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ALLELIC POLYMORPHISM OF K-CASEIN GENE (CSN3) IN THREE MONTENEGRIN CATTLE BREEDS

SUMMARY

Genetic polymorphism of κ-casein gene was investigated in three cattle breeds reared in Montenegro: Brown Swiss, Busha and Gray breeds of cattle. The DNA of these breeds was genotyped for polymorphisms at the kappa-casein gene by a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) essay. A 351 bp fragment of κ-casein was amplified and digested with HinfI restriction endonuclease. Three genotypes were identified (AA, AB and BB) with frequencies of 0.162, 0.501 and 0.356 (respectively) in Brown Swiss breed, 0.267, 0.501 and 0.233 in Busha breed and 0.236, 0.500 and 0.264 in Gray cattle breed. The frequencies of alleles A and B were 0.403 and 0.597 (respectively) in Brown Swiss, 0.517 and 0.483 in Busha cattle and 0.486 and 0.514 in Gray cattle. The genotyping of κ-casein alleles (A and B) is of practical importance, since B allele is in positive correlations with commercially valuable parameters of cheese yielding efficiency. The results of high frequency of B allele in Brown Swiss cattle confirmed a good performance of this breed in Montenegrin population. Results could be used for possible increasing the frequency of desired alleles and genotypes by including it in the breeding program for Brown Swiss breed as well as in the preservation program for Gray cattle and Busha as the important animal genetic resources in Montenegro.

Keywords: *κ-casein, PCR-RFLP, Busha, Brown Swiss, Gray cattle*

INTRODUCTION

Molecular biology and genomic selection techniques development have enabled to obtain more detailed information on the individual genome structure. The discovery of PCR-RFLP (*Polimerase Chain Reaction - Restriction Fragment Lenght Polymorphism*) generated renewed interest in the use of gene marker loci as an aid to selection programs and playing an increasingly important role as genetic markers in many fields of animal breeding. By the PCR-RFLP technique it is possible to determine the variability of milk protein fractions.

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Cow milk contains two classes of specific proteins, i.e., the group of caseins and the group of whey proteins. Caseins constitute about 78-82% of bovine milk proteins and subdivided into four main groups: αS1-casein, αS2-casein, β-casein, and κ-casein (Azevedo *et al.,* 2008, Smiltiņa and Grīslis, 2010, Hristov *et al.*, 2012).

Genetic variants of κ-casein have been extensively studied in cattle at the protein and DNA levels, and numerous alleles have been revealed (Tanaskovska *et al.,* 2016). K-casein (κ-CN) is being determined by the gene positioned at the 6th bovine chromosome. The genomic DNA encoding the CSN3 milk protein is about 13 kb that is divided into 5 exons and intervening sequences (Azevedo *et al.*, 2008, Smiltiņa and Grīslis, 2010). Some authors reported fourteen polymorphic types of κ-CN (A, A1, B, B2, C, D, E, F1, F2, G1, G2, H, I, J). The A and the B alleles are the most frequent among all the species of the genus Bos (Djedović *et al.,* 2015, Dokso *et al.,* 2014, Hristov *et al.,* 2012). The existence of the E allele in some dairy cattle breeds was reported in the papers of Soria *et al.* (2003), Caroli *et al.* (2010), Taha and Punan (1993).

The genetic variants of milk proteins are resulting as a consequence of substitution or deletion of amino acids within the polypeptide chain (El Rafey and Darwish, 2008). A and B variants of κ-casein differ in the amino acids 136 and 148. At position 136, threonine is replaced by isoleucine, while at position 148, aspartic acid is replaced by alanine, for A and B, respectively (Azevedo *et al.,* 2008, Deb *et al.,* 2014, Dogru and Ozdemir, 2009). The restriction endonucleases HindIII, HaeIII, and MaeII could be used for distinguishing κ-casein alleles (Soria *et al.,* 2003).

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 and cheese production. In most of the κ-casein studies, the association of the B allele variant with some quantitative and milk processing traits was identified. The B allele found to be associated with thermal resistance, shorter coagulation time, better curdles and micelles of different sizes, which are preferable in cheese making (Azevedo *et al,* 2008, Dokso *et al.,* 2014). In majority of recent studies, a positive effect of κ-CN-B allelic variant on the share of casein and total milk proteins has been observed (Molina *et al.,* 2006, Djedović *et al.,* 2015). The cheese yield from cows with genotype BB is 10% higher than in cows with AA genotype (Deb *et al.,* 2014, Tanaskovska *et al.,* 2016).

Identification of the polymorphic gene variants of milk proteins associated with productive traits opens additional possibilities for improvement of production of different breeds (Djedović *et al.,* 2015). B allele of κ-casein gene is integrated into cattle breeding programs in many countries. In recent years, some countries started trading milk and dairy products with desirable kappa and/or beta casein as well as breeding animals with genotype for kappa and/or beta casein. In some countries the genotype for κ-casein is present in sire catalogues for a long time (Potočnik, 2015).

Cattle husbandry in Montenegro is the most important branch of animal husbandry and agriculture in general. Annual production of cow's milk is about 180 thousand tons. Breed composition of Montenegrin cattle population is quite unfavorable because various crossbreeds dominate, about 50% of the total population. In the last two decades share of high productive breeds Holstein and Simmental has been increased, at the same timeBrown Swiss and Grey cattle sharply decreased, while autochthonous Busa breed is in risk of extinction.

The aim of this study was to identify the genetic variants of κ-casein in three cattle breeds reared in Montenegro for a longer period (Busha, Grey cattle and Brown Swiss) by use the PCR-RFLP technique. The 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 future. Genotyping is very important for the evaluation of biodiversity and preservation of these cow breeds.

MATERIAL AND METHODS

Animals

Blood samples were obtained from 96 cows of three breeds: 30 samples were collected from Busha cattle 31 samples from Brown Swiss and 35 samples were collected from Gray cattle.

Busha is an autochthonous breed of cattle, which was the most dominant breed in Montenegrin cattle population till the seventies of XX century, (approximately 90%). In the second half of XX century, Busha breed had been crossed or just replaced by more productive breeds. Today, Busha is endangered breed, with less than 300 animals and because of that it is involved in the program of in situ conservation (Marković *et al.,* 2020).

The Gray cattle in Montenegro today is a local breed originated from Tyrolean grey breed. First time it was imported on beginning of XX century as well as after Second World War, till the sixties, and used for improvement of production traits of Busha cattle. This breed is very adaptable, resistant and very suitable for hilly mountain region of Montenegro. However, its population is permanently decreased and now its share in total cattle population is about 5% (Marković *et al.,* 2020).

Brown cattle in Montenegro originated from Brown Swiss breed, which is occasionally imported since the seventies of last century (Radonjić *et al.,* 2017). The population of the Brown breed in Montenegro participates by 12% in the total cattle population (Marković *et al.,* 2020).

DNA extraction from blood

Blood samples were collected from v. jugularis into vacuum tube K2EDTA (3ml). The blood samples were maintained at -20° C until used for DNA extraction. DNA extraction was performed using DNA isolation kit (ZYMO research, USA) according to manufacturer's instructions, while phenol-chloroform standard protocol used for some samples (Marković *et al.,* 2018). The quality of DNA was checked by Nano-Vue Spectrophotometer taking ratio of

optical density (OD) value at 260 and 280 nm. Also, the quality of the DNA samples was examined on 1% agarose gel.

Genotyping of kappa-casein gene by PCR-RFLP method

The κ-casein genotype was determined using PCR-RFLP. Amplification of 351 bp fragment of κ-casein was done using the polymerase chain reaction (PCR) with primers: forward (5'-TT TAT GGC CAT TCC ACC AA-3') and reverse (5'-ATT AGC CCA TTT CGC CTT CT-3') as reported by Dogru and Ozdemir (2009). The amplification was performed in 30 µl of final volume with 100 ng of genomic DNA. The reaction mix contained 10xPCR buffer, MgCl2 (1.5mM), dNTP nucleotides (100 µM), 1 µM of each primers and AmpliTaq Gold polymerase (0.5 U). Amplification was performed in Mastercycler Pro (Eppendorf) gradient programmed for initial denaturation at 94°C for 5 min (initial denaturation), followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 60 sec, and final extension at 72°C for 7 min. PCR products were checked for amplification by electrophoresis on 1% agarose gel, in parallel with 50 and 100 bp DNA marker (Đokić *et al.,* 2020).

For genotyping, PCR product was digested with Hinf I restriction enzyme which was used for the determination of κ-casein alleles. The total reaction mixture of 15 µL comprised of 10 µL of PCR product, 1.5 µL of enzyme buffer, 5 U of restriction enzyme and 3.2 µL of dH$_2$O. After digestion at 37°C for three hours the digested products were resolved by electrophoresis on 3% agarose gel in parallel with a 100 bp DNA marker. The genotype patterns were visualized under UV light by Quantum ST400. The genotypes of cattle κ-casein gene were identified on the base of restricted fragments size.

Statistical analysis

Allele and genotype frequencies for the genetic variants of κ-casein gene were analyzed by Hardy-Weinberg equilibrium calculator including analysis for ascertainment bias (Rodriguez *et al.*, 2009). The chi-square statistic used to check whether the populations were in Hardy-Weinberg equilibrium: http://www.husdyr.kvl.dk/htm/kc/popgen/genetik/applets/kitest.htm.

RESULTS AND DISCUSSION

Frequencies of genotypes and allele of κ-casein (CSN3) were detected in 96 individuals, in Brown Swiss, Busha and Gray cattle. PCR amplification of the 351-bp fragment of the κ-casein gene produced a clear and distinct band on the gel, which is of major importance for successful digestion step of PCR products (Figure 1). Digestion of the PCR products with endonuclease HinfI generated presence of three fragments (Table 1).

The genotype AB had highest frequencies of all genotypes in the investigated breeds: 0.501 in Brown Swiss and Busha and 0.500 in Gray cattle. The frequency of BB genotype was higher in Brown Swiss than in Busha breed and Gray cattle (0.356 and 0.233, 0.264, respectively).

According to χ2 test, all breeds were in Hardy-Weinberg equilibrium, suggesting that the κ-casein gene in investigated population was not influenced by selection (Table 2). Higher share of observed AB and BB genotypes determined in Brown Swiss breed could be a consequence of use of homozygous bulls (semen) to "desirable" allelic polymorphic B form. The frequency of AA genotype of Brown Swiss breed was the lower (0.162), than for Busha and Gray cattle (0.267 and 0.236).

Many other authors (Taha and Punan, 1993, Dogru and Ozdemir, 2009, Dokso *et al.,* 2014, Djedović *et al.,* 2015) found that the frequency of the AB genotype in the Brown Swiss breed was higher compared to other genotypes, while Potočnik (2015) determined a higher share of BB genotypes compared to AA and AB in this breed. The higher frequency of BB genotype in Jersey cattle (0.772) with absent of opposite AA genotype is reported by Ren *et al.,* 2011.

Table 1. Expected /observed fragment sizes (in base pairs, bp) after restriction PCR products with *Hinf*I

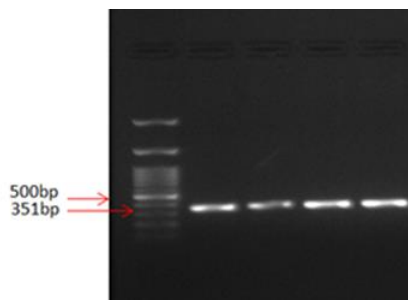| PCR Product (bp)	Restriction Fragments Sizes (bp) and determined genotypes		
	AA	AB	BB
351	131; 89	261; 131; 89	261; 89

Figure 1. PCR products of κ-casein gene polymorphism visuelised on 1% agarose gel
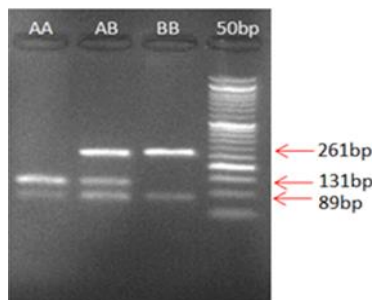

Figure 2. PCR-RFLP of κ-casein gene using *Hinf* I on 3% agarose gel

Table 2. Frequencies of genotypes and alleles and Hardy Weinberg equilibrium test for κ-casein locus of Brown Swiss, Busha and Gray cattle breeds

Breed	N	Parameters	Kappa – casein genotypes			Allele frequencies		χ^2
			AA	AB	BB	A	B	
Brown Swiss	31	Ho	6	13	12	0.403	0.597	0.513
		He	5.04	14.91	11.04			
		Frequency	0.162	0.501	0.356			
Busha	30	Ho	8	15	7	0.517	0.483	0.000
		He	8.01	14.98	7.01			
		Frequency	0.267	0.501	0.233			
Gray cattle	35	Ho	7	20	8	0.486	0.514	0.723
		He	8.26	17.49	9.26			
		Frequency	0.236	0.500	0.264			

Ho: observed heterozygotes, He: expected heterozygotes on the basis of Hardy-Weinberg, χ2 =chi-square value

Similar to our research, Djedović *et al.* (2015), Maletić *et al.* (2016), Brka *et al.* (2010) also obtained that heterozygote genotype of κ-casein (AB) was dominant in Busha breed. Only in the research of Ivanković *et al.* (2011) determined dominance of BB homozygote. Brka *et al.* (2010) found BC genotype at a low frequency (0.143), which was not found in Busha cattle in Montenegro.

For high productive Holstein Friesian breed some authors (Volkandari *et al.,* 2017, Tanaskovska *et al.,* 2016, Khaizaran and Razem, 2014, Dokso *et al.,* 2014, Ren *et al.,* 2011) determined higher frequency of AA genotype than AB and BB genotypes, what is similar to result obtained for Brown Swiss breed. Other authors, as Doosti *et al.* (2011), Gouda *et al.* (2013), Maletić *et al.* (2016) reported for Holstein breed the highest frequency of AB genotype κ-casein.

Different to our results, Djedović *et al.* (2015), Ivanković *et al.* (2011), Dokso *et al.* (2014), Taha and Punan (1993) identified very low frequency of BB homozygous of κ-casein gene for Simmental breed, predominantly lower compared to AA and AB genotypes. The frequency of BB genotype in the Simmental breed ranged from 0.096 (Djedović *et al.,* 2015) to 0.220 (Taha and Punan, 1993).

On the other side, the results of genotyping k-caseine of Czech Fleckvieh, Bulgarian Rhodopean Cattle, Shorthorn Rhodopean Cattle as old autchthonous breed (Kučerová *et al.,* 2006 and Hristov *et al.,* 2012), as well as results for Gatačko cattle reared in Bosnia and Herzegovina (Brka *et al.,* 2010) show strong

domination of the AB genotype, as it obtained for Gray cattle breed in Montenegro.

Table 3. The frequency of genotypes κ-casein in some cattle breeds reported by other authors)

Breed	Frequency of k-casein genotypes			Source
	AA	AB	BB	
Brown Swis (Slovenia)	0.069	0.387	0.544	Potočnik, 2015
Swiss Brown	0.279	0.480. AC 0.039	0.167. BC 0.034	Taha *et al.,* 1993
Crosses Brown cattle x American Brown Swiss	0.168	0.466. AC 0.016	0.334. BC 0.016	Taha *et al.,* 1993
Brown Swiss	0.194	0.602	0.204	Dogru and Ozdemir, 2009
Brown Swiss	0.392	0.449	0.159	Dokso *et al.,* 2014
Busha	0.417	0.500	0.083	Djedović *et al.,* 2015
Busha	0.444	0.555	0.00	Maletić *et al.,* 2016
Busha	0.206	0.294	0.500	Ivanković *et al.,* 2011
Busha	0.286	0.357	0.214. BC 0.143	Brka *et al.,* 2010
Gatačko*	0.308	0.538	0.154	Brka *et al.,* 2010

*Gatačko cattle is local name for population of Gray cattle breed reared in South East Bosnia and Herzegovina
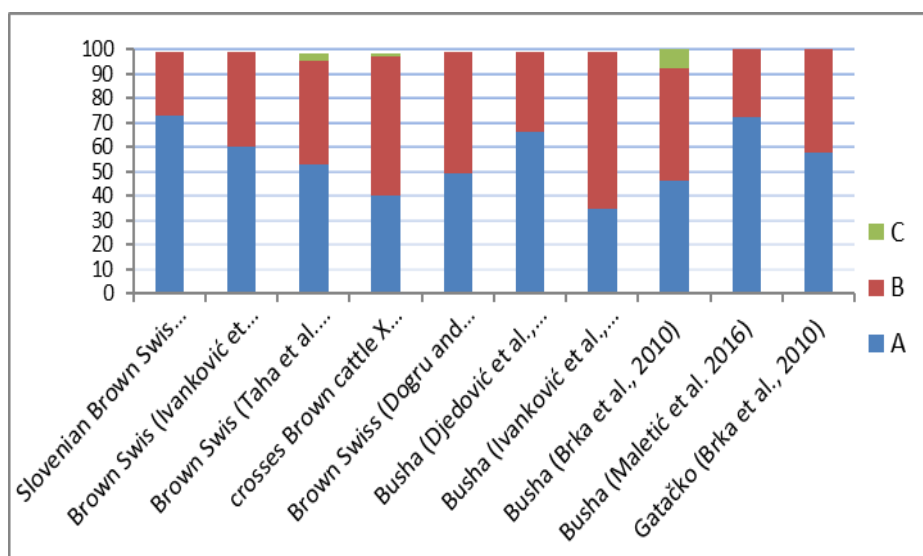
Graph 1. Percentage of allelic CSN3 variants in cattle breeds (different authors)

In regarding allele frequencies of κ-casein gene for all three investigated breeds, slightly higher frequencies of A allel than B allele were determined. It was for Busha breed 0.517, Brown Swiss breed 0.597 and for Gray cattle 0.514.

Brka *et al.* (2010) obtained the similar ratio of A and B allele for Gatačko cattle (A - 0.58, B - 0.42) and Dogru and Ozdemir (2009) for Brown Swiss breed reared in Turkey. Very high frequency of allele A was reported by Potočnik (2015) for population of Brawn Swis in Slovenia (0.737) and Ivanković *et al.* (2011) for Brown Swiss in Croatia (0.607), Graph 1.

The higher frequency of A allele than allele B of Montenegrin Busha breed in our present study is in agreement with the earlier observations for Busha breed reared in other countries of Western Balkan, as it was reported for Busha in Serbia (Djedović *et al.* 2015, Maletić *et al.,* 2016) and for Busha reared in Bosnia and Herzegovina (Brka *et al.,* 2010). However, our results are not in accordance with the results for Busha breed in Croatia where lower frequency of A than B allele was found (Ivanković *et al.,* 2011).

Allele B of κ-casein was a favorable in cattle population because it significantly affected both milk and milk protein yield of Holstein and Native Iranian breed (Doosti *et al.,* 2011). Kučerová *et al.* (2006) also reported higher frequency of A allele (0.62) than allele B (0.38) in population of Czech Simmental cattle breed. Similar results for allele and genotype frequencies found in Czech Simmental (Fleckvieh) cattle population Kučerová *et al.* (2006), Holstein Friesian cattle in Macedonia and for some commercial cattle breeds in Croatia (Tanaskovska *et al.,* 2016, Ivanković *et al.,* 2011).

CONCLUSIONS

The results of PCR-RFLP analysis showed that three genotypes (AA, BB and AB) were found in the population studied for κ-casein gene. The results of very high frequency of B allele in Brown Swiss cattle implied that this breed should have a good performance in the milk traits related to the B allele of κ-casein gene. Hence, future studies should be focused on the association between cattle genotype and milk composition characteristics.

Determination of A and B allele frequencies in population of Busha and Grey cattle breeds could be used in the programs of selection and preservation, as important animal genetic resources in Montenegro, in order to increase frequency of desired alleles and genotypes.

Knowledge about genetic profile of breeds due to studied polymorphic variants of milk proteins is useful in further breeding development and economic valorization of cattle breeds, especially autochthonous and local ones.

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