Original Paper

The Genotypic Profile of Milk Proteins in Cattle Populations Created on the Mother Base of the Lebedinian Breed

Alona Malikova[1], Yuliia Pavlenko[1], Yuriy Skliarenko*[2], Tatyana Chernyavska[1], Olha Korzh[1], Marina Shkurko[1]
[1]*Sumy National Agrarian University, Ukraine*
[2]*Institute of Agriculture in the North East of the National Academy of Agrarian Sciences of Ukraine, Ukraine*

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A study of polymorphism of individual genes of milk proteins (kappa-casein and beta- casein) was carried out on the population of cows of the ukrainian brown dairy breed and the sumy interbreed type of the ukrainian black and white dairy breed. Animals of the ukrainian brown dairy breed prevailed in terms of BB genotypes, both for the kappa-casein and genotypes A2A2 for beta-casein genes. According to the complex genotypes of kappa-casein and beta-casein, a difference was established between the animals of the studied breeds. In animals of the ukrainian brown dairy breed a higher frequency is characteristic of the complex genotype A2A2/BB (0.32), and in cows of the sumy interbreed type of the ukrainian black and white dairy breed – A2A2/AA (0.24) and A1A2/AA (0.24).

Keywords: genetic polymorphism, kappa- casein, beta- casein, genotype frequency, cattle breeds

1 Introduction

Modern methods of selection are aimed at the formation in animals of the herd of expressiveness of the production direction of the breed, high metabolic status of the organism, strength of the body structure and high natural resistance. This requires the introduction of new science-based genetic methods for evaluating and selecting animals, because increasing the genetic potential of cattle is largely determined by the availability of information on the genetic nature of productivity traits. Therefore, modern genetic research is aimed, in particular, at identifying the association of polymorphism of allelic variants of milk protein genes (Gubarenko, 2020).

Such genetic markers include genes for: κ-casein (CSN3), which is responsible for the technological properties of milk; β-casein (CSN2), which affects the digestibility of milk and can cause certain diseases in humans (Mitioglo et al., 2021; Ramakrishnan et al., 2023).

Over the past 20 years, scientists around the world have repeatedly studied the impact of the A1 genetic variant of the beta-casein protein on human health. It was established that an opioid-like peptide – casomorphin-7 (-CM-7) is released during its digestion. It takes part in the dysregulation of many physiological processes in the human body. Studies conducted by scientists indicate a negative effect of the A1 variant of beta-casein on digestion indicators and cognitive signs in children. It is considered desirable to eliminate the A1 allele in dairy cattle populations and promote the production of dairy products based on the milk of cows with the A2A2 genotype (Cie´sli´nska et al., 2022).

The studies of many researchers established a significant difference in the frequencies of kappa-casein and beta-casein genotypes between animals of different breeds. Thus, the frequency of beta-casein genotypes in ukrainian red and white dairy breed cows was, respectively 13% A1A1, 61% A1A2 and 26% A2A2, of ukrainian black and white dairy breed – 42% A1A1, 48% A1A2, 10% A2A2 (Kulibaba et al., 2023), holstein breed – 0–15% A1A1, 30–60% A1A2, 20–40% A2A2 (Mumtaz et al., 2021; Antonopoulos et al., 2021).

***Corresponding Author:** Yuriy Skliarenko, Sumy National Agrarian University, Herasima Kondratieva St, 160, Sumy, Sumy Oblast, Ukrajina, 40000, Ukraine, sklyrenko9753@ukr.net

The frequency of kappa-casein genotypes in holstein cows was, respectively 56.8% AA, 39.8% AB and 3.4% BB (Pilonetto et al., 2022), of simmental breed – 35.8% AA, 51.6% AB and 14.6% (Niksic et al., 2018), busha – 44.4% AA, 55.6% AB (Maletić et al., 2016), swiss cattle – 38.6% AA, 44.3% AB and 17.1% BB, slavonsko-srijemski podolac – 9.1% AA, 13.6% AB and 77.3% BB, istrian cattle – 18.5% AA, 66.7% AB and 17.8% BB (Ivanković et al., 2011).

The lebedinian breed has been bred for more than a hundred years in the Sumy region of Ukraine. The creation of this breed was started at the beginning of the 20th century, by crossing local cattle with breeders of the swiss breed (Sklyarenko et al., 2018).

The ukrainian brown dairy breed was created by crossing cows of the Lebedinian breed with breeders of the swiss breed of American selection. The sumy intrabreed type of the ukrainian black and white dairy breed was created by transforming lebedinian cattle using Holstein breeders (Ladyka et al., 2019). Scientists have established that animals of the lebedinian breed have a frequency of 20% of the BB genotype for the kappa-casein gene, and 74% of the A2A2 genotype for the beta-casein gene (Eremenko & Oblyvantsov, 2004). The polymorphism of the kappa-casein and beta-casein genes in breeding formations created on the basis of the Lebedinian breed remains unexplored.

The objective of our study was to evaluate the cattle populations created on the mother base of the lebedinian breed according to the individual milk protein genes

2 Material and Methods

Cows were genotyped according to kappa-casein and beta-casein genes. The experimental herd consisted of: ukrainian brown dairy breed (UBD) (n = 50), sumy interbreed type of the ukrainian black and white dairy breed (SITUBWD) (n = 50). All experimental animals belong to State Enterprise Research Farm of the Institute of Agriculture of Northern East of National Academy of Agrarian Sciences (Sumy district of Sumy region).

Monovets were used to collect blood samples. The amount of blood collected was 3 ml from each animal. Potassium salt was used as an anticoagulant. After blood sampling, the samples were stored at -20 °C. Isolation of DNA samples was carried out according to the appropriate protocol, using equipment of Monarch® Genomic DNA Purification Kit New England BioLab (USA).

TaqMan®Custom was used to perform allelic discrimination. The TaqMan® SNP Genotyping Assays use TaqMan® 5´-nuclease chemistry for amplifying and detecting specific polymorphisms in purified genomic DNA samples. All assays are developed using Life Technologies robust bioinformatics assay design process relying on a pipeline using heuristic rules deduced from both manufacturing and assay performance data. These assays use TaqMan® minor groove-binding (MGB) probes for superior allelic discrimination, improved signal-to-noise ratios, and design flexibility. TaqMan real-time PCR Two primers were designed to amplify the 101 bp product involving SNP rs43703011 (genomic DNA: X14711 (http://www.ncbi.nih.gov); forward primer, 5´-AAG CAG TAG AGA GCA CTG TAG CTA-3´; reverse primer, 5´-TGA TCT CAG GTG GGC TCT CAA TAA-3´). Two fluorogenic TaqMan probes were designed with different fluorescent dye reporters to allow single-tube genotyping. The first probe was targeted at the wild type allele A (5´-VIC-CTTCTGGAGAAGCTTCTA-3´) and the second one at the mutated allele B (5´-FAM-CTTCTGGAGAATCTTCTA-FAM-3´) of the CSN3 gene. The NFQ quencher was linked to the 3´ end of both probes. Primers and probes were designed using Primer Express software, version 3.0 (Applied Biosystems, CA, USA) and were obtained from Applied Biosystems. The accuracy of the used sequence source was verified by comparison with sequences from the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Real-time PCR was performed in 20 µl reactions with 10 µl of TaqMan universal PCR master mix containing AmpliTaq Gold DNA Polymerase (Applied Biosystems, CA, USA), 200 nM concentration of forward and reverse primer, 100 nM of each probe and 2 µl (50–100 ng) of sample DNA. The PCR reaction was obtained using the FAST 7500 Real Time PCR System (Applied Biosystems). The time and temperature profile of the PCR reaction consisted of the following steps: 2 min at 50 °C for UNG activation, 10 min at 95 °C for starting AmpliTaq Gold activity, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. As a negative control, we used a sample without a template. An allelic discrimination experiment consisted of three steps: a pre-read run, an amplification run and a post-read run. Each sample was visually verified by analysing the generated PCR curves. Analyses of amplification products were performed using SDS software, version 4.2 (Ladyka et al., 2022).

The TaqMan@Genotyping real-time PCR system was used to perform allelic discrimination. Two primers were designed to amplify the 101 bp product involving SNP rs43703011 (genomic DNA: X14711 (http://www.ncbi.nih.gov); forward primer, 5´-CCCAGACACAGTCTCTAGTCTATCC-3´; reverse primer, 5´-GGTTTGAGTAAGAGGAGGGATGTTT-3´). Two fluorogenic TaqMan probes were designed with different fluorescent dye reporters to allow single-tube genotyping. The first probe was targeted to the wild type allele A (5´-VIC-CCCATCCATAACAGCC-3´) and the second one to the mutated allele B (5´-FAM-CCATCCCTAACAGCC-

FAM-3´) of the CSN2 gene. The powerful NFQ quencher was linked to the 3´ end of both probes. Primers and probes were designed using Primer Express software, version 3.0 (Applied Biosystems, CA, USA) and were obtained from Applied Biosystems. The accuracy of the used sequence source was verified by comparison with sequences from the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Real-time PCR was performed in 20 µl reactions with 10 µl of TaqMan universal PCR master mix containing AmpliTaq Gold DNA Polymerase (Applied Biosystems, CA, USA), 200 nM concentration of forward and reverse primer, 100 nM of each probe and 2 µl (50–100 ng) of sample DNA. The PCR reaction was realized using the FAST 7500 Real Time PCR System (Applied Biosystems). The time and temperature profile of the PCR reaction consisted of the following steps: 2 min at 50 °C for UNG activation, 10 min at 95 °C for starting AmpliTaq Gold activity, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. As a negative control, we used a sample without template. An allelic discrimination experiment consisted of three steps: a pre-read run, an amplification run and a post-read run. Each sample was visually verified by analyzing the generated PCR curves. Analyses of amplification products were performed using SDS software, version 4.2 (Ladyka et al., 2023).

Statistical analysis was performed in the R (www.R-project.org, V.4.0).

3 Results and Discussion

We have conducted studies to determine the genotype of animals of the studied breeds by the capa-casein gene. The figures show amplification curves and allelic discrimination by polymorphism of the CSN3 gene genotype (Figs 1 and 2).

According to the results of our research, it was established that the desired BB genotype of the kappa-casein gene was more common in cows of the UBD breed (0.34). Animals of this breed are also characterized by a higher frequency of heterozygous AB genotype (0.44). Animals of the SITUBWD are characterized by a higher frequency of homozygous AA genotype (0.54).

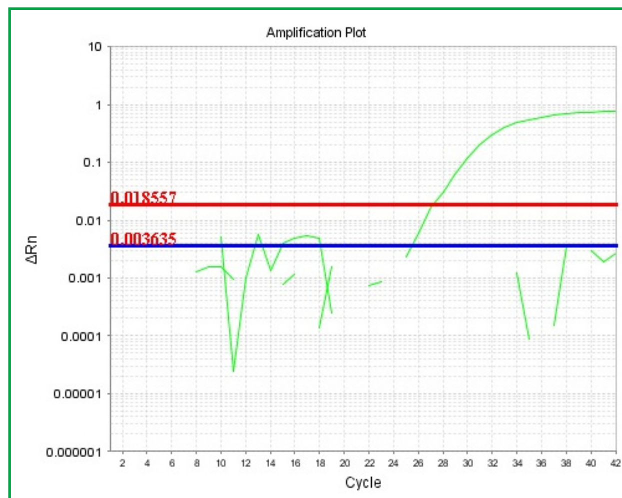

Fig. 1 Amplification curves of genotype determination by CSN3 gene

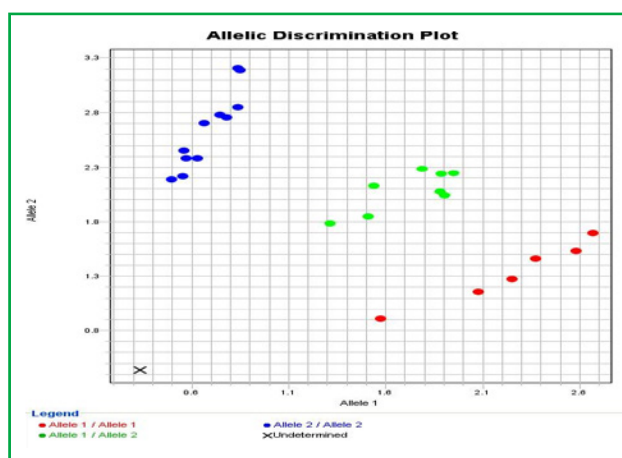

Fig. 2 Allelic discrimination by genotypes polymorphism of the CSN3 gene

Table 1 Frequency of alleles and genotypes by locus of the kappa-casein gene in cows

Distribution*	Genotype						Allele (pcs.)		χ^2
	AA		AB		BB		A	B	
	n	frequency	n	frequency	n	frequency			
UBD									
A	11	0.22	22	0.44	17	0.34**	0.44 ±0.05	0.56 ±0.05	0.57
T	–	0.20	–	0.49	–	0.31			
SITUBWD									
A	27	0.54	17	0.34	6	0.12a	0.71 ±0.05	0.29 ±0.05	1.52
T	–	0.50	–	0.41	–	0.09			

* A – actual number of genotypes; T – theoretical number of genotypes; **P – level of significance according, to Fisher's test: P <0.01; a – the difference is likely to be relative to the indicator indicated by the superscript

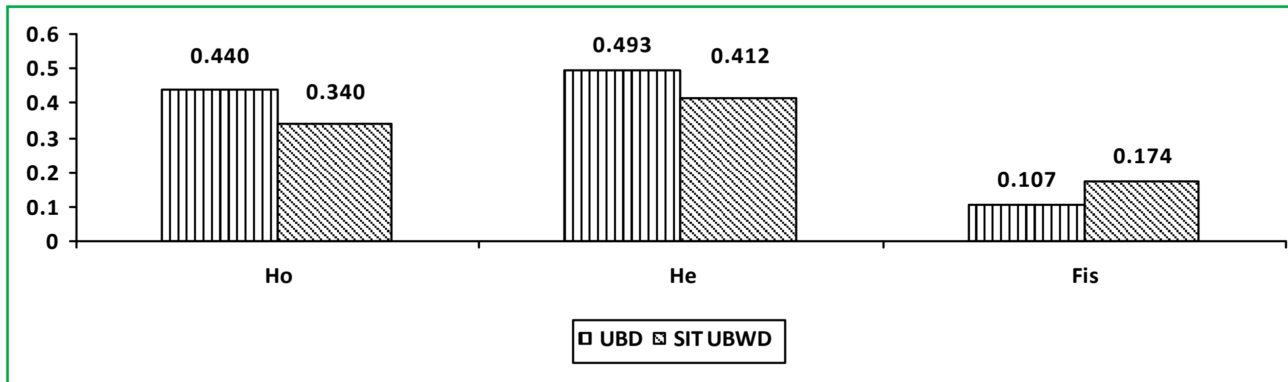

Fig. 3 Heterozygosity and fixation index for the kappa-casein gene in the studied animals
Ho – actual heterozygosity, He – expected heterozygosity, Fis – fixation index

As a result, the frequency of the desired B allele was higher in animals of the UBD breed (0.56). In animals of the SITUBWD, the frequency of allele A was 0.71. The frequency of B alleles is 1.3 times higher in animals of the UBD, and the frequency of the A allele is 2.5 times higher in animals of the SITUBWD. It was established that allele frequencies in animals of both studied breeds are statistically significant (reliability criterion tA and tB >3) (Table 1).

The degree of conformity of the actual distribution of genotypes with the expected values was determined by the use of criterion χ^2. It is found that the actual distribution of the number of different phenotypes corresponds to the theoretically expected one with a high degree of reliability (*P* <0.01).

In animals of both studied breeds, the actual heterozygosity was lower than expected. This confirms the positive value of the fixation index (Fig. 3).

The figures show the amplification curves and allelic discrimination by the CSN2 gene polymorphism (Figs 4 and 5).

According to the results of the genotyping of animals for the beta-casein gene, it was established that the homozygous A1A1 genotype was more common in cows of the SITUBWD (0.26). UBD cows were characterized by a higher frequency of another homozygous A2A2 genotype (0.60). The proportion of heterozygous A1A2 genotypes was approximately the same in animals of both studied breeds (0.34 and 0.40, respectively).

According to the results of the calculations, it was established that the share of allele A1 was greater in cows of the SITUBWD (0.46), and allele A2 – of the UBD (0.77). The frequency of A2 alleles is 3.3 times greater in animals of the UBD, and in animals of the SITUBWD – 1.2 times. It was established that the frequencies of alleles in animals

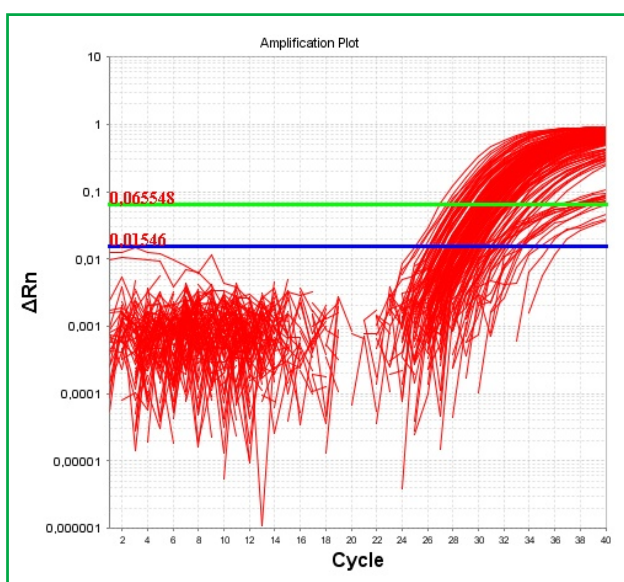

Fig. 4 Amplification curves of genotype determination by CSN2 gene polymorphism (rs43703011)

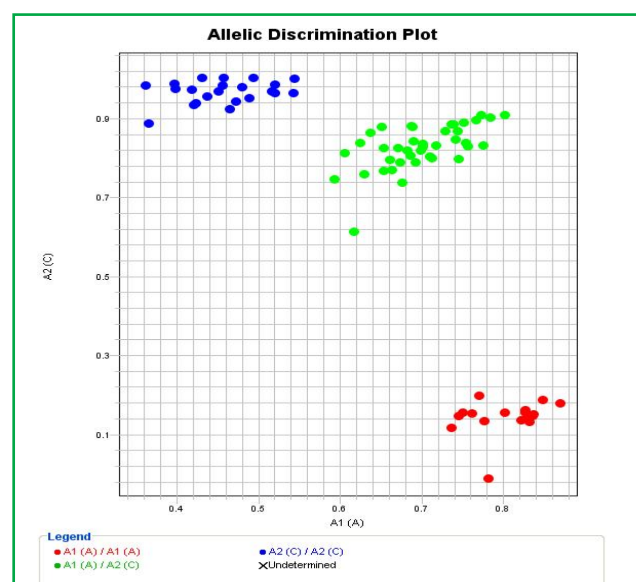

Fig. 5 Allelic discrimination by genotypes polymorphism of the CSN2 gene (rs43703011)

Table 2 Frequency of alleles and genotypes by locus of the beta-casein gene in cows

Distribution*	Genotype						Allele (pcs.)		χ^2
	A1A1		A1A2		A2A2		A1	A2	
	n	frequency	n	frequency	n	frequency			
UBD									
A	3	0.06	17	0.34	30	0.60**	0.23 ±0.04	0.77 ±0.04	0.08
T	–	0.05	–	0.36	–	0.59			
SITUBWD									
A	13	0.26	20	0.40	17	0.34a	0.46 ±0.05	0.54 ±0.05	1.89
T	–	0.21	–	0.50	–	0.29			

* A – actual number of genotypes; T – theoretical number of genotypes; **P – level of significance according, to Fisher's test: P <0.01; a – the difference is likely to be relative to the indicator indicated by the superscript

Table 3 Frequency of complex genotypes of CSN2/CSN3 genes

Combinations of CSN2 and CSN3 milk protein genotypes(CSN2/CSN3)	Breed	
	UBD	SITUBWD
A1A1/AA	0.02	0.06
A1A1/AB	0.02	0.14
A1A1/BB	0.02	0.06
A1A2/AA	0.14	0.24
A1A2/AB	0.20	0.12
A1A2/BB	0.00	0.04
A2A2/AA	0.06	0.24
A2A2/AB	0.22	0.08
A2A2/BB	0.32	0.02

of both studied breeds are statistically significant (criterion of reliability tA_1 i tA_2 >3) (Table 2).

The degree of conformity of the actual distribution of genotypes with the expected values was determined by the use of criterion χ^2. It is found that the actual distribution of the number of different phenotypes corresponds to the theoretically expected one with a high degree of reliability (P <0.01).

The actual heterozygosity in animals of both studied breeds for the beta-casein gene was higher than expected. Accordingly, the fixation index had a positive value (Fig. 6).

Between the animals of both researched breeds, a difference was found in the proportion of complex genotypes for beta-casein and kappa-casein. Complex genotypes were more common in animals of the UBD A2A2/BB (0.32), A2A2/AB (0.22), A1A2/AB (0.20). Whereas

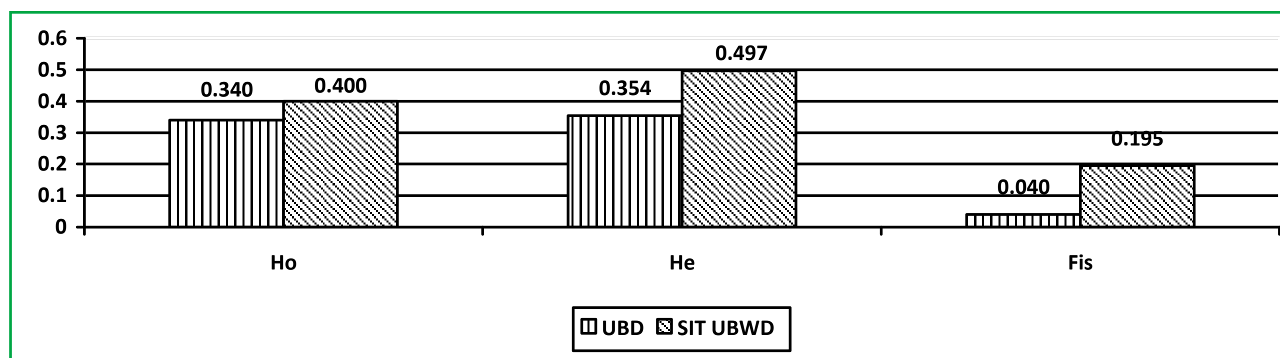

Fig. 6 Heterozygosity and fixation index for the beta-casein gene in the studied animals
Ho – actual heterozygosity, He – expected heterozygosity, Fis – fixation index

in cows of the SITUBWD – A2A2/AA (0.24), A1A2/AA (0.24) and A1A1/AB (0.14). In animals of the UBD, the complex A1A2/BB genotype was not found (Table 3).

Our study showed that the beta-casein A2 allele was more common in the UBD cattle population (0.77) compared to the A1 allele (0.23). In the SITUBWD animals, the frequency of the A2 allele (0.54) was higher than the frequency of the A1 allele (0.46). These results are almost in line with previously published studies, in which the frequency of the A2 allele in the Swiss breed was about 0.80, while in the Holstein breed the frequency of the A1 allele was 0.42–0.51 (Antonopoulos et al., 2021; Cie´sli´nska et al., 2022).

The frequency of the B allele of the kappa-casein gene was higher in UBD animals (0.56) compared to SITUBWD animals (0.29). The obtained results are in line with the previously reported data, in which the frequency of the B allele in Swiss breed animals was (0.60), and in Holstein breed animals (0.35) (Ladyka et al., 2022).

4 Conclusions

Interbreed differentiation by polymorphism of the kappa-casein and beta-casein genes was established. UBD animals are distinguished by a higher frequency of the desired BB genotype and the B allele of the kappa-casein gene, and the A2A2 genotype and the A2 allele for beta-casein.

It was found that general population genetic equilibrium among the studied species coincides with other researchers' data and indicates a lack of directed selection and selection on these genotypes.

According to the complex genotypes of beta-casein and kappa-casein a difference was established between the animals of the studied breeds.

Further research should be directed to researching the genotypes of the breeders used in the brood stock in order to obtain animals with the desired genotype.

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