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Genetic polymorphism of milk proteins in Holstein cattle population from Serbia

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INTRODUCTION

Previous research has shown that genetic polymorphism of milk proteins significantly affects the quality of milk, milk yield, and composition. Cows with the BB genotype of κ-casein and β-lactoglobulin produce milk that is more favorable for cheese production, compared to cows of the AA genotype. Consumption of milk from cows that possess the A1 allele of the β-casein gene can have negative health effects on human health. For this reason, the aim of this study is to examine the polymorphisms of genes encoding the synthesis of κ-casein, β-casein, and β-lactoglobulin in the population of the Holstein cattle breed from Serbia.

MATERIALS AND METHODS

In order to determine polymorphism of milk proteins, blood samples from 32 Holstein Friesian cows were taken. Blood was collected using a vacutainer and then stored at a temperature of 4 °C until DNA extraction.

Isolation of DNA from blood was performed using a method based on the use of silica membrane columns. The GenElute™ Blood Genomic DNA Kit, manufactured by Sigma-Aldrich (Merck), was used for isolation. The entire isolation procedure was carried out according to the instructions specified by the manufacturer.

Checking the quantity and quality of isolated DNA was performed using a NanoPhotometer N60 spectrophotometer. In order to evaluate the purity of the isolated DNA, the absorption ratios A260/A280 and A260/A280 were calculated.

The agarose-gel electrophoresis method was used to check the integrity of isolated DNA, the length of PCR products and the length of fragments after restriction digestion of PCR products.

Amplification of the three investigated genes was carried out using the PCR reaction. The total lengths of the amplified genes for κ-casein (exon IV), β-casein (exon VII), and β-lactoglobulin (parts of exon IV and intron IV) genes were 453, 251, and 247 base pairs (bp), respectively. For β-casein gene amplification, primers designed to create amplification-created restriction sites (ACRS) were used. The primers used for gene amplifications in this research are shown in Table 1.

Table 1. The primers used for gene amplifications

κ-casein	Forward – CASKF: 5'-TGTGCTGAGTAGGTATCCTAGTTATGG-3' Reverse – CASKR: 5'-GCGTTGTCTTCTTTGATGTCTCCTTAG -3'
β-casein	Forward – CASB122: 5' – GAGTCGACTGCAGATTTTCAACATCAGTGAGAGTCAGGCCCTG - 3' Reverse – CASB67: 5' - CCTGCAGAATTCTAGTCTATCCCTTCCCTGGGCCCATCG - 3'
β-lactoglobulin	Forward – JBLG2: 5' - TGTGCTGGACACCGACTACAAAAAG - 3' Reverse – JBLG3: 5'- GCTCCCGGTATATGACCACCCTCT - 3'

Determination of polymorphisms of three genes was performed using the Restriction fragment length polymorphisms (RFLP) method. After amplification, DNA was cut using restriction enzymes according to the manufacturer's instructions (New England Biolabs). Table 2 shows for each gene the restriction enzymes that were used, the DNA cutting sites, possible fragments resulting from cutting and possible genotypes that are determined based on the specific combination of fragments.

Table 2. Parameters used for RFLP analysis

Gene	Restriction enzyme	Cutting site	Possible fragment lengths (bp)	Possible genotypes
κ-casein	*HinfI*	5'-G/ANTC-3'	426, 326, 100 and 27	326, 100, 27 (AA) 426, 326, 100, 27 (AB) 426, 27 (BB)
β-casein	*TaqI-v2*	5'-T/CGA-3'.	251, 213 and 38	213, 38 (A1A1) 25, 213, 38 (A1A2) 251 (A2A2)
β-lactoglobulin	*HaeIII*	5'-GG/CC-3'	148, 99 and 74	148, 99 (AA) 148, 99, 74 (AB) 99, 74 (BB)

Statistical analysis of the data was performed using the "R Programming Language and Environment for Statistical Analysis" (Version 4.0.0) (R Foundation for Statistical Computing, R Core Team). Allele frequencies and genotype frequencies were calculated. The exact test was used to calculate whether the population for the examined loci is in Hardy-Weinberg equilibrium, using the "HWExact" function from the "HardyWeinberg" package (Graffelman, 2015).

RESULTS AND DISCUSSION

DNA was detected in each of the samples. The average DNA concentration in tested isolates was 27.7 ng/µl, and the average value of the A260/A280 ratio was 1.74. After agarose gel electrophoresis, it was determined that the DNA was visible and complete. The results for each gene are presented separately below.

κ-casein

After checking through agarose-gel electrophoresis, it was determined that out of a total of 32 samples, 31 samples were successfully amplified. The restriction digestion reaction was also successful with the same number of samples. After cutting with the restriction enzyme *HinfI*, three fragments of lengths 426, 326 and 100 bp were visible on the gel. The 27 bp fragment was not visible on the gel, due to its short length.

In the tested sample of cattle, genotype AB had the highest frequency (0.51), and genotype BB had the lowest frequency (0.10). Allele A with a frequency of 0.64 was more prevalent than allele B, whose frequency was 0.36

Given that the p-value of the exact test for determining Hardy-Weinberg equilibrium was 0.6987 (p>0.05), it was concluded that the studied population for this locus was in Hardy-Weinberg equilibrium. All results for κ-casein locus are shown in the table 3.

Table 3. Results of PCR-RFLP analysis for the κ-casein locus

κ-casein	Genotypes			Alleles		HWE, p-value
	AA	AB	BB	A	B	
Sum	12	16	3	40	22	0,6987
Frequency	0.39	0.51	0.10	0.64	0.36	

β-casein

After control using agarose-gel electrophoresis, it was determined that out of a total of 32 samples, 27 samples were successfully amplified. After restriction digestion of the PCR product with the enzyme *TaqI-v2 and* agarose-gel electrophoresis, only fragment 251 was visible. This means that the restriction endonuclease did not find a site for cutting and the fragments remained intact. Based on this, it can be concluded that all cows are of the A2A2 genotype and that only one allele (A2) is present, and for that reason there was no need for by counting the frequencies and checking the Hardy-Weinberg equilibrium.

β-lactoglobulin

Using agarose-gel electrophoresis for verification, it was determined that out of a total of 32 samples, 29 samples were successfully amplified. The restriction digestion reaction was also successful with the same number of samples. After cutting with the *HaeIII* restriction enzyme and and agarose-gel electrophoresis, presence of three DNA fragments of lengths 148, 99 and 74 was observed.

In the tested sample of cattle, genotype AB had the highest frequency (0.41), and genotype BB had the lowest frequency (0.24). Allele A with a frequency of 0.55 was more prevalent than allele B, whose frequency was 0.45. Given that the p-value of the exact test for determining Hardy-Weinberg equilibrium was 0.4545 (p>0.05), it was concluded that the studied population for this locus was in Hardy-Weinberg equilibrium. All results for β-lactoglobulin locus are shown in the table 4.

Table 4. Results of PCR-RFLP analysis for the β-lactoglobulin locus

β-lactoglobulin	Genotypes			Alleles		HWE, p-value
	AA	AB	BB	A	B	
Sum	10	12	7	32	26	0,4545
Frequency	0,35	0,41	0,24	0,55	0,45	

CONCLUSIONS

In the examined population, it was found that the genotype frequencies of κ-casein were as follows: AB (0.51), AA (0.39), and BB (0.10). The frequency of alleles A and B was 0.64 and 0.36, respectively. After analysis of the β-casein locus, it is determined that only the A2A2 genotype was present in the examined cows. Analysis of the β-lactoglobulin locus showed that the heterozygous AB genotype was the most common with a frequency of 0.41. It was followed by the AA and BB genotypes with a frequency of 0.35 and 0.24, respectively. The frequency of alleles A and B was 0.55 and 0.45, respectively, which indicates high variability in the study population. Using an exact test, it was determined that the population was in Hardy-Weinberg equilibrium at the loci for κ-casein and β-lactoglobulin. Equilibrium was not calculated for β-casein because only allele A2 was present. Although a relatively small sample was used, this research is a good basis for further, more extensive research of this type and selection in the Holstein cattle population from Serbia.

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