

## SIGNIFICANCE OF MILK PROTEIN GENES POLYMORPHISM FOR BULGARIAN RHODOPEAN CATTLE: COMPARATIVE STUDIES

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### ABSTRACT

*The aim of the present research was to reveal the genotype profile of the local Bulgarian Rhodopean cattle population with respect to  $\alpha$ 1-casein, kappa-casein and  $\beta$ -lactoglobulin genes, by PCR-RFLP assay and conformational 2D PAGE. According to these profiles, it is possible to determine the association between each genotype and milk qualitative and quantitative traits and to establish the position of the breed with regard to the genetic diversity other European cattle breeds. The investigation also revealed the high significance of the genetic variants of the three above-mentioned milk protein genes for:*

*Clarification of the influence of other breeds on the Bulgarian Rhodopean cattle population;*

*Obtaining data for genetic drift among the Bulgarian Rhodopean cattle population, the indigenous Shorthorn Rhodopean cattle population as a predecessor breed and the Jersey cow as a main improvement breed;*

*Gathering the necessary information about preservation of this local breed, which is important for the Bulgarian biodiversity gene fund.*

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### Introduction

The Bulgarian Rhodopean cattle (BRc) is a local Bulgarian cattle breed included in a national programme for selection and reproduction because of its continuously decreasing number of animals. The present-day BRc originated from native Shorthorn Rhodopean cattle (one of two indigenous cattle breeds in Bulgaria), upgraded mainly with Sofia Brown cattle and Jersey cow. Several other breeds also participated in the creation of the BRc cattle: the Bulgarian Grey cattle, Oberinntal Grey, Brown Alpine, Ayrshire, American Brown, etc. (20). The breed is characterized by high viability and fertility, calving ease, resistance to diseases, and low requirements for feed.

There are, nowadays, less than 1000 typical BRc representatives in Bulgaria (although minimum 7500 are necessary for survival of the breed). They are located mainly in Southwestern Rhodope Mountains. About 100 cows are maintained at the Agricultural Academy (Agricultural and Stockbreeding Experimental Station, Smolyan city) and about 800 animals are dispersed in several private farms in the whole country. The BRc breed is included in the National Livestock Breeds Preservation Programme, which is a part of the national strategy for genetic resources preservation. Because of all its features, the BRc cattle breed gene fund is valuable for Bulgaria and its preservation and improvement is of high significance.

Since the discovery of milk protein genes polymorphism, the development of this field has been targeted at: understanding the biological significance of genetic variants; in the practice, clarifying the association between genetic variants and milk traits; in applied fields, selection and breeding techniques; and, in the last decades, achieving better understanding of the origin and domestication of cattle breeds (2, 8, 13).

Clarification of the association between genetic variants and milk traits is connected to the fact that the variants of milk protein genes differ among themselves mostly in single nucleotide polymorphisms which could cause amino acid substitutions in mature proteins, with potential biological effect. There are different opinions about the existence of such an association and its dependence on cattle breed and/or environmental conditions (19, 22). The biological effect of milk protein genetic variants could affect primary milk constituents and technological properties used in the milk industry. Consequently, milk products could be affected and, hence, even human nutrition. As milk products are an integral part of the human diet, they could affect the health, since some of them might act as allergens (e.g.  $\alpha$ s1-casein). This targets the studies towards searching of milk protein variants and production of milk with hypoallergenic properties (4).

Another area in which the polymorphism of milk protein genes is of significant importance, is the selection, breeding and genetic improvement of the dairy cattle breeds. All these activities are important steps in the search of an economically efficient way to increase milk production and achieve the desired milk quality (3). This is directly connected with

identification of allelic variants determining valuable milk features and increasing their frequency in the dairy cattle populations.

It is of great importance to preserve the gene fund of the local Bulgarian dairy cattle populations. These populations, their genetic structure, origin and domestication processes are not studied at all. That is why, the most recent researches seek to explain the genetic diversity of present-day cow breeds with respect to milk protein genes and/or mitochondrial gene polymorphism (1, 13).

The aim of the present research was to reveal the genotype profile of the BRc population with respect to  $\alpha$ s1-casein (*CSN1S1*), kappa-casein (*CSN3*) and  $\beta$ -lactoglobulin (*LGB*) genes, to determine the association between each genotype and milk qualitative and quantitative traits, and to make a referent comparison with other European cattle breeds.

## Materials and Methods

### Animals and sample collection

The experiments were performed in 2010–2011 in a farm located in Southwestern Bulgaria (Smolyan city) with cows 2 to 7 years of age. The animals were fed with a diet balanced in terms of energy and protein. The feed ration contained maize silage, haylage and grain fodder. A total of 90 cows of BRc were genotyped for the *CSN1S1*, *CSN3* and *LGB* genes. Blood samples (5 mL) were obtained from *v. jugularis* into K<sub>2</sub>EDTA vacuum tube (BD Vacutainer®). Individual milk samples were taken monthly, the milk production of each animal was recorded monthly (for 305-day lactation) averaged for two consequent lactations, and protein and fat content were determined with MilkoScan 133-B (Foss Electric).

### Gene selection

***CSN1S1***. The gene is localized on Chromosome 6 (7). Recent data indicates 9 genetic variants of it in the genus *Bos* (4). The most common alleles of the *CSN1S1* gene, *B* and *C*, differ from each other in one amino acid substitution 192Glu(B)/Gly(C) due to a transition at position 26181 bp (A/G). This variation was used for differentiation of the allelic forms by RFLP assay after PCR amplification of the polymorphic region located between the 5' end and the first exon of the *CSN1S1* gene.

***CSN3***. The gene is situated on Chromosome 6 and 12 genetic variants have been recently determined (8, 11). The *A* and *B* alleles are most frequent for the genus *Bos* (11) and they differ from each other in two amino acid substitutions (36Thr(A)/Ile(B) and 48Asp(A)/Ala(B)) due to a transversion at position 13068 bp (A/C) and a transition at position 13104 bp (T/C). According to them the polymorphic region chosen for PCR–RFLP analysis is located between exon IV and intron IV.

***LGB***. The gene is situated on Chromosome 11 (5) and a total of 11 alleles are known up to date (4). The genetic variants *A* and *B* occur with the highest frequency in most cattle breeds (10). These two main genetic forms of *LGB* differ in the amino acid substitutions 64Gly(A)/Ala(B) and 118Asp(A)/

Val(B) in the mature protein due to transitions in the gene at positions 3984 bp (G/A) and 5263 bp (C/T), respectively. The polymorphic gene region chosen for PCR amplification is located between exon IV and intron IV and covers the first above-mentioned nucleotide variation characteristic for identification of the allelic forms.

### DNA extraction and amplification

Total genomic DNA was extracted using GeneJet™ Genomic DNA Purification Kit (Fermentas) according to the manufacturer's protocol. Primers for *CSN1S1* polymorphic region amplification were described by Koczan et al. (14). PCR amplification of the polymorphic region of *CSN3* and *LGB* genes was performed with primers described by Medrano and Cordova (16, 17). All PCR reactions were accomplished by LittleGenius thermocycler (BIOER Technology Co., Ltd) under the following conditions: initial denaturation at 94 °C for 5 min; 35 cycles (denaturation at 94 °C for 30 s; primer annealing at 50 °C for 30 s; extension at 72 °C for 1 min); and final extension at 72 °C for 10 min. PCR products were visualized in a 1 % agarose gel with ethidium bromide under UV light. Fragment size was determined using GeneRuler™ 1 kb Ladder Plus (Fermentas).

### Genotyping

Animals were genotyped with respect to milk protein genes by PCR–RFLP assay. 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 results obtained from the PCR–RFLP analysis.

### PCR–RFLP assay

The amplified *CSN1S1* gene fragment was 310 bp and it was digested with *Tsp45I* endonuclease (BioLabs®Inc.) for 1 h at 65 °C. Restriction products were separated in a 2 % agarose gel with ethidium bromide and visualized under UV light.

The amplified *CSN3* gene fragment was 350 bp and it was restricted with *HinfI* endonuclease (Fermentas) at 37 °C for 1 h. The RFLP profile was visualized the same way as for *CSN1S1*.

The amplified *LGB* gene fragment was 252 bp in length. Digestion was performed with *HaeIII* endonuclease (Fermentas) at 37 °C for 1 h. Restriction products were visualized in a 12 % polyacrylamide gel after silver staining.

The fragments' size was determined using GeneRuler™ 100 bp Ladder Plus (Fermentas). According to the restriction profile, the allelic variants of the *CSN1S1*, *CSN3* and *LGB* genes were determined.

### Electrophoretic separation of milk proteins

The rough milk samples were diluted 100 times and protein concentrations were estimated by BCA kit (Thermo Scientific) according to the manufacturer's protocol. Equal amount of protein was loaded on each lane after 3 min incubation at 100 °C with 4x Laemmli non-reducing sample buffer. One-dimensional SDS PAGE was performed according to the standard system (15) in 12.5 % polyacrylamide gels on VS10

minigel system (Cleaver Scientific Ltd.) and visualized after 0.1 % Coomassie Brilliant Blue G250 staining. For 2D PAGE the first dimension – isoelectrofocusing (IEF) in denaturing conditions was performed on VS10 vertical minigel system according to the protocol of Robertson et al. (21) with a final concentration of 6 mol/L urea added to the gel. Carrier ampholytes (Sigma–Aldrich®) were high resolution pH 3.0–10.0. Gels were run with 0.1 mol/L NaOH as the cathode buffer and 0.1 mol/L CH<sub>3</sub>COOH as the anode buffer, and subsequently stained with Coomassie Brilliant Blue G250. For the second dimension, lanes were excised, equilibrated for 15 min in SDS equilibration buffer (60 mmol/L Tris-Cl, pH 8.8; 2 % SDS; 10 % glycerol; 2 % bromphenol blue). The lanes were applied to a 14 % Laemmli running gel without a stacking gel and overlaid with 0.5 % agarose dissolved in Laemmli electrophoresis running buffer. Gels were stained with Coomassie Brilliant Blue G250.

### Data analysis

Descriptive statistics was used concerning the milk productivity and qualitative milk traits data. The calculated mean values (shown as mean value ± SEM) for milk productivity and qualitative traits were compared within different genotypes and evaluated by Student's *t*-test. These statistical assays were performed with GraphPad Prism version 5.04 for Windows (9). Genotype and allele frequencies were determined and the validity of the Hardy–Weinberg equilibrium for the BRC population was evaluated using Co-dominant Diploid data analysis from the program POPGENE, version 1.31.

## Results and Discussion

### CSN1S1 gene

**Restriction profile.** The animals of the BRC breed were examined for variants of the *CSN1S1* gene. Three genotypes were obtained, two homozygous ones (*BB* and *CC*) and a heterozygous one (*BC*). About 71 % of the animals were heterozygous and their RFLP profiles showed three electrophoretic bands (310 bp, 214 bp and 96 bp). Only 26 % 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, and was expressed with one unrestricted fragment in the electrophoregram (310 bp). Genotype and allele frequencies were estimated and shown in **Table 1**.

The chi-square test for the Hardy–Weinberg equilibrium, at degree of freedom – 1, showed a value of  $\chi^2 = 22.46$  ( $p = 0.000002$ ), thus confirming the validity of the Hardy–Weinberg equilibrium for the BRC population.

**Association between genotype and milk traits.** 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 kg ± 114.67 kg). This exceeded with c. 12 % the milk yield of the *CC* homozygous animals (3412.00 kg ± 103.09 kg) and with 7 % that of the *BB*

homozygous cows (3600.81 kg ± 153.79 kg). Similar results were obtained for fat yield, where the *BC* animals had better values and the lowest values were those of the *CC* cows. These observations allowed the assumption for the superiority of the *B* allele of the *CSN1S1* gene relative to both above-mentioned milk features. The values for the protein content were similar and only a slight superiority of the *CC* genotype was detected. The differences were more obvious for the fat content. With respect to the fat and protein contents, there was predominance of the *C* allele of the *CSN1S1* gene. The results about the influence of each genotype on qualitative and quantitative milk traits were summarized and presented in **Table 2**.

**TABLE 1**

Genotype and allele frequencies for the *CSN1S1*, *CSN3* and *LGB* genes in the Bulgarian Rhodopean cattle breed

Gene	Genotype	Genotype frequencies		Allele frequencies
		Observed	Expected	
<i>CSN1S1</i>	<i>BB</i>	0.264	0.385	<i>B</i> – 0.621 <i>C</i> – 0.379
	<i>CC</i>	0.023	0.144	
	<i>BC</i>	0.713	0.471	
<i>CSN3</i>	<i>AA</i>	0.318	0.345	<i>A</i> – 0.587 <i>B</i> – 0.413
	<i>BB</i>	0.143	0.170	
	<i>AB</i>	0.540	0.485	
<i>LGB</i>	<i>AA</i>	0.395	0.439	<i>A</i> – 0.686 <i>B</i> – 0.314
	<i>BB</i>	0.023	0.099	
	<i>AB</i>	0.581	0.416	

### CSN3 gene

**Restriction profile.** Of the genotyped animals, those heterozygous (*AB*) regarding the *CSN3* gene were with the highest prevalence (about 54 %). Four electrophoretic bands characterize this genotype (266 bp; 134 bp; 132 bp and 84 bp). About 32 % of the studied cows were defined as homozygous for the *A* allele (*AA*) and were visualized with three electrophoretic bands (134 bp; 132 bp and 84 bp). With least representatives were the homozygous *BB* animals (about 14 %), identified with two electrophoretic bands (266 bp and 84 bp). The genotype and allele frequencies were obtained (**Table 1**).

The chi-square test for the Hardy–Weinberg equilibrium, at degree of freedom – 1, showed a value of  $\chi^2 = 0.70$  ( $p = 0.40$ ), e.g. that locus was found to be at Hardy–Weinberg equilibrium for the BRC population.

**Association between genotype and milk traits.** The milk productivity (305-day lactation) of the heterozygous *AB* cows (4112 kg ± 149.4 kg) was found to be about 600 kg higher than that of the homozygous *BB* animals (3495 kg ± 290.4 kg) and about 300 kg higher than that of the *AA* representatives (3838.2 kg ± 160.2 kg). This shows the superiority of the *A*

allele with respect to milk productivity. A similar tendency was observed for the fat yield as well.

The fat and protein content of the cow milk were with similar mean values among the three genotypes. Nevertheless, there was slight predominance of the *AA* genotype.

The results about the qualitative and quantitative milk traits are summarized in **Table 2**.

### LGB gene

**Restriction profile.** About 58 % of the animals were established to be heterozygous (*AB*) and they were with the highest prevalence. The *AB* genotype was characterized by four electrophoretic bands (144 bp, 108 bp, 74 bp, and 70 bp). Animals homozygous for the *A* allele (*AA*) were observed with a frequency of about 40 % and this genotype was electrophoretically visualized with two bands (144 bp and 108 bp). With the lowest frequency (about 2 %) were homozygous *BB* animals which were identified with three electrophoretic bands (108 bp; 74 bp, and 70 bp). The distribution of genotype and allele frequencies among the studied animals is presented in **Table 1**.

The chi-square test for the Hardy–Weinberg equilibrium, at degree of freedom – 1, showed a value of  $\chi^2 = 10.21$  ( $p = 0.001$ ), thus confirming the validity of the Hardy–Weinberg equilibrium for the BRc population with regard to the *LGB* locus.

**Association between genotype and milk traits.** The *LGB* polymorphism results showed that milk productivity was highest in homozygous *BB* animals (4240.5 kg  $\pm$  33.5 kg) and was about 660 kg higher than that of homozygous *AA* cows (3581.48 kg  $\pm$  154.14 kg). The heterozygous *AB* genotype defined average milk production (3955.24 kg  $\pm$  125.45 kg). This demonstrates the superiority of the *B* allele with respect to quantitative milk traits. A similar tendency was observed for the fat content and fat yield as well. The differences were more

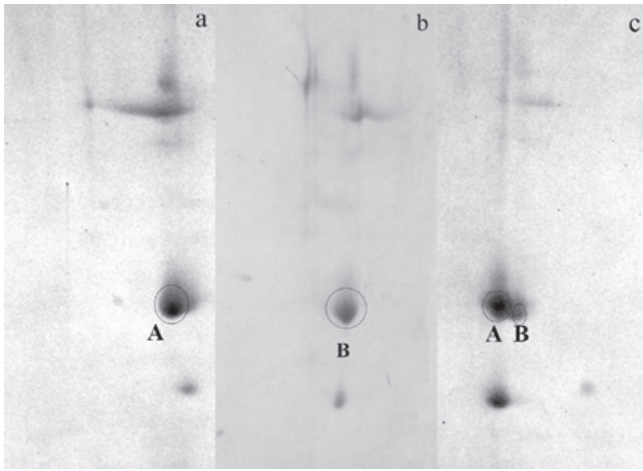
obvious for the fat yield mean values. The protein content of cow milk was with almost equal mean values among the three genotypes. Nevertheless, there was slight predominance of the *AA* genotype. The results about the qualitative and quantitative milk traits are summarized in **Table 2**.

**IEF and 2D PAGE.** Bovine milk proteins were separated by SDS PAGE, showing several differences, both in quantity and quality, of the available proteins (data not shown). The predominant LGB and casein fractions were identified as compared to standards (Sigma Aldrich®). According to the standard, the LGB fraction was determined to be about 15 kDa. Previous reports showed an expected molecular mass of c. 18 kDa (6). The casein fraction was determined to be between 25 kDa and 35 kDa as compared to the  $\alpha$ -S casein standard. The differences between LGB protein isoforms could be observed 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 different bands could be observed. Further experiment showed that a one-dimensional IEF in a wide (3–10) pH gradient 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, have an isoelectric point (pI) around 4.0 to 5.5 (6). Therefore, we conducted 2D PAGE analyses, allowing successful resolving of the predominantly low-molecular weight LGB by pI and molecular weight (Mw) (**Fig. 1**). The 2D PAGE results confirmed what was observed on SDS PAGE, as two spots, differing by pI (4.9–5.1) and Mw (15 kDa and 14.8 kDa), could be distinguished in the presumable heterozygous animals (**Fig. 1c**) and one spot was observed in the homozygous animals (**Fig. 1a,b**). 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.

**TABLE 2**

Influence of the *CSN1S1*, *CSN3* and *LGB* genotypes on milk qualitative and quantitative traits (shown as mean value  $\pm$  SEM) in cows from the Bulgarian Rhodopean cattle breed

Milk protein gene	Genotype	Bovine milk features			
		Milk production, kg	Fat yield, kg	Fat content, %	Protein content, %
<i>CSN1S1</i>	<i>BB</i>	3600.81 $\pm$ 153.79	170.06 $\pm$ 8.00	4.72 $\pm$ 0.08	3.68 $\pm$ 0.06
	<i>CC</i>	3412.00 $\pm$ 103.09	167.01 $\pm$ 7.35	4.88 $\pm$ 0.05	3.72 $\pm$ 0.04
	<i>BC</i>	3877.32 $\pm$ 114.67	179.93 $\pm$ 5.12	4.66 $\pm$ 0.04	3.63 $\pm$ 0.03
<i>CSN3</i>	<i>AA</i>	3838.20 $\pm$ 160.22	180.27 $\pm$ 7.28	4.70 $\pm$ 0.06	3.66 $\pm$ 0.06
	<i>BB</i>	3495.00 $\pm$ 290.40	161.17 $\pm$ 10.55	4.66 $\pm$ 0.18	3.56 $\pm$ 0.10
	<i>AB</i>	4112.00 $\pm$ 149.40	191.45 $\pm$ 6.97	4.66 $\pm$ 0.05	3.63 $\pm$ 0.04
<i>LGB</i>	<i>AA</i>	3581.48 $\pm$ 154.14	168.40 $\pm$ 7.26	4.70 $\pm$ 0.05	3.72 $\pm$ 0.04
	<i>BB</i>	4240.50 $\pm$ 33.50	203.00 $\pm$ 1.00	4.79 $\pm$ 0.02	3.60 $\pm$ 0.23
	<i>AB</i>	3955.24 $\pm$ 125.45	184.30 $\pm$ 5.57	4.69 $\pm$ 0.05	3.58 $\pm$ 0.03



**Fig. 1.** 2D PAGE (Coomassie staining) of milk protein samples from the Bulgarian Rhodopean cattle breed for identification of LGB genetic variants. Homozygous *AA* (a); homozygous *BB* (b); heterozygous *AB* animals (c).

### Comparative analysis

The present-day BRC breed originated from the indigenous Shorthorn Rhodopean cattle native to Bulgaria. Since 1963 during the genetic elaboration, selection and breeding programs, BRC has been upgraded mainly with Jersey cow, with the aim to create a high productive dairy cattle breed adapted to the Bulgarian mountain areas it inhabits. The results of these studies make it possible to compare the studied animals from the BRC breed with Shorthorn Rhodopean cattle and Jersey cow as a predecessor and improvement breed, respectively.

The research focus was mainly on genetic variants of the *CSN1S1*, *CSN3* and *LGB* genes. If the cattle screening for desirable allelic forms is successful, this would have a direct scientific and practical value for stock-breeding farms both in the public and the private sector. The available data about the relationships between different genotypes of milk protein genes and milk traits is contradictory and controversial. Usually, these relationships depend on the cattle breed and the country of origin. The Shorthorn Rhodopean cattle breed is important only for gene fund preservation on the Balkan Peninsula. This explains the lack of data about the milk features of this breed. That is why our studies on BRC could only be compared with reports about the Jersey cow.

The obtained results about the *CSN1S1* genotype and its association with the milk production and fat yield of the BRC breed could be summarized as follows:  $BC > BB > CC$ . This is in agreement with the study of Miciński et al. (19) on the Jersey cow, where the tendency is the same, except that the authors did not reveal presence of the homozygous *CC* genotype. The presence of BRC animals homozygous for the *C* allele could be attributed to the origin of the breed from the native Shorthorn Rhodopean cattle, where this genotype is observed (12). As regards the fat and protein content, our study established the following relations with *CSN1S1* genotypes:  $CC > BB > BC$ . For the Jersey cow the association between the genotype and these two milk traits could be summarized as  $BB > BC$  (18).

The dominance of the *CC* homozygous genotype in that case is explainable again with the influence of the indigenous breed (Shorthorn Rhodopean cattle) because this breed, like all the native cattle breeds, is characterized with higher content of milk fat and protein as compared to modern cattle breeds.

Concerning the *CSN3* gene, the summarized results for the qualitative and quantitative milk traits are as follows: milk production and fat yield –  $AB > AA > BB$ ; fat and protein content –  $AA > AB \geq BB$ . The comparative analysis with the Jersey cow, according to reported data for the *CSN3* gene (19), showed no supportive results. Both investigations (ours and that of Miciński et al. [19] for the Jersey cow) revealed no significant effect of any particular genotype on the qualitative and quantitative milk traits. The differences between the data about the BRC and the Jersey cow, with respect to milk features, could again be discussed with the influence of the indigenous Shorthorn Rhodopean cattle breed where the *B* allele has predominance (12).

The 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 and are as follows: milk production and fat yield –  $BB > AB > AA$ ; fat content –  $BB > AA > AB$ ; protein content –  $AA > BB > AB$ . Having in mind the extremely low frequency of the *BB* genotype (about 2 %), it could be considered as insufficient and could be ignored. In that case the results are similar with those reported by Miciński et al. (19) for the Jersey cow.

It could be concluded that, as regards the association between different genotypes of milk protein genes and milk traits, there is an extremely high and distinct influence of genetic drift from the Jersey cow as a main improvement breed to the BRC breed. Still there also exists the effect of the indigenous Shorthorn Rhodopean cattle breed as a predecessor breed.

Milk protein genes haplotypes are also of great significance in the researches for clarification of the origin, biogeography, evolution and domestication of cattle breeds. According to the description of Jann et al. (13), for geographic distribution of haplotype diversity at the bovine casein locus (mainly *CSN1S1* and *CSN3* genes in particular), there are three geographic regions of cattle breeds' origin (Northern and Central Europe (NC); Southern Europe and Africa (SE); and the Near East). For the NC and SE groups of cattle breeds' the predominant *CSN1S1* allele is *B* but the *CSN1S1* *C* allelic frequency for the SE group (about 40 %) is far more higher than that for the NC group (less than 10 %). The frequencies for the BRC breed observed by us were about 40 % for the *CSN1S1* *C* allele, suggesting genetic similarity with the SE group of cattle breeds. This is not unexpected, having in mind the cattle breeds that have influenced the BRC genetic profile (Shorthorn Rhodopean cattle and Jersey cow). These two breeds have similar allelic frequencies for *CSN1S1* *C*: over 30 % for the Shorthorn Rhodopean cattle (12) and about 40 % for the Jersey cow (13).

Concerning the *CSN3* gene allelic frequency, in contrast to *CSN1S1*, the *A* allele is characteristic of the NC group (presented with about 70 % to 80 %), whereas for the SE cattle breeds *CSN3 B* is predominant (allelic frequencies of over 50 % to 60 %) (13).

The established allele frequencies for the BRc breed are about 60 % for *CSN3 A* and 40 % for *CSN3 B*. The information about the indigenous Shorthorn Rhodopean cattle breed shows that the *CSN3 B* allelic frequency is slightly over 50 % (12). According to Miciński et al. (19), the frequency of the *A* allele for the Jersey cow breed is close to 70 %. This explains the relatively high frequency of the *A* allele in the BRc population. The effect of the indigenous Shorthorn Rhodopean cattle breed is also unambiguous, manifested through a comparatively high *CSN3 B* allelic frequency. However, there are some fluctuations from the expected allelic frequencies due to the simultaneous influence of both the predecessor breed and the improvement breed. Still, the BRc breed population could be assigned to the SE cattle breeds.

The *LGB* milk protein gene has not been studied with respect to the genetic diversity, origin and biogeography of cattle breed populations. That is why a comparative allelic frequency assay cannot be performed with respect to BRc's belonging to the NC or SE group.

## Conclusions

The genetic polymorphism of the *CSN1S1*, *CSN3* and *LGB* milk protein genes has been investigated for many years. Nevertheless, there is no sufficient data about the Bulgarian local and indigenous cattle breed populations. The present study on the Bulgarian Rhodopean cattle breed revealed high significance of the polymorphic variants of the three above-mentioned genes both with respect to their influence on milk traits and defining the proper position of the BRc population with regard to the origin and biogeography of European cattle breeds (i.e. genetically similar with the SE group of cattle breeds).

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