exon 4 and intron 4), primers described by Medrano & Cordova (1990a) were used (in total 350 bp fragment). For the RFLP assay, *HinfI* specific restrictase was used. For the BRC breed, 63 animals were genotyped by the CSN3 gene. There were 34 heterozygous individuals (AB), which represented more than half of the sample (c. 54%). Four electrophoretic bands characterized that genotype (266 bp, 134 bp, 132 bp and 84 bp). Twenty animals (c. 32%) were identified as homozygous on the A allele (AA), which was visualized with three electrophoretic bands (134 bp, 132 bp and 84 bp). The homozygous BB animals were few (9 cows, c. 14%); they were identified by the presence of two electrophoretic bands, i.e. 266 bp and 84 bp (Figure 2).

From the frequency data for the CSN3 gene (Table 2), it is obvious that the A allele frequency is higher in comparison with that of B allele. This finding is in agreement with previous results, which have recognised the B allele as exhibiting lower frequency in the majority of cattle breeds (Tsiaras et al. 2005; Heck et al., 2009).

Gene	CSN3 variants													
Protein	A	A <sup>1</sup>	B	B <sup>2</sup>	C	D	E	F <sup>1</sup>	F <sup>2</sup>	G <sup>1</sup>	G <sup>2</sup>	H	I	J
12690	<b>CGC</b>								<b>CAC</b>					
10	Arg								His					
12940	<b>ACT</b>			<b>ACC</b>										
93	Thr			Thr										
12950	<b>CGT</b>									<b>TGT</b>				
97	Arg									Cys				
12951	<b>CGT</b>				<b>CAT</b>	<b>CAT</b>								
97	Arg				His	His								
12971	<b>TCA</b>												<b>GCA</b>	
104	Ser												Ala	
13065	<b>ACC</b>									<b>ATC</b>		<b>ATC</b>		
135	Thr									Ile		Ile		
13068	<b>ACC</b>		<b>ATC</b>	<b>ATC</b>	<b>ATC</b>									<b>ATC</b>
136	Thr		Ile	Ile	Ile									Ile
13096	<b>ACT</b>							<b>ACG</b>						
145	Thr													
13104	<b>GAT</b>		<b>GCT</b>	<b>GCT</b>	<b>GCT</b>			<b>GTT</b>			<b>GCT</b>			<b>GCT</b>
148	Asp		Ala	Ala	Ala			Val			Ala			Ala
13111	<b>CCA</b>	<b>CCG</b>												
150	Pro													
13119	<b>ATT</b>			<b>ACT</b>										
153	Ile			Thr										
13124	<b>AGC</b>							<b>GGC</b>						?
155	Ser							Gly						Arg
13162	<b>ACT</b>			<b>ACC</b>							<b>ACC</b>			
167	Thr													
13165	<b>GCA</b>		<b>GCG</b>	<b>GCG</b>	<b>GCG</b>	?					<b>GCG</b>			
168	Ala													

**Table 4.** Position of the CSN3 genetic variants within the gene and the mature protein in the genus *Bos* (Caroli et al., 2009). Mutations are presented in bold. F<sup>1</sup> = F of Sulimova et al. (1992); F<sup>2</sup> = F of Prinzenberg et al. (1996), GenBank no. AF123250; G<sup>1</sup> = G of Erhardt (1996), Prinzenberg et al. (1996),

GenBank no. AF123251; G<sup>2</sup> = G of Sulimova et al. (1996); D = GenBank No. AJ619772. ? = Information not available.



**Figure 2.** PCR-RFLP assay (2% agarose gel electrophoresis) of the CSN3 gene after restriction of the polymorphic region with *HinfI* restrictase. 1, 7 – AA genotype; 2, 5 – BB genotype; 3, 6 – AB genotype; 4 – GeneRuler™ 100 bp Ladder Plus (Fermentas). The size of the restriction fragments is shown (white letters).

The validity of Hardy-Weinberg equilibrium for BRC population was confirmed by the almost equal values of the observed and the expected genotype frequencies. The dominant frequency of the A allele and the heterozygous AB genotype allowed the assumption that animals with the AA and/or the AB genotypes have been used during the selection and reproduction of the breed. The extremely low frequency of the homozygous BB individuals also agreed with the above-mentioned assumption.

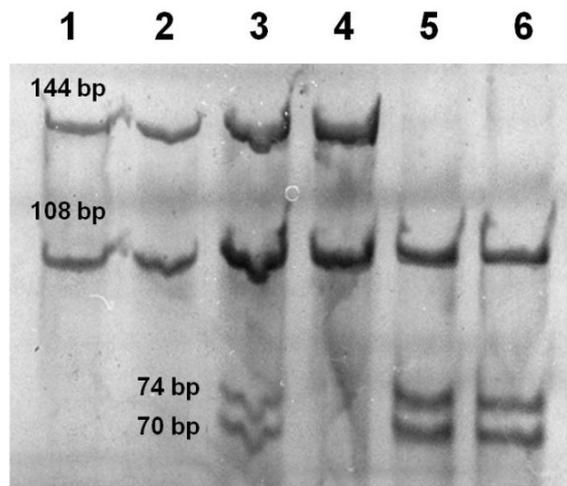
The CSN3 gene polymorphism was also studied for the SRC population (Table 3). A total of 36 animals were genotyped. The presence of the homozygous AA (9 cows) and BB (10 cows) representatives was found to be with almost equal frequencies (25% and 28%, respectively). The heterozygous AB genotype was presented by the highest frequency (47%). Regardless of the comparatively small sample, the present study allowed to conclude that the homozygous genotypes are almost uniformly distributed among the SRC population. This is in contrast with the results for BRC breed, where the frequency of the AA genotype is double than that of the BB genotype (Table 2). For the CSN3 gene, the observed and the expected genotype frequencies were also similar, thus confirming the validity of Hardy-Weinberg equilibrium for the SRC population.

### 2.1.3. LGB gene

The LGB gene is mapped on Chromosome 11 (Hayes and Petit, 1993b). The genomic DNA encoding LGB milk whey protein is much smaller (c. 4 kb) than that encoding the two

caseins described. The LGB is the major whey protein of ruminants and is also present in the milks of many other species. Its physiological function has been reported to be implicated in hydrophobic ligand transport and uptake, enzyme regulation and the neonatal acquisition of passive immunity (Kontopidis et al., 2004). A recent review of the milk protein nomenclature (Caroli et al., 2009) indicated eleven LGB genetic variants (A, B, C, D, E, F, G, H, I, J and W) in the genus *Bos* (Table 5). For this gene, similarly to CSN3, the most common alleles are A and B. These allele forms can be found in all cattle breeds. The E allele is also one of the most common alleles of the LGB gene in the genus *Bos*; however, it is not specific for *Bos taurus* but occurs in other *Bos* spp. (*Bos grunniens* and *Bos javanicus*).

The total DNA extraction and the PCR amplification of the LGB gene were performed following the same protocol and under the same conditions as described in section 2.1.1. For amplification of the polymorphic region of the LGB gene (located between exon 4 and intron 4), primers described by Medrano & Cordova (1990b) were used (in total 252 bp fragment). For the RFLP assay, *HaeIII* specific restrictase was used.



**Figure 3.** PCR-RFLP assay (10% acrylamide gel electrophoresis) of the LGB gene after restriction of the polymorphic region with *HaeIII* restrictase. 1, 2, 4 – AA genotype; 5, 6 – BB genotype; 3 - AB genotype. The numbers above the bands show the size of restriction fragments.

For the BRC breed, 86 animals were genotyped by the LGB gene. Fifty animals were recognised as heterozygous (AB) and they prevailed in the population (c. 58%). The AB genotype was characterized by four electrophoretic bands, i.e. 144 bp, 108 bp, 74 bp and 70 bp (Figure 3). The homozygous on the A allele (AA) animals (34 cows) were recorded with frequency of c. 40%; electrophoretically, that genotype was visualized with two bands (144 bp and 108 bp). The lowest frequency (c. 2%) was exhibited by the homozygous BB animals (2 cows), which were identified by three electrophoretic bands (108 bp, 74 bp and 70 bp).

From data shown on Table 2 about the genotype and the allele frequencies of both CSN3 and LGB genes, it is obvious that the A allele is prevailing in comparison with the B allele for both genes. These data are in agreement with previous observations for the majority of the cattle breeds (Tsiaras et al. 2005; Heck et al., 2009).

Gene	LGB variants										
Protein	B	A	C	D	E	F	G	H	I	J	W
3065	<b>GAG</b>			<b>CAG</b>							
45	Glu			Gly							
3080	<b>CCT</b>					<b>TCT</b>					
50	Pro					Ser					
3098	<b>ATC</b>										<b>CTC</b>
56	Ile										Leu
3109	<b>CAG</b>		<b>CAT</b>								
59	Gln		His								
3982	<b>AAC</b>	<b>AAT</b>			?	?	?	?	?	?	?
63	Asn										
3984	<b>GGT</b>	<b>GAT</b>						<b>GAT</b>			
64	Gly	Asp						Asp			
4003	<b>AAG</b>							<b>AA?</b>			
70	Lys							Asn			
4027	<b>ATC</b>							<b>ATG</b>			
78	Ile							Met			
5174	<b>AAT</b>	<b>AAC</b>	?	?	?	?	?	?	?	?	?
88	Asn										
5233	<b>GAG</b>								<b>GGG</b>		
108	Glu								Gly		
5263	<b>GCC</b>	<b>GTC</b>						<b>GTC</b>			
118	Ala	Val						Val			
5962	<b>CCG</b>										<b>CTG</b>
126	Pro										Leu
5970	<b>GAC</b>					<b>TAC</b>					
129	Asp					Tyr					
6280	<b>GAG</b>				<b>GGG</b>	<b>GGG</b>	<b>GGG</b>				
158	Glu				Gly	Gly	Gly				

**Table 5.** Position within the gene and the mature protein of the LGB genetic variants in the genus *Bos* (Caroli et al., 2009). In bold – mutations; ? – information not available.

The observed and the expected genotype frequencies were with similar values, which confirmed the validity of Hardy-Weinberg equilibrium for the BRC population with respect to the LGB gene. The genotype profiles of both CSN3 and LGB milk protein genes confirm

the assumption that the assisted selection used animals possessing the AA and/or the AB genotypes during the reproduction of the BRC breed.

The SRC population was also genotyped with regards to the LGB gene polymorphism (Table 3). A total of 32 animals were studied. The homozygous AA (2 cows) and BB (2 cows) representatives were with the same low frequency (c. 6%). The representation of the heterozygous AB genotype was found to be high (c. 88%). From the present study, it is obvious that the homozygous genotypes are uniformly distributed among the SRC breed.

That is in contrast with the results obtained for the BRC breed with respect to the LGB gene, where the frequency of the AA genotype is double than that of the BB genotype (Table 2). It is also in contrast with the published data (Tsiaras et al. 2005; Heck et al., 2009). These results coincided with those for the CSN3 gene. For the LGB gene, the observed and the expected genotype frequencies were with similar values, thus confirming the validity of Hardy-Weinberg equilibrium for the SRC population.

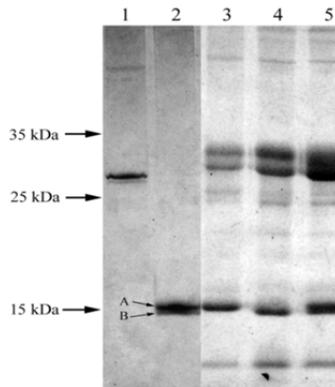
Genetic variations can be detected at the phenotypic level by various protein identification techniques as well. These techniques could be, e.g., acrylamide electrophoresis in denaturing (SDS PAGE) or native conditions, IEF, chromatography etc. The electrophoretic and IEF methods, mainly used for routine typing at the protein level, only allow the detection of variations resulting in aminoacid substitutions altering the electric charge, the molecular weight or the isoelectric point of proteins. For the screening of breeds and populations at the phenotypic level, if milk is available, profiling at the protein level by IEF is recommended because the method is cheap and fast as well as gives a simultaneous picture of the phenotype expression of the main milk protein genes (Caroli et al., 2009).

Profiling of the BRC population genotypes with respect to the LGB gene was performed with IEF and 2D PAGE as well for confirmation of the obtained results.

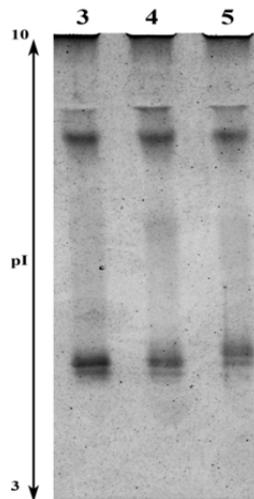
The total bovine milk protein was separated by SDS PAGE (Figure 4), showing several differences, both in quantity and quality, of the available proteins. The predominant LGB and casein fractions were identified as compared to standards (Sigma). According to the standard, the LGB fraction was determined to be around 15 kDa (Figure 4, line 2), a little lower than expected. Previous reports showed an expected molecular mass of c. 18 kDa (Farrell et al., 2004). The casein fraction was determined to be between 25 and 35 kDa as compared to the alpha-S casein standard (Figure 4, lane 1). The differences between LGB protein isoforms (A, B and AB) could be observed on 14%-SDS PAGE (Figure 4, lanes 3-5) as a difference in molecular mass. As the A and B alleles lead to a difference of around 100 Da in the molecular masses of the resulting proteins and two bands could be observed for the LGB standards, we speculated that the milk proteins shown were from homozygous A (Figure 4, lane 3), homozygous B (Figure 4, lane 4) and heterozygous AB (Figure 4, lane 5) animals, respectively.

Further experiment showed that a one-dimensional IEF in a wide (3-10) pH gradient (Figure 5) is not sufficient for the successful profiling of the LGB and casein fractions. The reason is that both LGB and caseins, along with other milk proteins, are characterized by isoelectric point (pI) around 4.0 to 5.5 (Farrell et al., 2004).

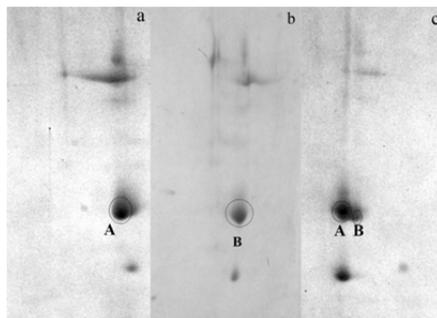
Therefore, we conducted 2D PAGE analyses combining a vertical IEF and 14%-SDS PAGE for the second dimension, allowing a successful resolving of the predominantly low-molecular weight LGB and caseins by pI and molecular weight (Mw) (Figure 6). The results from 2D PAGE confirmed what was observed on SDS PAGE as two spots, differing by pI (4.9 – 5.1) and Mw (15 and 14.8 kDa) could be distinguished in the presumable heterozygous animals (Figure 6c) and one spot was observed in homozygous animals (Figure 6a and 6b). The casein fraction in all samples was shown to be highly heterogeneous and difficult to interpret. Further experiments, involving IEF in a narrow pH gradient should be performed in order to characterize the casein fraction.



**Figure 4.** 14%-SDS PAGE (Coomassie staining) of bovine milk proteins. 1 - alfa-S casein standard; 2 - LGB standard; 3-5 - bovine milk protein samples. A and B indicate the A and the B alleles of the LGB gene.



**Figure 5.** Vertical IEF (Coomassie staining) in a wide (3-10) pH range. Lanes 3-5 corresponds to the lanes in Figure 4.



**Figure 6.** 2D PAGE (Coomassie staining) of milk protein samples. 6a - homozygous AA; 6b - homozygous BB; 6c - heterozygous AB animals.

### 3. Milk protein genes polymorphism and cattle selection and breeding

One of the most important effects of the milk protein polymorphisms on milk traits of economic importance is their relation to the technological properties of milk. The research was mainly concentrated on genetic variants of the CSN1S1, CSN3 and LGB genes. Some variants of these genes have a significant influence on the production of cheese, human nutrition etc. The constant monitoring of the milk protein variations in various breeds of cattle is an essential practice aiming to increase the frequency of genetic variants with favourable effects and to adapt their utilization for marker-assisted selection and breeding of cows. The application of genetic markers in dairy cattle breeding is a new stage in the selection practice in Bulgaria. If the cattle screening for desirable allelic forms is successful, it will have a direct scientific and practical value for stock-breeding farms in both public and private sectors. The marker-assisted selection is reliable and economically efficient way for increasing the farm production. The advantages of that approach are directly related to the genetic improvement of cattle populations, the increase of quality and quantity of production, the decrease of production loss and the improvement of the competitiveness of Bulgarian milk industry. Regardless multiple studies on milk protein genes polymorphism application for marker-assisted selection of dairy cattle, studies for Bulgarian cattle populations are scarce. The first focused research for Bulgaria has been initiated by our team (Zlatarev et al., 2008). Subsequently, results were published concerning genetic variants of milk protein genes, their relationships with milk traits and importance for selection and breeding for a Bulgarian breed, i.e. the Black Pied Cattle (Hristov et al., 2011a). Recent investigations concern the Bulgarian Rhodopean Cattle Breed (BRC).

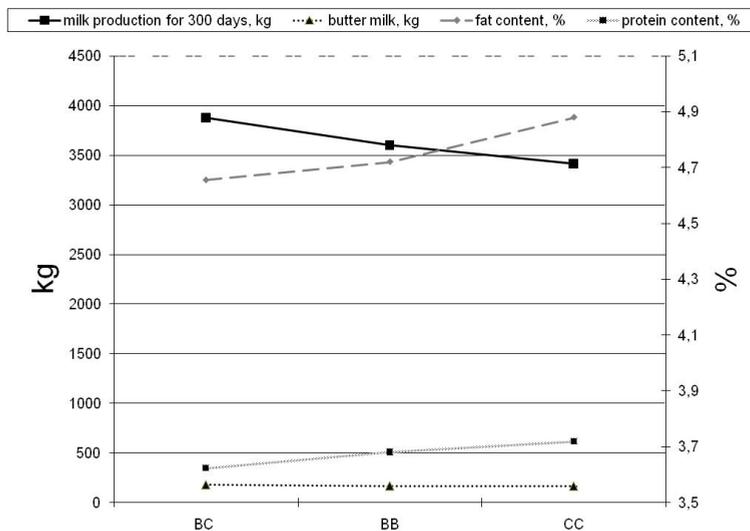
Although the SRC breed was genotyped by all the three important milk protein genes (CSN1S1, CSN3 and LGB), the relationships between recorded genetic variants and the milk quantitative and qualitative features were not examined. This is due to the specific way of the maintenance of this breed, which is targeting only the SRC population gene fund preservation, without its participation in milk industry.

With respect to the milk traits for the BRC breed, the milk productivity, the butter milk, the fat content and the protein content were examined monthly for 300 days of lactation. Data

were analyzed by Statistical tool Descriptive statistics (Microsoft Excel, 2007) for each studied milk protein gene. The calculated mean values (shown as mean value  $\pm$  SE) for milk productivity and qualitative traits were compared within each genotype.

### 3.1. CSN1S1 gene polymorphism

With respect to the importance of the CSN1S1 gene polymorphism for the milk production, it was found that the heterozygous BC animals had the highest values ( $3877.32 \pm 114.67$  kg). This exceeded with c. 12% the milk yield of the CC homozygous animals ( $3412.00$  kg  $\pm$  103.09 kg) and with 7% that of the BB homozygous cows ( $3600.81 \pm 153.79$  kg). Similar results were obtained for butter milk data, where the BC animals had better values and the lowest values were those of the CC cows (BC -  $179.93 \pm 5.12$  kg; BB -  $170.06 \pm 8.00$  kg; CC -  $167.01 \pm 7.35$  kg). These observations allowed the assumption for the superiority of the B allele of the CSN1S1 gene relative to both above-mentioned milk features. The milk fat and protein contents were affected mainly by the CC genotype. The values of the protein contents (CC -  $3.72 \pm 0.04\%$ ; BB -  $3.68 \pm 0.06\%$ ; BC -  $3.63 \pm 0.03\%$ ) were similar and only a slight superiority of the CC genotype was detected. The differences were more obvious with respect to the fat contents (CC -  $4.88 \pm 0.05\%$ ; B -  $4.72 \pm 0.08\%$ ; BC -  $4.66 \pm 0.04\%$ ). With respect to the fat and protein contents, there was predominance of the C allele of the CSN1S1 gene. The results about qualitative and quantitative milk traits were summarized as follow: milk production and milk butter, BC>BB>CC; fat and protein contents, CC>BB>BC (Figure 7).



**Figure 7.** Influence of the CSN1S1 gene polymorphism on the milk production and the milk quality traits in cows of Bulgarian Rhodopean Cattle Breed. BB, CC, BC – genotypes.

The correlations between the CSN1S1 gene polymorphism and the milk traits obtained by other researchers have not been straightforward, partly due to the differences in parameters

used and/or depending on cattle breeds. E.g., the CSN1S1 BB genotype correlated with higher milk production in some cases (Ng-Kwai-Hang et al., 1984; Aleandri et al., 1990; Sang et al., 1994) but there was also an evidence for the superiority of the heterozygous BC genotype (Micinski et al., 2007). Our results support the positive effect of the BC genotype on the milk yield being about 9.5% higher than the homozygous genotypes. In general, the results presented and the published data confirm the dominance of the B allele over the C allele relative to the milk production. No publications were found about the influence of the genotypes of the CSN1S1 gene on the butter milk values; however, the present study revealed a positive effect of the B allele of this gene.

The data concerning the protein content are controversial. According to some reports, the BB genotype is linked to high protein content (Ng-Kwai-Hang et al., 1984; Aleandri et al., 1990; Sang et al., 1994) but the same genotype was associated with low protein values in other studies (Ng-Kwai-Hang et al., 1986, 1992). Micinski et al. (2007) reported that the CSN1S1 BC genotype affected the increase of the protein content of milk. Our observations coincided with data presented by Pečiulaitienė et al. (2007) that had demonstrated the superiority of the CC genotype relative to the protein content.

With regard to the fat content, all publications claim that the homozygous BB genotype is associated with higher values (Micinski et al., 2007; Kamiński, 1996; Pečiulaitienė et al., 2007). This contrasted with the present results exhibiting c. 4% of higher fat content in the milk of the CC animals compared to the BB cows from BRC breed.

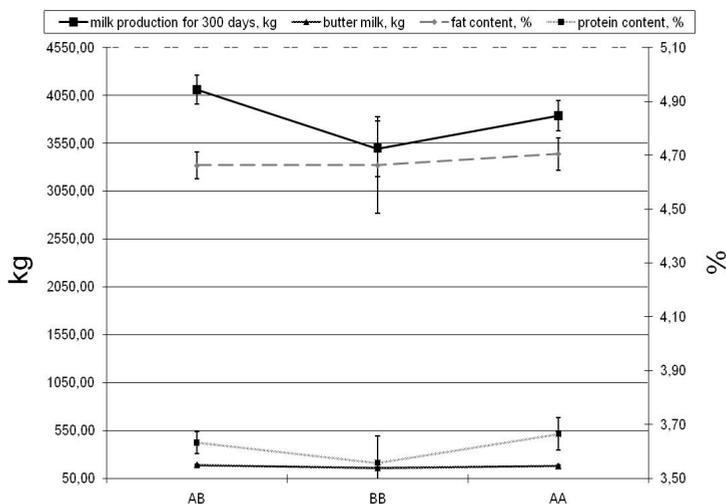
### 3.2. CSN3 gene polymorphism

The present results for the milk productivity show that the heterozygous AB cows have c. 600 kg higher milk yield ( $4112.00 \pm 149.40$  kg) than the homozygous BB animals ( $3495.00 \pm 290.40$  kg) and c. 300 kg more than the AA representatives ( $3838.20 \pm 160.22$  kg). These data indicate the superiority of the A allele with respect to the milk productivity. Similar trends were also observed for the butter milk (AB -  $191.45 \pm 6.97$  kg; AA -  $180.27 \pm 7.28$  kg; BB -  $161.17 \pm 10.55$  kg). Fat (AA -  $4.70 \pm 0.06\%$ ; BB -  $4.66 \pm 0.18\%$ ; AB -  $4.66 \pm 0.05\%$ ) and protein contents (AA -  $3.66 \pm 0.06\%$ ; AB -  $3.63 \pm 0.04\%$ ; BB -  $3.56 \pm 0.10\%$ ) were with similar mean values among the three genotypes. Nevertheless, there was a slight predominance of the AA genotype. The summarised results for the qualitative and quantitative milk traits were as follows: milk production and milk butter, AB>AA>BB; fat and protein contents, AA>AB≥BB (Figure 8).

Available data for the relationships between variants of the milk protein genes and the milk traits are contradictory. Usually, these relationships depend on cattle breeds and the country of origin. Some studies claim that the BB genotype was associated with higher (Van Eenennaam & Medrano, 1991) or lower (Bovenhuis et al., 1992) milk yield whereas other studies indicated no effect (Ng-Kwai-Hang et al., 1990; Lundén et al., 1997). The present results support data by Bovenhuis et al. (1992) suggesting a 15% decrease of the milk production of the BB homozygous animals compared to the AB heterozygous cows. According to our observations, there is a slight difference between butter milk mean values

of the AB genotype and the BB genotype of the CSN3, with the latter being about 16% higher. Most of the previous authors (van Eenennaam & Medrano, 1991; Lundén et al., 1997) reported insignificant differences between the variants of the CSN3 gene or a slight prevalence of the AA genotype (Miciński et al., 2007).

With respect to the fat and the protein contents, the differences between the three genotypes were insignificant. This allows concluding that the CSN3 gene polymorphisms have no effect on these two milk traits. There is only a slight prevalence of the AA genotype as it concerns these milk traits, i.e. less than 1% for the fat content and about 3% for the protein content. Data about the fat content are in contrast with previous studies where an advantage of the AB genotype compared to the AA and BB genotypes has been shown but are in agreement relative to the protein content (Miciński et al., 2007).



**Figure 8.** Influence of the CSN3 gene polymorphism on the milk production and the milk quality traits in cows of the Bulgarian Rhodopean Cattle Breed. AA, BB, AB – genotypes.

### 3.3. LGB gene polymorphism

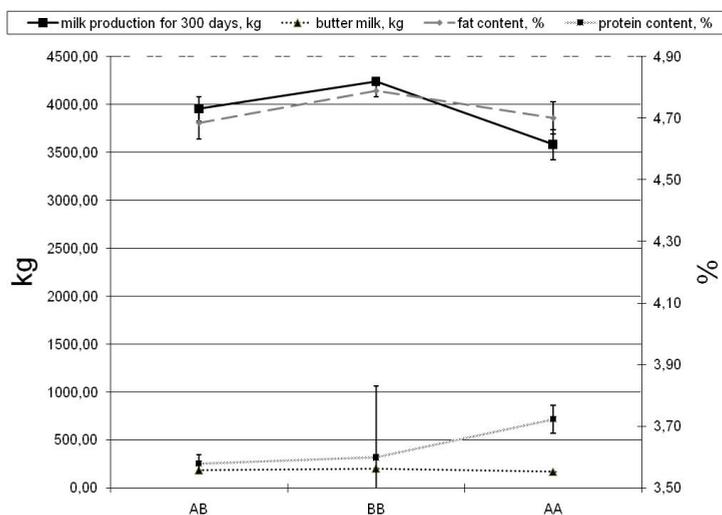
The milk productivity of the homozygous BB animals was the highest one ( $4240.50 \pm 33.50$  kg). This is c. 660 kg more than that of the homozygous AA cows ( $3581.48 \pm 154.14$  kg). The heterozygous AB genotype defined an intermediate level of the milk production ( $3955.24 \pm 125.45$  kg). These results indicated the superiority of the B allele with respect to the quantitative milk traits. The fat content exhibited a similar trend (BB -  $4.79 \pm 0.02\%$ ; AA -  $4.70 \pm 0.05\%$ ; AB -  $4.69 \pm 0.05\%$ ). The differences were more obvious for the butter milk mean values (BB -  $203.00 \pm 1.00$  kg; AB -  $184.30 \pm 5.57$  kg; AA -  $168.40 \pm 7.26$  kg). The protein content (AA -  $3.72 \pm 0.04\%$ ; BB -  $3.60 \pm 0.23\%$ ; AB -  $3.58 \pm 0.03\%$ ) was with almost equal mean values among the three genotypes. Nevertheless, there was a slight predominance of the AA genotype. The summarised results about qualitative and quantitative milk traits (Figure 8)

were as follows: milk production and milk butter,  $BB > AB > AA$ ; fat content,  $BB > AA > AB$ ; protein content,  $AA > BB > AB$ .

Our results for the influence of the LGB gene polymorphism on the milk traits are more unambiguous than those for the effect of the CSN3 gene. In studies of the LGB genotype effects on the milk production, several authors reported no significant associations (Lundén et al., 1997; Ojala et al., 1997). Nevertheless, there are also reports for the positive influence on the milk quantity of all the genotypes, i.e. AA (Aleandri et al., 1990; Bovenhuis et al., 1992), AB (Pupkova, 1980) or BB (Jairam & Nair, 1983). The present results show that the BB genotype determines higher milk production.

Previous studies suggested the advantage of the AA genotype of the LGB gene on the protein content (Aleandri et al., 1990; Bovenhuis et al., 1992), are similar to our results. That is the only milk feature that is affected by the AA genotype of LGB gene. This result coincides with that for the CSN3 gene.

Positive effects of the BB genotype on the fat content (Aleandri et al., 1990; Bovenhuis et al., 1992; Hill, 1993) and of the AA genotype on the butter milk (Miciński et al., 2007) have been reported. Our observations for BRC breed are similar and have revealed the favourable influence of the BB homozygous genotype on these milk traits. The differences were much more significant relative to the butter milk than to the fat content. The distinction between fat content values associated with the BB and AB genotype is c. 2% whilst between the butter milk values are 17%.



**Figure 9.** Influence of the LGB gene polymorphism on the milk production and the milk quality traits in cows of the Bulgarian Rhodopean Cattle Breed. AA, BB, AB – genotypes.

The analysis of the genetic polymorphisms of the CSN1S1, CSN3 and LGB genes of the Bulgarian Rhodopean Cattle Breed revealed their influence on the quantitative and

qualitative milk traits. This allows selection of proper animals (cows and bulls) with desirable genotypes and increasing the frequency of favourable alleles within the population, thus improving milk composition and increasing economic efficiency of dairy cattle farms.

#### **4. Future research**

The research plans of our team include various aspects of the milk protein genotyping. Primary experiments will be related to milk proteomics. The casein fraction in all the samples revealed to be highly heterogeneous and difficult to interpret with the IEF technique are to be studied in a narrow pH gradient and with 2D PAGE method in order to achieve their better characterization. The CSN1S1, CSN3 and LGB proteins and their variants will be separated and quantified by reversed-phase high-performance liquid chromatography for higher precision and resolution. At the genomics level, these protein genes will be examined in the light of the single nucleotide polymorphism analysis and the population sequence datasets will be completed for the BRC and the SRC breeds aiming population profiling of these native Bulgarian cattle breeds. The genetic diversity of milk proteins could serve as a criterion of selection and as an informative marker in studies of the phylogenetic relationships and evolution of breeds. Finally, similar investigations will be carried out at the protein and DNA levels for other Bulgarian indigenous cattle breeds as well as for small ruminants, which have not been studied in Bulgaria up to date.

#### **5. Conclusion**

The present studies bring forward a novel approach for genotyping of two Bulgarian native cattle breeds specific for the Rhodope mountain area, i.e. Bulgarian Rhodopean Cattle (BRC) and Shorthorn Rhodopean Cattle (SRC). They also show the relationships between the genotypes of the BRC breed and qualitative and quantitative milk traits. This gives the opportunity for application, improvement and development of genetically-based marker-assisted selection and breeding of cows with desirable genotypes and increasing of dairy farms economic and financial gains. The BRC and SRC breeds are of high significance for the Bulgarian cattle biodiversity and their genotyping with respect to the studied genes could be utilized also for preservation of that native breed's gene fund.

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