Bulgarian Journal of Veterinary Medicine, 2014, **17**, No 1, 18–24
ISSN 1311-1477; online at http://tru.uni-sz.bg/bjvm/bjvm.htm

Original article

POPULATION STRUCTURE OF TWO NATIVE BULGARIAN CATTLE BREEDS WITH REGARD TO *CSN3* AND *CSN1S1* GENE POLYMORPHISM

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Summary

Hristov, P. I., D. R. Teofanova, B. S. Neov, L. I. Zagorchev & G. A. Radoslavov, 2014. Population structure of two native Bulgarian cattle breeds with regard to *CSN3* and *CSN1S1* gene polymorphism. *Bulg. J. Vet. Med.*, **17**, No 1, 18–24.

The application of gene specific molecular markers in genotyping and genetic identification is of essential significance for preserving genetic diversity. The aim of study was to reveal the genotype profile of two native for Bulgaria cattle breeds – Shorthorn Rhodopean cattle breed (SRc) and Bulgarian Grey cattle breed (BGc). These breeds were genotyped with respect to alfaS1-casein gene (*CSN1S1*) and kappa casein gene (*CSN3*) polymorphism by PCR-RFLP assay. The results showed high frequency of the homozygous BB genotype and B allele (70%) of *CSN1S1* in SRc breed. Analysis of *CSN3* polymorphism revealed that the heterozygous AB genotype was presented with the highest frequency and prevalence of the B allele (54%). Analysis of polymorphism of the *CSN1S1* gene in the BGc breed displayed high frequency of the heterozygous BC genotype and B gene allele (57%). The results for *CSN3* locus showed superiority of the heterozygous AB genotype and prevalence of the uncommon B allele (51%). Genetic profiles of the Bulgarian local breeds were compared to other European cattle populations to establish the position of the breeds with regard to cattle diversity. It may be concluded that SRc and BGc breeds are with specific genotype profiles similar to other cattle population in South-Eastern Europe.

Key words: Bulgarian Grey cattle, *CSN1S1, CSN3*, genotyping, PCR-RFLR, Shorthorn Rhodopean cattle

INTRODUCTION

During the last few decades modern breeding techniques have greatly improved the production potential of farm animal species. Due to pressure of crossbreeding, the number of native breeds is rapidly decreasing. These trends lead to the use of intensive crossbreeding of local breeds with imported breeds. As a result the indigenous breeds are completely replaced by high producing animals. How-

ever, when livestock breeds become extinct, their unique genes and genetic profiles are lost forever. The native cow breeds possess numerous valuable features: they are a unique source of genes for improved stability of modern breeds; initial material for creation of new breeds with specific characteristics; abundant source of adaptivity in environment changes and a reserve for hereditary features. Consequently, the indigenous breeds represent a source of precious genetic base which may be used for "construction" material for development of animal husbandry.

Shorthorn Rhodopean cattle (SRc) is one of the two local breeds in Bulgaria. The breed is the last remainder of the prehistoric cattle in Europe, along with the South Albanian, Montenegro and Ilyric Dwarf cattle (Nikolov, 1999). Nowadays the population size in Bulgaria is about 300 cows and its status is in danger of extinction due to the uncontrolled crossbreeding with modern breeds which have almost completely replaced it. The area of distribution of the population is the Eastern and Southeastern Rhodopa mountains. The SRc breed represents a valuable genetic resource for our country. Because of that a national strategy for *in situ* conservation of the breed has been developed.

The Bulgarian grey cattle (BGc) represents one of the cattle breeds called Grey cattle (or Bos Steppe cattle), believed to be the ancestor or a relative of the same European Grey cattle living in Italy, Bulgaria and Hungary (Kök & Soysal, 2006). The Grey Steppe originated in the steppe of the Ukraine from where it moved west and south into Italy, Hungary, the Balkans and Turkey in ancient times. BGc is the first one of the indigenous breeds in Bulgaria, which biodiversity is protected at a national level (Gorinov, 2004). Nowadays the population size is about 600–700 cows and its status is in danger of extinction due to the mechanisation of agriculture and crossbreeding with highly productive breeds. The main area of distribution of the population is the Sakar and the Strandzha mountains, the Central Balkan mountain, and the Sredna Gora mountain.

The genetic variability of SRc and BGc populations has been investigated by microsatellite markers (Dalvit *et al.*, 2009) and milk protein gene polymorphism (Panajotova *et al.*, 1998; Hristov *et al.*, 2013).

Since milk protein genes polymorphism have been discovered, its development is associated with several main goals: to understand the biological significance of genetic variants, to clarify the association between genetic variants and milk traits in practice. In applied fields it concerns the selection and breeding techniques and in the last decades researchers are focused on the origin and domestication of cattle breeds (Formaggioni *et al.,* 1999; Jann *et al.,* 2004; Botaro *et al.,* 2008).

The objective of this study is to determine the genetic profile of Shorthorn Rhodopean and Bulgarian Grey cattle according to milk protein genes and to compare it with other cattle populations in order to establish the position of the breed with regard to the other European cattle breeds genetic diversity.

MATERIALS AND METHODS

Sample collection and DNA extraction

A total of 40 and 47 nasal swab samples were collected from SRc cows (Momchilgrad region) and BGc cows (Starosel and Hisar regions), respectively. The animals were chosen from distinct herds as unrelated typical representatives of the breeds.

DNA was extracted by commercial GeneJet™ Genomic DNA Purification Kit (Fermentas). The DNA concentration was determined spectrofotometrically and the quality of the DNA samples examined on 1% agarose gel electrophoresis.

Gene selection

The *CSN1S1* gene is localized on chromosome 6 (Ferretti *et al.,* 1990). Recent data indicates 9 genetic variants of it in the genus *Bos* (Caroli *et al.,* 2009). 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 transition at position 26181 bp (A/G). This variation was used for allelic forms differentiation through RFLP assay after PCR amplification of a polymorphic region located between 5'end and the first exon of the *CSN1S1* gene.

The *CSN3* gene is situated on chromosome 6 and recently 12 genetic variants have been determined (Formaggioni *et al.,* 1999). A and B alleles are the most frequent for the genus *Bos* and they differ from each other in two amino acid substitutions (36Thr(A)/Ile(B) and 48Asp(A)/Ala(B)) due to transversion at position 13068 bp (A/C) and 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.

PCR-RFLP analysis of milk protein genes

For amplification of the polymorphic region of the *CSN1S1* gene, specific primers were used (Koczan *et al.,* 1991). PCR amplification of the polymorphic region of *CSN3* gene was performed with primers described by Medrano & Cordova (1990). All PCR reactions were carried out using LittleGenius thermocycler (BIOER Technology Co., Ltd) under the following conditions: initial denaturation 94 ºC for 5 min; 35 cycles (denaturation 94 ºC for 30 s; primer anealing 50 ºC for 30 s; extension 72 ºC for 1 min) and final extension 72 ºC for 10 min. PCR products were visualised on 1% agarose gel with ethidium bromide under UV light. Fragment size was determined using GeneRuler™ 100 bp Ladder Plus (Fermentas).

Amplified fragments of *CSN1S1* gene (in total 310 bp) were restricted with *Tsp*45I (New England BioLabs Inc.) endonuclease for 1 hour at 65 ºC according to manufacturer's instructions. PCR products of the *CSN3* gene (in total 350 bp fragment) were digested with *Hinf*I specific endonuclease (Fermentas) for one hour at 37 ºC according to manufacturer's instructions. Restriction products of *CSN1S1* and *CSN3* genes were visualized on 2% agarose gel with ethidium bromide under UV light. Fragments size was determined using GeneRuler™ 100 bp Ladder Plus (Fermentas).

Data analysis

Genotype and allele frequencies were determined and validity of Hardy-Weinberg equilibrium for BGc and SRc populations were evaluated by POPGENE, version 1.31(Raymond & Rousset, 1995) using Co-dominant Diploid data analysis.

RESULTS

CSN1S1 gene polymorphism

In Bulgarian Grey cattle, three genotypes were obtained according this locus, two homozygous (BB and CC) and one heterozygous (BC). About 77% of the animals (36 cows) were heterozygous BC cows and their RFLP profiles showed three electrophoretic bands (310 bp, 214 bp and 96 bp). The homozygous CC ani-

mals (2 cows) represented the lowest frequency (4%) and they were expressed with one unrestricted fragment on the electrophoregram (310 bp). About 19% of animals (9 cows) were defined as homozygous by B allele (BB) and two electrophoretic bands were characteristic for them (214 bp and 96 bp). Genotype and allele frequencies were estimated and shown on Table 1. The frequency of B allele (57%) of the gene was higher than C allele (43%).

The chi-square test for Hardy-Weinberg equilibrium, at degree of freedom – 1, showed a value of $\chi 2 = 12.60$ (P value 0.0003), thus confirming the validity of the Hardy-Weinberg equilibrium for the BGc population.

In studied Shorthorn Rhodopean cattle, about 53% (20 cows) were homozygous BB animals, while CC animals were presented with the lowest frequency – 13% (5 cows). The heterozygous BC genotype was presented by frequency of 34% (13 cows). Genotype and allelic frequencies are presented on Table 1. The frequency of B allele (about 70%) of the gene was more than twice higher than that of the C allele (30%).

The chi-square test for Hardy-Weinberg equilibrium, at degree of freedom – 1, showed a value of $\chi 2 = 10.51$ (P value 0.0004), thus confirming the validity of Hardy-Weinberg equilibrium for the SRc population.

CSN3 gene polymorphism

Genotyping of Bulgarian Grey cattle animals by *CSN3* gene showed that heterozygous individuals (AB) were with the highest prevalence (about 39%). Four electrophoretic bands characterised that genotype (266 bp; 134 bp; 132 bp and 84 bp). About 32% of the studied cows were defined as homozygous by B alleles (BB) which were visualised with two electrophoretic bands (266 bp and 84 bp). Least represented were the homozygous AA

Table 1. Genotype and allele frequencies for the *CSN1S1* and *CSN3* genes in Bulgarian Grey cattle and Shorthorn Rhodopean cattle populations

	Gene	Genotype	Genotype frequencies		Allele frequencies
			Observed	Expected	
Bulgarian Grey cattle	*CSN1S1*	BB	0.04	0.18	B – 0.57 C – 0.43
		CC	0.19	0.32	
		BC	0.77	0.49	
	CSN3	AA	0.29	0.24	A – 0.49 B – 0.51
		BB	0.32	0.26	
		AB	0.39	0.49	
Shorthorn Rhodopean cattle	*CSN1S1*	BB	0.52	0.48	B – 0.70 C – 0.30
		CC	0.13	0.09	
		BC	0.34	0.42	
	CSN3	AA	0.23	0.21	A – 0.46 B – 0.54
		BB	0.31	0.29	
		AB	0.44	0.49	

animals (22%), identified with three electrophoretic bands (134 bp; 132 bp and 84 bp). Genotype and allele frequencies were obtained. Distribution of genotype and allele frequencies among the studied animals was presented on Table 1. The frequency of B allele (51%) of the gene was slightly higher than that of allele A (49%).

The chi-square test for Hardy-Weinberg equilibrium, at degree of freedom – 1, showed a value of $\chi 2 = 1.03$ (P value 0.0028), e.g. that locus was found to be at Hardy-Weinberg equilibrium for the BGc population.

It was established that in Shorthorn Rhodopean cattle the heterozygous AB genotype was presented by the highest frequency: 47% (17 cows). The presence of the homozygous AA (9 cows) and BB (10 cows) animals was found to be with almost equal frequencies (25% and 28%). Genotype and allelic frequencies of SRc population are shown on Table 1. As for BGc, the frequency of the B allele (54%) in SRc was higher than that of the A allele (46%).

The chi-square test for Hardy-Weinberg equilibrium, at degree of freedom – 1, showed a value of $\chi 2 = 11.53$ (P value 0.00002), i.e. validity of Hardy-Weinberg equilibrium for the SRc population.

DISCUSSION

Milk protein genes polymorphism is of a great importance to researches for clarification of the origin, biogeography, evolution and domestification of cattle. Genotyping of 30 cattle breeds from four continents revealed a geographically associated distribution of haplotypes, mainly defined by frequencies of alleles at *CSN1S1* and *CSN3* locuses (Jann *et al.,* 2004). According to this basic study there are three differentiated geographic regions of cattle breeds' origin (Northern and Central Europe (NC); Southern Europe and Africa (SE) and the Near East). As *CSN1S1* allelic frequencies distribution is concerned, it was established that for both groups (NC and SE) the *CSN1S1*-B allele is predominant but *CSN1S1*-C allelic frequencies for SE group (about 40%) are far more higher than for NC group (less then 10%). Established frequencies of C allele of the gene in our study were 45 % for BGc breed and 30% for SRc (Table 1). Thus these results give the opportunity to assume genetic similarity of the two native Bulgarian cow breeds with the SE group. Another explanation of the distribution of *CSN1S1* allelic frequencies among NC and SE groups is directly connected with the selection and breeding scheme. The *CSN1S1* alleles seem to be an indicator of artificial selection on the distribution of haplotypes (Jann *et al.,* 2004). Most breeds selected for milk yield (e.g. Angler, Holstein-Friesian, Ayshire), which originated from Northern and Western Europe are with high prevalence of the B allele of the gene. In comparison, Central European breeds are mostly dual-purpose breeds and have a slightly higher frequency of the C allele. Otherwise unselected breeds and those used in extensive production systems, which originated from Southern Europe, showed much higher frequencies of C allele of the gene.

In contrast to the *CSN1S1* locus, for the *CSN3* gene allelic frequency, the A allele is characteristic for the NC group (presented with about 70-80%) but for the second group (SE) of cattle breeds, *CSN3*-B is predominant (allelic frequencies over 50-60%) (Jann *et al.,* 2004). Established allele frequencies for BGc and SRc breeds are about 50% for the B allele of the gene and about 40% for the A allele (Table 1). These results confirm the close genetic

similarity of the Bulgarian local breeds to the SE group. The allelic frequencies of the *CSN3* gene did not seem to be influenced by the way the breed has been selected, indicating that its distribution could be caused by a natural, rather than by artificial selection (Jann *et al.,* 2004).

CONCLUSIONS

According to the results of the study, the B allele of *CSN1S1* gene was predominantly found in populations of Shorthorn Rhodopean cattle breed (70%) and Bulgarian grey cattle breeds (57%). The B allele of *CSN3* gene showed higher frequency in the populations of Shorthorn Rhodopean cattle breed (54%) and Bulgarian grey cattle breeds (51%). These results show that this ancient cow populations may be defined as genetically similar to other cattle populations in South-Eastern Europe. Allelic variants of milk protein genes could be utilized as markers for the origin of *Bos taurus*.

ACKNOWLEDGMENTS

This study was supported by grant DMU № 02/23 28.12.2009 from the National Science Fund of the Bulgarian Ministry of Education and Science, Sofia, Bulgaria.

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Paper received 18.06.2013; accepted for publication 11.10.2013

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