

**RELATIONSHIP BETWEEN GENETIC POLYMORPHISM OF K- CASEIN AND
QUANTITATIVE MILK YIELD TRAITS IN CATTLE BREEDS AND CROSSBREDS
IN SERBIA**

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The quality and yield of milk, and therefore of dairy products as well, depends directly on their protein composition. Milk gene proteins represent structural locuses that are functionally associated with milk production. In cattle selection a special importance is attached to the variability of kappa casein (κ -CN) as one of the four casein milk proteins. Genotypes of 21 Simmental breed cows, 20 crossbred cows obtained by crossing of Simmental and Red Holstein breed, and 25 cows of autochthonous Busha breed were determined for kappa casein by restriction fragment length polymorphism analysis (PCR-RFLP) of amplified DNA.

The κ -CN genotype frequencies for Simmental breed were: 42.8; 47.6 and 9.6 % for AA, AB and BB genotype, for crossbreds: 75.0; 25.0 and 0.0% and for Busha individuals: 41.7; 50.0 and 8.3 %, respectively. Frequencies of A and B alleles, assessed on the basis of genotypic frequencies were 0.667 and 0.333 for Simmental breed, 0.875 and 0.125 for crossbreds and 0.667 and 0.333 for autochthonous Busha cattle breed, respectively. For confirming the phenotypic variability of milk yield in standard lactation, yield of milk fat and the content of milk fat per κ -CN genotypes within breeds observed the fixed model which included the breed effect, lactation in order and effect of κ -CN genotype (AA, AB and BB) was used. The κ -CN genotype statistically significantly ($p \leq 0.05$) influenced milk yield and highly significantly ($p \leq 0.01$) milk fat yield, while the content of milk fat was not statistically significantly ($p > 0.05$) influenced in examined animals.

Key words: cattle, genetic polymorphism, κ - casein, milk traits

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INTRODUCTION

Bovine milk traits in quantitative and qualitative respect are influenced partially by structural and functional genetic changes having an effect on milk gland physiology. The yield and quality of milk in modern breeding programs can often be expressed through the quantity of milk fat and proteins. Identifying the polymorphic gene variants that can affect milk secretion physiological process and their association with productive traits opens the possibilities of further improvement of production predispositions both in individuals and populations which a dairy processing industry is particularly interested in (DOSKO, 2012).

Molecular biology and genomic selection techniques development have enabled to obtain more detailed information on the individual genome structure (RIAZ *et al.*, 2008; ĆINKULOV *et al.*, 2008; BRKA *et al.*, 2010; DJEDOVIĆ *et al.*, 2012). By the application of PCR and RFLP techniques it can be possible to determine the variability of milk protein fractions. A special importance in dairy processing has been given to determining the casein and lactoglobuline variants as the most important milk proteins (BESKOROVAJNI *et al.*, 2002, IVANKOVIĆ *et al.*, 2011; GOUDA *et al.*, 2013).

Kappa casein (κ -CN) as one of the four milk casein proteins is being determined by the gene positioned at the 6th bovine chromosome. CAROLI *et al.* (2009) reported fourteen polymorphic types of κ -CN (A, A1, B, B2, C, D, E, F1, F2, G1, G2, H, I, J) out of which two polymorphic types (A and B) are particularly dominant in their frequency. For polymorphic κ -CN A type the Thr and Asp amino acids on positions 136. and 148. are typical, while for κ -CN B type, at the same positions, Ile and Ala amino acids are typical (MERCIER *et al.*, 1973; EIGEL *et al.*, 1984).

In majority of recent studies a positive effect of κ -CN-B allelic variant on the share of casein and total milk proteins (BOVENHUIS *et al.*, 1992; MOLINA *et al.*, 2006) has been observed. Bovine milk of κ -CN BB genotype is very important in cheese production technology. In addition, the milk with κ -CN BB genotype demands shorter curdling time (SAVIĆ *et al.*, 1996; BESKOROVAJNI *et al.*, 2000; STEVANOVIĆ *et al.*, 2000) and produces more cheese containing higher proteins share (IKONEN *et al.*, 1999, MOLINA *et al.*, 2006). High proteins and milk fat content in milk have a positive effect on the cheese quality and quantity, and on the relationship between casein and total proteins (IVANKOVIĆ *et al.*, 2011).

The milk fat and casein percent are in positive correlation with the quantity of the cheese obtained. It is confirmed that κ -CN BB genotype in milk contains 0.13% proteins more than milk of AA genotype. The advantage of BB genotype is explained by the fact that more casein, more fat and other elements can be "caught" in casein net. It has been assessed that BB type casein milk produces about 10% more cheese than AA type (AZEVEDO *et al.*, 2008). Different variants of k casein have an effect on the milk yield as well. Thus the individuals of AB genotype produce more than 150 kg milk per lactation in comparison with AA genotype individuals (SAVIĆ *et al.*, 1996; KASTONINA *et al.*, 2004; KONOVALOVA *et al.*, 2004). Also, in the study of BOVENHUIS *et al.* (1992), κ -CN genotype had a significant effect on milk production ($p < 0.001$), with cows of the BB genotype producing 173 kg less milk than AA cows. Furthermore, κ -CN genotypes had a highly significant effect on protein content ($p < 0.001$), such that κ -CN BB cows produced milk containing 0.08 percent higher protein content than that of AA cows. The same authors found that, for fat content, the effect of κ -CN genotype was not significant, while for fat yield the B allele it was associated with a significantly lower production of fat when compared with the A allele.

The objective of this study has been to define the frequencies of κ -CN primary polymorphic variants in Simmental breed population, crossbreds obtained by crossbreeding of Simmental and Red Holstein breed and autochthonous Busha breed of Serbia. The results obtained

will enable to define their comparative advantages associated with qualitative and quantitative dairy traits and milk suitability for further processing.

MATERIALS AND METHODS

A total of 21 samples of Simmental breed cows, 20 samples of crossbreds obtained by crossing between Simmental and Red Holstein and 25 samples of Busha breed have been genotyped.

Isolation of DNA samples

Peripheral blood was sampled from 66 cattle and stored with 3.8% sodium citrate as anticoagulant. DNA was isolated by CTAB-DNA precipitation method (DEL SAL *et al.*, 1989). Briefly, 300µl of blood samples are gently mixed with 600µl of pre-warmed solution A (CTAB 8%, NaCl 1.5M, Tris pH 8.5 100mM, EDTA pH 8 50mM) and incubated at 68°C for 5 minutes. After adding one volume of chloroform, the mixture is centrifuged for 5 minutes on 10 000 rpm. Then, the upper phase is precipitated with solution B (CTAB 5%, 0.1M NaCl) to a final concentration of 0.1% and one volume of water. After centrifugation the DNA pellet is resuspended in 300µl of 1.2M NaCl and precipitation was performed by adding 2.5 volumes of the ethanol. DNA pellet was resuspended in ultrapure water and used in subsequent PCR reactions.

PCR-RFLP

In order to analyze natural polymorphisms of k-casein gene, a 311-bp fragment covering the polymorphic sequence was amplified by PCR method, according to the procedure proposed by MEDRANO and AGUILAR-CORDOVA (1990). The sequence of primers used in PCR reactions was: K346A:5'CATTATGGCCATTCCACCAAAG3' and casR1:5'GCTCCCGGTATATGAC CACCCTCT3'.

The PCR reactions were performed with 10 pmol of each primer, 0.1 µg of genomic DNA, 400 µM dNTPs, 1.5 mM MgCl₂ and 2.5U of *Taq* polymerase (Promega) in a total volume of 50 µl. PCR amplifications carried out according to the following program: hot start 98°C 5min, 72°C 6min, 35 successive cycles of 96°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec followed by the final elongation on 72°C for 7min. Obtained PCR products are visualized on 2% agarose gel electrophoresis and concentrated by ethanol precipitation.

Subsequent digestion of PCR products with restriction endonucleases were performed in 1X restriction buffer containing 10µl of PCR product and 5U of *Pst*I enzyme (Fermentas). Obtained fragments were analyzed on 2% agarose gel electrophoresis and genotyping was performed according to specific fragment pattern. Expected fragment sizes are shown in Table 1.

Table 1. Expected fragment sizes

PCR product (bp)	Restriction fragments expected sizes (bp)		
	AA	AB	BB
311	194.117	311,194.117	311

Daily records for 305-day milk, milk fat and milk fat content yields were obtained from relevant expert services performing the control of production capacities as well as from the records kept on the farms.

Data statistical processing

Allele frequencies were determined by gene counting. Statistical calculations were performed using SAS procedures (SAS, 1997, Version 9.1.3.). The data obtained were analysed by the SAS procedure according to the following model:

$$Y_{ijk} = \mu + R_i + L_j + C_k + e_{ijk}$$

Where:

Y_{ijk} – is a phenotypic manifestation of studied trait;

μ -population general average;

R_i - effect of i-breed ($i= 1,3$);

L_j - effect of j-lactation in order ($j= 1,3$),

C_k = effect of κ -CN genotype ($k= AA, AB$ and BB);

e_{ijk} = random error.

RESULTS AND DISCUSSION

In order to perform genotyping of the amplified gene segment Restriction Fragment Length Polymorphism (RFLP) was performed. PCR products were subjected to digestion with restriction endonuclease and obtained fragments analysed on agarose gel electrophoresis. Summary of the PCR-RFLP analysis is presented in Table 2. The representative image of gel electrophoresis is shown in Figure 1.

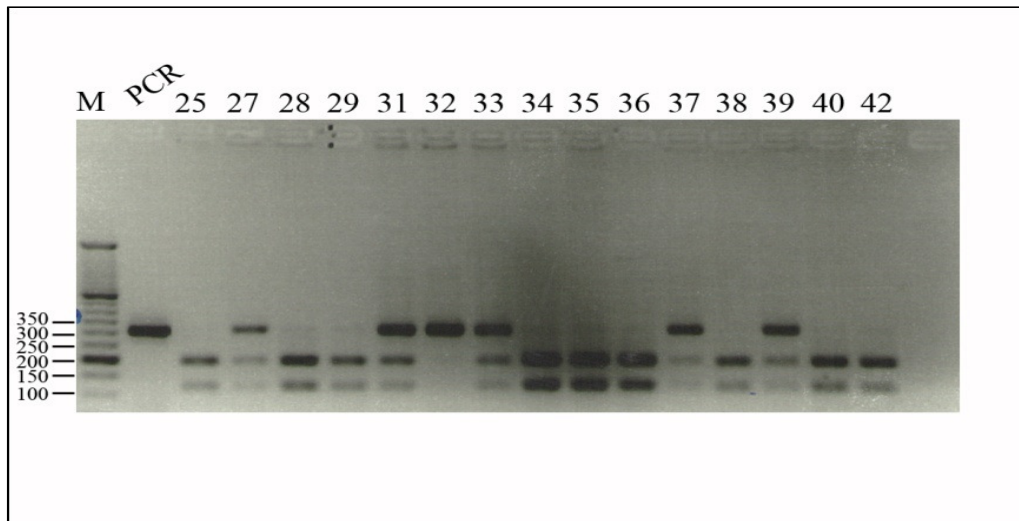


Figure 1. Determination of κ -casein gene variants by PCR-RFLP. M: O'range Ruller DNA

Ladder (50bp, Ferments); PCR: Undigested PCR product; 25, 28, 29, 34, 35, 36, 38, 40, 42 represent samples with *AA* genotype; 27, 31, 33, 37, 39 represent samples with *AB* genotype and 32 represents samples with *BB* genotype.

Table 2. Summary of the PCR-RFLP analysis

Genotype	AA	AB	BB	Total no. of samples
	35	27	4	66

Table 3. The frequency of kappa-casein alleles and genotypes in the investigated populations

Breed		Number of animals	Genotypic frequency (%)	Allele frequency (%)		
Simmental	AA	21	0.428	A	0.667	
	AB		0.476	B		0.333
	BB		0.096			
Crossbreds	AA	20	0.750	A	0.875	
	AB		0.250	B		0.125
	BB		0			
Busha	AA	25	0.417	A	0.667	
	AB		0.500	B		0.333
	BB		0.083			

The results in Table 3 suggest that κ -CN genotypic frequencies for Simmental breed were: 42.8; 47.6 and 9.6 % for AA, AB and BB genotype, for crossbreds: 75.0; 25.0 and 0.0%, and for Busha individuals: 41.7; 50.0 and 8.3 %, respectively. Frequencies of A and B alleles, in examined breeds, assessed on the basis of genotype frequencies, were 0.667 and 0.333 for Simmental breed, 0.875 and 0.125 for crossbreds, and 0.667 and 0.333 for autochthonous Busha cattle, respectively.

By determination of κ -CN polymorphism the domination of κ -CN-A allelic variant was confirmed in all examined breeds, particularly in the crossbreds obtained by crossing between Simmental and Red Holstein (Table 3). The frequency of desirable κ -CN-B allelic variant was either lower or the same in Simmental and Busha breeds.

SULIMOVA *et al.*, (2007) and DINC *et al.*, (2013) reported that, besides κ -CN A and B allelic variants that were displayed only in small number of cattle breeds, particularly autochthonous, some other forms could appear as well, and that in fairly low frequencies.

In relation to previous studies on the frequency of κ -CN allelic variants in Simmental population in Croatia, the share of allelic A κ -CN variant increased, what can be deemed undesirable for the milk processing traits (IVANKOVIĆ *et al.*, 2011). A significant domination of κ -CN A allelic variant in the Simmental and Holstein populations in other countries and regions was observed as well, while in the populations of autochthonous cattle breeds the κ -CN B allelic variant prevails (BRKA *et al.*, 2010; DOOSTI *et al.*, 2011; IVANKOVIĆ *et al.*, 2011; DINC *et al.*, 2013).

DOSKO (2012) also suggests that the *A* allelic variant was confirmed as a κ -*CN* dominant variant wherein *AB* genotype prevailed in Simmental and Brown breed populations, while *AA* genotype was prevalent in Holstein populations.

In comparison with the studies conducted on Holstein Frisian breed cows, representing at the same time the most dominant dairy breed, there are little official records on polymorphic forms of κ -*CN* in Simmental breed, particularly Simmental crossbreds obtained by crossing with Red Holstein cows. According to the small number of trials conducted on Simmental breed it was confirmed that the frequency of κ -*CN-A* allele was greater than the frequency of κ -*CN-B* allele. However, several different studies (BONFATI *et al.*, 2010; IVANKOVIĆ *et al.*, 2011; DOSKO, 2012; AKYÜZ *et al.*, 2013) showed that, contrary to HF breed, in Simmental cows, difference between the frequencies of the two alleles (*A* and *B*) was not so great. Thus, in the study of AKYÜZ *et al.*, (2013), the frequency of κ -*CN-A* allele in Simmental cows (0.72) was higher than that of *B* allele. However, it was confirmed that this frequency was lower than in HF cows (0.82). The results obtained in our study on the frequency of *A* and *B* alleles of κ -*CN* genes in Simmental breed are in harmony with the results previously reported from different European and worldwide regions.

On the contrary, IVANKOVIĆ *et al.*, (2011), confirmed that the frequency of *B* alleles in Busha cows (0.65) was greater than that of *A* alleles (0.35).

According to the study of JANN *et al.* (2004), the frequency of desirable κ -*CN-B* allele in autochthonous breeds in Europe was (0.298- 0.345) in the Turkish, (0.130-0.460) Croatian, (0.330) Polish and (0.190-0.280) Belgium autochthonous breeds. In most cases these results agree with the results obtained in this study.

The population of Busha and of other autochthonous breeds will certainly be reaffirmed through the production of milk and milk products what is being favored by a high frequency of κ -*CN-B* variant.

In the studies on bovine breeds genetic characterization, it was found out that the *k-CN-B* allele occurs at higher frequencies in breeds originating from *Bos taurus* than in those of *Bos indicus* origin (KEMENES *et al.*, 1999).

Table 4 shows the mean values of milk yield traits per genotypes *k-CN* and p-value of studied effects. The cow breed influenced statistically significantly ($p \leq 0.01$) all studied traits, genotype κ -*CN* statistically significantly ($p \leq 0.05$) influenced the milk yield and highly significantly ($p \leq 0.01$) the milk fat yield, but it did not influence significantly ($p > 0.05$) the milk fat content, while the lactation in order significantly ($p \leq 0.05$) influenced the milk yield and milk fat yield, but it did not significantly ($p > 0.05$) influence the milk fat content.

Simmental cows of *AB* genotypes have higher milk yield (+191 kg and +787 kg, respectively, than *AA* and *BB* individuals producing at the same time higher milk fat yield (+32 kg and +40 kg, respectively) than *AA* and *BB* animals.

AB genotype crossbred animals have higher milk yield (+560 kg) than *AA* genotype individuals. In crossbred animals no individuals of *BB* genotype have been detected by *BB* genotyping.

The results obtained for Busha individuals showed that the *BB* genotype cows have lower milk yield (-149 kg and - 289 respectively) than *AA* and *AB* individuals.

AB genotype cows throughout all examined breeds and crossbreds in this study had higher milk fat content in relation to *AA* and *BB* genotype, this record being confirmed also by BOTARO *et al.*, 2009.

Table 4. Mean values of milk yield per *k-CN* genotypes and *p*-value of studied effects

Breed		Milk yield (kg) ± SD	Milk fat content (%) ± SD	Milk fat yield (kg) ± SD
Simmental	AA	5966±802	3.98±0.06	222.1±52.9
	AB	6157±801	4.00±0.07	254.2±41.6
	BB	5370±536	3.98±0.04	214.4±20.7
Crossbreds	AA	4583±612	3.90±0.13	178.8±21.6
	AB	5143±533	3.78±0.12	194.3±18.6
	BB	-	-	-
Busha	AA	904±81	4.46±0.35	40.4±5.4
	AB	1044±95	4.72±0.42	49.2±5.5
	BB	755±32	4.88±0.14	36.8±2.0
Effect			p-value	
Breed		0.001**	0.0001**	0.0001**
Genotype <i>κ-CN</i>		0.033*	0.330 ^{nz}	0.008**
Lactation		0.013*	0.980 ^{nz}	0.037*

***- $p \leq 0.001$; ** - $p \leq 0.01$; * - $p \leq 0.05$; ^{nz} - $p > 0.05$

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AB genotype cows throughout all examined breeds and crossbreds in this study had higher milk fat content in relation to *AA* and *BB* genotype, this record being confirmed also by BOTARO *et al.*, 2009.

According to SITKOWSKA *et al.* (2013) *k-CN* *AA* genotype cows obtained the highest yield of milk (6414kg), fat (217kg) and proteins (209kg), while the lowest content of milk fat and proteins was recorded in the milk produced by *BB* genotype cows.

However the milk of *BB* genotype cows produces 10% higher yield of cheese in relation to the milk of *k-CN* *AA* genotype cows (MARZALI and NG-KVAJ-HANG, 1986; AZEVEDO *et al.*, 2008).

ALIPANAH *et al.* (2008), indicated that in Black Pied breed the effect of *k*-casein polymorphism was significant for fat percentage. The milk produced by the *BB* genotype cows showed higher fat percentage (+0.37% and +0.49%, respectively) than *AA* and *AB* individuals. In Red-pied cows, statistically significant differences for milk yield and fat yield were found. *AA* genotype cows had higher milk yield (+93.28 kg and 204.02 kg, respectively) than *AB* and *BB* individuals, and higher fat content (+3.10 kg and +5.29 kg, respectively) than *AB* and *BB* animals.

CONCLUSION

It can be concluded that by determination of *κ-CN* polymorphism the domination of *A* allelic variant was confirmed in all breeds included in this study, what particularly applies to crossbreds obtained by crossing of Simmental and Red Holstein. The frequency of desirable *κ-CN-B* allelic variant was lower and equal in Simmental and Busha cows. By identification of cattle populations and breeds with increased share of *κ-CN-B* allelic variant and their including into selection programmes the possibility of increasing the frequency of desirable genes is more attainable and therefore, the improvement of technological quality of bovine milk, what could

increase the economical value of livestock production as well. The study gives the starting point for breeders to decide both on the breeds to be raised and on the qualitatively and quantitatively defined milk production in accordance with the needs of dairy processing industry.

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POVEZANOST GENETSKOG POLIMORFIZMA K- KAZEINA I KVANTITATIVNIH OSOBINA MLEČNOSTI RASA I MELEZA GOVEDA U SRBIJI

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Izvod

Kvalitet i prinos mleka kao i proizvoda od mleka direktno zavisi od njegovog proteinskog sastava. Geni proteina mleka predstavljaju strukturne lokuse, koji su funkcionalno povezani sa proizvodnjom mleka. U selekciji goveda poseban značaj ima varijabilnost kapa kazeina (κ -CN) kao jednog od četiri kazeinske belančevine mleka. Genotipovi 21 krava simentalske rase, 20 krava meleza dobijenih ukrštanjem simentalca sa crvenim holštajnom i 25 krava autohtone rase buše su određene za kapa kazeina pomoću PCR-RFLP analize. Genotipske frekvencije κ -CN za simentalsku rasu bile su: 42,8; 47,6 i 9,6 % za AA, AB i BB genotip, za meleze: 75,0; 25,0 i 0,0% i za individue buše: 41,7; 50,0 i 8,3 %, respektivno. Frekvencije alela A i B, za posmatrane rase procenjene na osnovu genotipske frekvencije, imale su vrednosti 0,667 i 0,333 za simentalsku rasu, za meleze 0,875 i 0,125 i 0,667 i 0,333 za goveda autohtone rase buša, respektivno. Za utvrđivanje fenotipske varijabilnosti prinosa mleka u standardnoj laktaciji, prinosa mlečne masti i sadržaja mlečne masti po genotipovima κ -CN unutar ispitivanih rasa korišćen je fiksni model koji je obuhvatao uticaj rase, laktacije po redu i efekta genotipa κ -CN (AA, AB i BB). Genotip κ -CN statistički značajno ($p \leq 0.05$) je uticao na prinos mleka i visoko značajno ($p \leq 0.01$) na prinos mlečne masti, ali nije statistički značajno ($p > 0.05$) uticao na sadržaj mlečne masti ispitivanih životinja.

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