Preliminary Report on *CSN3* and *LGB* genes Polymorphism among Two Romanian Cattle Breeds

Daniela Elena Ilie[1*], Radu Ionel Neamț[1], Cristina Popescu[2], Gheorghe Săplăcan

[1]*Research and Development Station for Bovine - Arad, 310059, Arad, Bodrogului 32, Romania*
[2]*Instiutute of Life Sciences, Vasile Goldis Western University of Arad, Rebreanu Street 86 Arad, Romania*

Abstract
The aim of the current study was to evaluate the genetic polymorphism of *A* and *B* variants at the *CSN3* (κ-casein) and *LGB* (β-lactoglobulin) loci in Romanian Spotted and Romanian Brown breeds using PCR-RFLP and PCR-HRM genotyping methods. Altogether, 280 cattle from Arad county were genotyped. The most frequent allele at the *CSN3* locus was *A* in Romanian Spotted breed (0.723) while the *B* allele was the most common in the Romanian Brown (0.694). For *LGB* locus the *A* and *B* alleles recorded similar frequencies for Romanian Spotted (0.5141 and 0.4859) compared to Romanian Brown breed where the *B* allele was the most prevalent (0.5970). Knowledges regarding genetic information of breeds related to milk protein are valuable for economic reassertion of cattle breeds, particularly autochthonous ones. Further, the results would be useful in future breeding programs for the studied population, aimed to improve cattle genetic structure for milk quality traits.

Keywords: κ-casein, β-lactoglobulin, genetic polymorphism, milk production traits, Romanian cattle breeds

1. Introduction

Romania is among the top ten countries in the EU regarding the cattle population, with 2,092,000 heads according to the National Institute of Statistics report for the year 2015 [1]. From the total breed structure, 30.96% are represented by the Romanian Spotted (RS) and 13.58% by the Romanian Brown (RB) breed according to the reports of the Romanian National Agency for Animal Improving and Reproduction [2]. Both breeds are considered as middle performing, however the breeds highlighting a remarkable resistance, adaptive capacity to environment and high longevity (7-10 lactations). Studies obtained from Official Performance and Recording Scheme (from 2010) regarding productive performance of Romanian Spotted showed an average milk production of 4754 kg / standard lactation with 3.92% fat and 3% protein content [3]. In Romanian Brown breed was recorded an average milk production of 3537 kg / standard lactation with 3.91% fat and 3.02% protein content [3].
The milk proteins polymorphism that proved to exert significant influences on quantitative and qualitative milk production and cheesemaking properties, have been thoroughly investigated [4]. The contribution of *CSN3* (κ-casein) and *LGB* (β-lactoglobulin) genetic variants to the milk production traits improvement was shown previously by several authors [5,6]. In the Caroli *et al.*, review for *Bos* genus were indicated 14 *CSN3* genetic variants (*A, A[1], B, B[2], C, D, E, F[1], F[2], G[1], G[2], H, I, J*) and 11 for *LGB* (*A, B, C, D, E, F, G, H, I, J, W*), the most common variants being *A* and *B* in all breeds [4]. Generally, the *CSN3*B* was associated with higher protein percentage [7] while *LGB*B* increased casein and fat content [8]. However, there is a limited information regarding the genetic background of cattle breed reared in Romania and very few studies were performed on a larger number of animals [9,10] aiming the genetic polymorphism of milk protein genes. Considering the high influence of milk protein

* Corresponding author: Daniela E. Ilie, danailie@animalsci-tm.ro

genetic variants on milk chemical composition, all new information regarding the genotypes of cattle populations might be useful in the selection, breeding and genetic improvement of dairy cattle breeds. The aim of the current study was to evaluate the genetic polymorphism of *A* and *B* variants for *CSN3* and *LGB* loci in Romanian Spotted and Romanian Brown breeds using PCR-RFLP and PCR-HRM genotyping methods and further to use those molecular genetic markers for marker assisted selection in order to increase the rate of genetic improvement of milk production traits.

2. Materials and methods

Samples were collected from 280 animals belonging to Romanian Spotted (n=213) and Romanian Brown (n=67), randomly selected. All studied animals are included in Official Performance and Recording Scheme.

Genomic DNA was isolated from blood taken from the tail vein in vacutainers containing K3EDTA as anticoagulant (Vacutest Kima, Italy). For DNA extraction, the manual kit Wizard Genomic DNA Purification Kit (Promega, USA) was used. After extraction, all DNA samples were spectrophotometrically evaluated with NanoDrop-2000 (Thermo Fisher Scientific, MA, USA) and diluted to 50-100 ng.

The *CSN3* genotypes were identified based on PCR-RFLP and PCR-HRM (high resolution melting) techniques. For rapid genotyping of cattle for *CSN3* the PCR-HRM genotyping assay was used as described before [11]. Amplification was performed on LightCycler Nano Real-Time PCR System (Roche Diagnostics GmbH, Germany). The PCR-HRM data were analysed using automated quantification, *Tm* calling (melt temperature analysis) and high resolution melting analysis and genotyping provided by the LightCycler Nano Software 1.0. Samples were clustered according to their genotypes.

For the PCR-RFLP analysis of *CSN3* a 350 bp fragment was amplified [12]. For the identification of the *A* and *B* alleles of *LGB*, a 247 bp fragment was amplified [13] using PCR-RFLP assay. The PCR was performed in a 25 µl reaction mixture containing 50-100 ng of genomic DNA, 25 pmol of each primer and 2× Go *Taq*Green Master Mix (Promega, USA) for *CSN3* amplification and 2× Dream*Taq* Green Master Mix (Fermentas, USA) for *LGB* amplification. PCR conditions were as follows: 30s at 95°C, 30s at 60°C (*CSN3*) / 30s at 63°C (*LGB*) and 30s at 72°C for 35 cycles with a first denaturation for 5 min at 95°C and a final extension cycle of 7 min at 72°C. Allelic differentiation analyses were performed using the RFLP method. The *CSN3* and *LGB* amplicons were digested for three hours at 37°C with the *Hind*III and *Hae*III endonuclease. The PCR-RFLP fragments were separated on 3.5% agarose gel and TBE buffer. Images of the gels were taken using Vilber Loumart Print II Systems.

The direct counting method was used to determine alleles frequencies of *CSN3* and *LGB* genetic variants. The *Chi-square* test (χ^2) was used to determine whether the populations were in Hardy-Weinberg equilibrium (HWE).

3. Results and discussion

At the *CSN3* locus, two alleles were identified. The 280 cattle belonging to Romanian Spotted and Romanian Brown breed were genotyped for *A* and *B* alleles using PCR-RFLP method and PCR-HRM assay.

Genotypes and alleles frequencies for *CSN3* locus of Romanian Spotted and Romanian Brown breed are presented in Table 1. The both investigated breeds were found in Hardy-Weinberg equilibrium at the *CSN3* locus.

For Romanian Spotted cattle the most prevalent genotype was *AA* (0.5211) compared with *AB* (0.4038) and *BB* (0.0751), similar results being reported in Serbian Simmental [14], Romanian Simmental [10,15], Holstein Friesian [9,16,17], Czech Holstein [18] and Estonian Native cattle [19]. Frequencies of *CSN3 A* and *B* alleles in Romanian Spotted were 0.723 and 0.277, respectively. Comparing with previous studies, a significant prevalence of *CSN3*A* allele was observed in other countries for dairy milk breeds, like Holstein [17,20,21] or for dual purpose cattle like Simmental [22,23], whereas in autochthonous cattle breeds the *CSN3*B* allelic variant predominates [15,24,25].

Table 1. Genotypes and alleles frequencies at the *CSN3* locus

		CSN3 genotype			*CSN3* allele frequency		
Breed	Total number	*AA*	*AB*	*BB*	*A*	*B*	*P* value
RS	213	111 (0.5211)	86 (0.4038)	16 (0.0751)	0.7230	0.2770	0.9067[a]
		111.34 (0.5227)	85.31(0.4005)	16.34 (0.0767)			
RB	67	5 (0.0746)	31 (0.4627)	31 (0.4627)	0.3060	0.6940	0.4642[a]
		6.27 (0.0936)	28.46 (0.4247)	32.27 (0.4817)			

[a]Test of Hardy-Weinberg equilibrium; NS, not significant
[b]Test of Hardy-Weinberg equilibrium; $P \leq 0.05$
Upper row: observed value; Lower row: expected value

Romanian Brown cattle recorded values of 0.0746, 0.4627, and 0.4627 for *AA*, *AB* and *BB* genotype frequencies (Table 1). Therefor, *CSN3*B* was the most common allele (0.6940) comparing with *CSN3*A* allele (0.3060). According to other surveys, *CSN3*B* seems to be more frequent than *A* allele in Romanian Grey, Montbeliarde, Bulgarian Grey, Shorton Rhodopean, Italian Maremmana, Busa, Jersey [10,15,24,26,27].

Results revealed that the *CSN3*B* allele was more frequent than the *CSN3*A* allele only in the Brown cattle from the two studied breeds. Successive studies conducted on *CSN3* gene influence on milk properties [28,29] revealed a positive relation between the *BB* genotype and the milk protein content. The *BB* genotype for *CSN3* determines favourable milk properties for cheese production compared with the *AA* genetic variant which determine lower casein contents and as a consequence, a lower efficiency of curd yield [30].

At the *LGB* locus, the *A* and *B* alleles were investigated trough PCR-RFLP method. For this locus, the genotypes and alleles frequencies are presented in Table 2. The *Chi-square* test indicated that the Romanian Spotted breed was not under Hardy-Weinberg equilibrium for *LGB* locus, recording significant deviation ($P < 0.0007$). No significant deviation from HWE was recorded for the Romanian Brown breed.

Table 2. Genotypes and alleles frequencies at the *LGB* locus

		LGB genotype			*LGB* allele frequency		
Breed	Total number	*AA*	*AB*	*BB*	*A*	*B*	*P* value
RS	213	44 (0.2066)	131 (0.6150)	38 (0.1784)	0.5141	0.4859	0.0007[b]
		56.29 (0.2643)	106.42 (0.4996)	50.29 (0.2361)			
RB	67	11 (0.1642)	32 (0.4776)	24 (0.3582)	0.4030	0.5970	0.9517[a]
		10.88 (0.1624)	32.24 (0.4812)	23.88 (0.3564)			

[a]Test of Hardy-Weinberg equilibrium; NS, not significant
[b]Test of Hardy-Weinberg equilibrium; $P \leq 0.05$
Upper row: observed value; Lower row: expected value

The *A* and *B* alleles frequencies showed close values for Romanian Spotted (0.5141 and 0.4859) at the *LGB* locus with *AA*, *AB* and *BB* genotype frequencies of 0.2066, 0.6150 and 0.1784, respectively. Similar results were obtained on Simmental [6,9] and Holstein cows [8]; however, according to other previous publications, *LGB*B* allele was more frequent than *LGB*A* allele in Nelore, Simmental [31], Red Danish, Danish Jersey, Finncattle, Finnish Ayrshire, Norwegian cattle, Swedish Mountain cattle, Swedish Red and White, Swedish Black and White cattle [32], Mexican Jersey [33].

The results obtained in Romanian Brown breed revealed that the *LGB*B* allele was the most prevalent (0.5970) compared with *A* allele (0.4030). The frequencies of *AA*, *AB* and *BB* genotypes were observed at 0.2066, 0.6150 and 0.1784, respectively. Similar results were also reported by other Romanian surveys (0.514) in the same breed [10]. However, for Brown breed, several authors reported a high frequency of *LGB*B* compared with *LGB*A* [34-36].

Literature data regarding the relationship between genetic variants of *LGB* and milk chemical composition revealed a positive correlation

between *LGB*B* allele and protein and fat content [28,34,37]. The similar positive correlation was reported by Celik *et al.* for total solids and *LGB*BB* genotype [34]. The *LGB*BB* genotype is also linked with casein content [38].

4. Conclusions

In the current preliminary study the main purpose was to evaluate the genetic polymorphism of *A* and *B* variants at the *CSN3* and *LGB* loci in two Romanian cattle breed (Romanian Spotted and Romanian Brown). Higher frequencies of the *CSN3*B* and *LGB*B* allele were founded in the Romanian Brown (0.694 and 0.597), similar to those previously reported in other Brown breed populations.

The results will be used for genetic improvement of Romanian cattle breeds through marker assisted selection in order to increase the rate of genetic improvement of milk production traits. The future work will be extended on association study between milk composition and *CSN3* and *LGB* polymorphism.

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