

The comparison of CSN2 (rs43703011) beta-casein gene options frequencies in different breeds of Ukraine cows and the prospect of creating herds with the A2/A2 genotype

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Abstract

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The purpose of this study was to perform genotyping of different Ukrainian breeds for the CSN2 gene (rs43703011), calculation, evaluation, and analysis of genotype and allele frequencies.

Using real-time polymerase chain reaction methods, we have researched the CSN2 polymorphism (GenBank M55158; SNP rs43703011) gene in 4 breeds of cows in the North-East of Ukraine. The observed distributions of genotype frequencies A1/A1, A1/A2, A2/A2 and A1 or A2 alleles varies considerably in different cattle breeds: Lebedyn cattle – 14.6%, 31.7%, 53.7% (0.3 and 0.7); Ukrainian brown dairy breed – 6.8%, 43.2%, 50% (0.28 and 0.72); Simmental cattle – 7.3%, 51.2%, 41.5% (0.33 and 0.67); Ukrainian black speckled milky – 38.5%, 34.6%, 26.9% (0.56 and 0.44). The ratio of genotypes and alleles were found significant differences when comparing Lebedyn cattle and Simmental cattle ($p = 0.01$), Lebedyn cattle and Ukrainian black speckled milky ($p < 0.0001$), Lebedyn cattle compared to all other breeds ($p = 0.04$), Ukrainian brown dairy breed and Ukrainian black speckled milky ($p < 0.0001$), Ukrainian brown dairy breed compared to all other Ukrainian breeds ($p = 0.008$); Simmental cattle and Ukrainian black speckled milky ($p < 0.0001$), Simmental cattle ($p = 0.002$) and Ukrainian black speckled milky ($p < 0.0001$) to all other breeds. The obtained results indicate the prospects of breeding work to create herds with genotype A2/A2 animals – specifically with the Lebedyn cattle.

Keywords: genetic polymorphism; beta-casein A2; genotype frequency; cattle breeds

Introduction

In all developed countries of the world, genotyping of cattle breeds has become routine practice. The search for gene variants that determine the formation of the optimal phenotype of a cow for industrial use is focused primarily on pairs of genes, for which significant associations

milk proteins: beta-casein (CSN2), kappa-casein (CSN3), beta-lactoglobulin (BLG) (Adamov et al., 2011; Askari et al., 2011; Molee et al., 2015). These include polymorphism of beta-casein, the second most abundant protein in cow's milk representing 27% of total protein, which is being studied most extensively given its medical significance. It is encoded by the CSN2 gene mapped on chromosome 6q31

(Gene ID: 281099) and consists of the 209-amino-acid single polypeptide chain and molecular mass of about 24 kDa (Barowska et al., 2012; Bech & Kristiansen, 1990). Beta-casein is expressed as 13 genetic variants (Anggraeni et al., 2010), which is the result of single nucleotide polymorphisms (SNP) in the CSN2 gene (Kyselová et al., 2019). The most frequent genetic variants in western dairy breeds are BC A1 and BC A2 (Nilsen et al., 2009). SNP of CSN2 exon VII allele A2 (201-CCT-203, GenBank: JX273429.1) and allele A1 (201-CAT-203, GenBank: JX273430.1) (Nilsen et al., 2009) produces the substitution of proline 67 (Pro67) of BC A2 for histidine (His67) in BC A1 (Costa et al., 2016; Molee et al., 2015).

The β -casein fraction is the most common protein fraction in cattle milk. There are 15 different genetic variants for β -casein (A1, A2, A3, B, C, D, E, F, G, H1, H2, I, J, K, L), the most common being A1, A2, and B. β -casein consists of 209 amino acids, and the A2 allele is the output (Gholami et al., 2016; Gorkhali et al., 2020).

Other variants differ from the A2 variant by one or more different amino acids at different positions. β -casein A1 results from a genetic mutation in cattle that is thought to have occurred about many years ago (Mumtaz et al., 2021).

The difference between variants A1 and A2 is only one amino acid, at position 67, the amino acid proline in A2 milk (wild type) and the amino acid histidine in A1 milk. The B, C, F and G alleles also have histidine at this point and are therefore A1-derived. These variants differ in another position from variant A1. Variants A3, D, E, H2, and I have the amino acid proline at position 67 and are therefore similar to A2; there is a difference between these variants in another site (Amalfitano et al., 2019; Gorkhali et al., 2021). In studies characterizing the genetic variability of the β -casein gene (β -CN) in the milk of two small local dairy breeds in Denmark, the Danish red and Jutland cows, a very high frequency of the F allele was found. This variant occurs only in shallow frequency in Danish breeds (Poulsen et al., 2016).

The frequency of the A2 allele in cattle breeds in the Guernsey, Brown Swiss, Jersey, Holstein, Ayrshire, and Danish Red breeds is about 96-98%, 66-70%, 50-63%, 44-53%, 40-49%, and 23%, respectively. In India, work has been done on screening dairy animals for β -casein variants. The study noted that with 231 buffalo (8 breeds of river buffalo), all were type A2. Also, the frequency of A2 alleles in 15 cattle breeds (a total of 618 animals were examined) was 98.7%. In another study, Sahiwal (a total of 15 animals examined) and Tharparkar (a total of 14 animals examined) cattle breeds were established. In crossbreeds (Karan Fries and Karan Swiss, a total of 38 animals examined), A2 allele

frequencies were 79–89% (Parashar, 2015).

About 80% of the milk comes from crosses between Holstein and Zebu (mainly gyr) breeds in Brazil. A genetic study at the University of São Paulo in São Carlos showed that Zebu breeds almost entirely produce A2 milk (about 100%) and are not affected by a genetic mutation. In European breeds, only the Guernsey breed exclusively makes A2 milk; the Jersey breed is in second place with 75% milk, and the Holstein breed with 50% A1 milk and 50% A2 milk (Rangel et al., 2016). The beta-casein A1 variant has become dominant in milk slowly. While dairy herds in much of Asia, Africa, and parts of Southern Europe remain naturally high in cows producing A2 milk, the A1 protein variant is common among cattle in the western world (ÇAK, 2018).

Studies by scientists that have studied the relationship between β -casein genotypes and milk productivity traits are very contradictory. Citing (Ng-Kwai-Hang et al., 1984), the authors note that a positive effect of Variant A2 compared to Variant A1 on the amount of milk in Holstein cows has been established (Bech & Kristiansen, 1990) proved that the A2/A2 genotype positively affects the amount of milk protein in first lactation cows and the fat and protein content in second lactation animals compared to the A1/A1 genotype. According to Ikonen et al. (1999), which the authors cite, animals with the A2/A2 genotype are characterized by more protein content. A higher fat content, on the contrary, was characteristic of animals with an A1/A1 genotype. So, variant A2 is positively associated with higher milk yield and protein content in milk, while variant A1 has a positive effect on fat content. According to (Heck et al., 2009; Ivanković et al., 2021), the authors also note that animals of Holstein breed of Dutch selection with variant A2 had higher productivity and protein content in milk than cows with variant A1. At the same time, the authors argue that (Olenski et al., 2010) found that Holstein steers with the A2/A2 genotype have a high breeding value in milk yield and protein content compared to A1/A1 genotype animals, but the low breeding value in fat content. At the same time, scientists have shown that as a result of research (Kučerová et al., 2011) found that Czech Simmental bulls with A2/A2 genotype had a negative breeding evaluation, while animals with A1/A1 genotype, on the contrary, had positive milk yield evaluation. With regard to suitability for cheese making, the researchers cite the results of (Poulsen et al., 2013), according to which milk from animals of genotype A2/A2 has longer clotting time and lower gel stability of Holstein cows of the Danish breed significantly compared to milk of animals of genotype A1/A1. Variant B had a substantially shorter clotting time and higher gel stability than

variant A1 (Amalfitano et al., 2019; Parashar, 2015; Park & Haenlein, 2021).

In recent years, the discussion about A2 milk has been intensifying. As mentioned at the beginning, the A2 variant is one of a total of 15 β -casein variants. There is only one difference between the A1 and A2 variants. At position 67, the amino acid proline in variant A2 is replaced by histidine in variant A1. During digestion, beta-casein A1 releases the peptide beta-casomorphin 7, not produced in milk A2. It is the beta-casomorphin 7 peptide (BCM). It was mentioned that BCM is a risk factor for some diseases and has been linked to digestive and other problems (Amalfitano et al., 2019; Parashar, 2015; Schettini et al., 2020).

Environmental studies conducted in nineteen countries (United Kingdom, Finland, Ireland, Sweden, Denmark, France, Germany, Iceland, Norway, Austria, Switzerland, United States, Japan, Israel, Australia, New Zealand, Hungary, Venezuela, and Canada) found a strong association between β -casein A1 consumption and the incidence of type 1 diabetes. Epidemiological studies reported in a patent application show a link between β -casein A1 consumption and worsening neurological disorders such as autism and schizophrenia (Amalfitano et al., 2019).

The role of beta-casein A1 as an undesirable variant led to an attempt to select dairy cows based on beta-casein polymorphism. Breeding programs have attempted to use bulls with the A2/A2 genotype. In New Zealand, this led to the selection of cows in the herd producing milk only with the A2 variant (Kamiński et al., 2007; Morris et al., 2005). Since 2003 A2 milk had been solved in New Zealand and Australia as a premium brand that proposes a natural selection of proteins. The company started marketing A2 milk in Asia and the USA (A2 Corporation 2006) (Amatyia et al., 2021; Schettini et al., 2020).

A separate task for geneticists is to determine the distribution of different beta-casein variants in local cattle breeds. For example, Iranian researchers analyzed the frequency of the A1 allele of the CSN2 gene in the following breeds: Holstein – 50 %, Simmental 51.57%, Sistani 54.5 %, 49.4%, and 46.6% in Taleshi and Mazandarani cattle populations, respectively (Morris et al., 2005).

In Ukraine, given the long tradition of cattle breeding, there is an excellent variety of different breeds, among which the unique Ukrainian Lebedyn breed, genetic studies of which began only recently (Ladyka et al., 2021).

The purpose of this study was to perform genotyping of different Ukrainian breeds for the CSN2 gene (rs43703011), calculation, evaluation, and analysis of genotype and allele frequencies.

Material and Methods

Genotyping of 193 cattle of the following breeds was carried out: Lebedyn cattle (n = 101), Ukrainian brown dairy breed (n = 44), Simmental cattle (n = 41), Ukrainian black speckled milky (n = 26). Blood samples were taken under sterile conditions into 2.7 mL Monovette containing EDTA potassium salt as an anticoagulant (“Sarstedt” Germany) with the following samples’ freezing and storage at -20°C. DNA for genotyping was extracted from the samples using Monarch® Genomic DNA Purification Kit New England BioLab kits (USA) according to the manufacturer’s protocol.

The TaqMan@Genotyping real-time PCR system was used to perform allelic discrimination (Figure 1).

Two primers were designed to amplify the 101 bp product involving SNP rs43703011 (genomic DNA: X14711 (<http://www.ncbi.nih.gov>); forward primer, 5'- CCCAGACA-CAGTCTCTAGTCTATCC-3'; reverse primer, 5'- GGTTC-GAGTAAGAGGAGGGATGTTT -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-CCCATC-CATAACAGCC-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 μ l reactions with 10 μ l 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 μ l (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 (Figure 2).

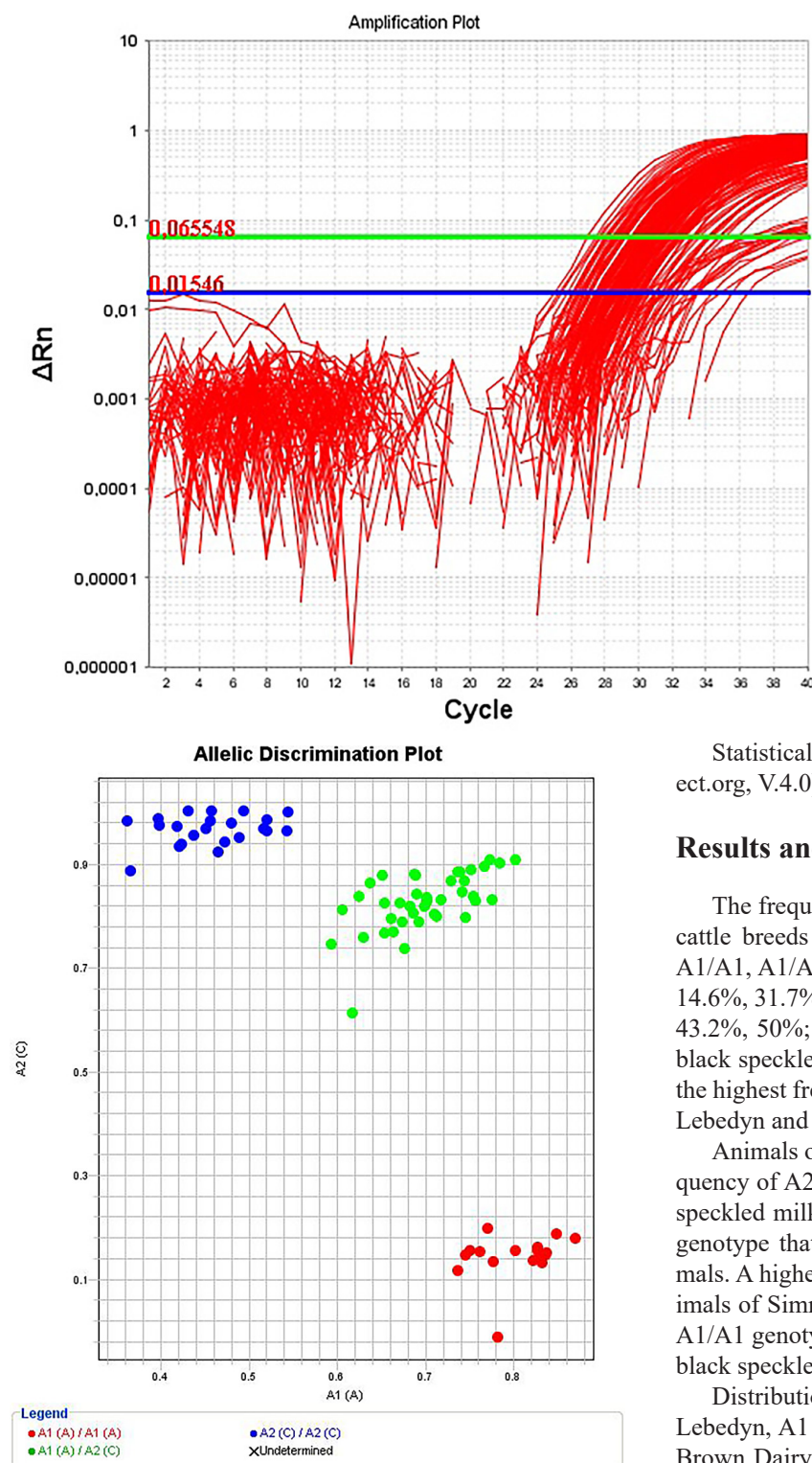


Fig. 2. Allelic discrimination by genotype polymorphism of the CSN2 gene (rs43703011)

Fig. 1. Amplification curves of genotype determination by CSN2 gene polymorphisms (rs43703011)

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

Results and Discussion

The frequency of allelic variants in 4 different Ukrainian cattle breeds gave the following information: the ratio of A1/A1, A1/A2, A2/A2 genotypes in the Lebedyn cattle was 14.6%, 31.7%, 53.7%; Ukrainian brown dairy breed – 6.8%, 43.2%, 50%; Simmental – 7.3%, 51.2%, 41.5%; Ukrainian black speckled milky – 38.5%, 34.6%, 26.9%. Accordingly, the highest frequency of A2/A2 genotype is characterized by Lebedyn and Ukrainian brown dairy breeds.

Animals of the Simmental breed had a slightly lower frequency of A2/A2 genotype, and animals of Ukrainian black speckled milky were characterized by the frequency of this genotype that was two times lower than brown cattle animals. A higher frequency of heterozygotes distinguished animals of Simmental and Ukrainian brown dairy breeds. The A1/A1 genotype was more frequent in animals of Ukrainian black speckled milky.

Distribution of alleles was also different in breeds: in Lebedyn, A1 allele frequency was 0.3, A2 – 0.7; Ukrainian Brown Dairy – 0.28 and 0.72; Simmental – 0.33 and 0.67; Ukrainian black speckled milky – 0.56 and 0.44, respectively. The results of allele discrimination on the CSN2 gene

(rs43703011) are shown in Table 1 and show significant differences in allele frequencies and genotypes in different Ukrainian breeds.

When comparing the ratio of genotypes in different breeds, there was a reliable difference by χ^2 between the Lebedyn and the Ukrainian black speckled milky ($p < 0.01$). Also, reliable differences in the distribution of genotypes were found when comparing Ukrainian Brown Dairy and Ukrainian Black Dairy ($p = 0.004$), Simmental and Ukrainian Black Dairy ($p = 0.007$), Ukrainian Black Dairy ($p < 0.001$) with all other breeds.

Analysis of the data obtained clearly shows that Lebedyn cattle, Ukrainian brown dairy breed, and Simmental have a significantly higher frequency of A2 allele than Ukrainian black speckled milky. That being said, cows of the Lebedyn breed have a slightly higher frequency of homozygous genotype A2/A2. On this basis, Lebedyn cattle can be considered the most promising producer of A2 milk under the condition of direct fertilization with sperm of A2/A2 bulls. Sufficient levels of productivity of Lebedyn cattle and other positive characteristics of this Ukrainian breed such as longevity (+ 1.5 lactations), breeding plasticity (dairy, dairy-meat, meat), high cheese making are additional arguments in the confirmation of the preliminary conclusion.

Our study found that the A2 beta-casein allele was more common in the Ukrainian brown dairy breed (0.695–0.716) and Simmental (0.671) cattle population compared to the A1 allele (0.284–0.305 and 0.329, respectively). In animals of the Ukrainian black speckled milky, in the breeding of which Holstein bulls are widely used, the frequency of allele A1 (0.558) was, on the contrary, higher than the frequency of allele A2 (0.442). These results correspond to the previously published studies, in which the frequency of A2 allele in

brown cattle was 0.66–0.705 (Ehrmann et al., 1997; Eenennam & Medrano, 1991; Swalssgood, 1992), while in the Simmental breed, it was slightly lower – 0.566–0.630 (Curik et al., 2007; Ehrmann et al., 1997). In Holstein cattle, contradictory data were obtained according to different authors (Amatya et al., 2021; Massella et al., 2017).

In addition, another allele (B) is observed in brown dairy breeds and Simmental breeds. The homozygous genotypes A1/A1 and A2/A2 were manifested in total in more than half of the studied livestock (69%) of Lebedyn cattle of the farm. Slightly more than half of the homozygous genotypes prevailed in the studied animals of Lebedyn cattle of Ukrainian black speckled milky (64%) and Ukrainian brown dairy breed (57%). As a result, a significant difference between the values of H_0 but not in cows of the Lebedyn cattle was established. The Wright fixation index indicates a deficit of heterozygotes in the population of Lebedyn and Ukrainian black speckled milky breeds and the presence of inbreeding. Heterozygous genotypes had an advantage in Simmental breed animals (51%).

Conclusions

As a result of this work, the frequencies of alleles and genotypes for the beta-casein locus were established. It was found that dairy cattle breeds bred in northeastern Ukraine significantly differ from each other by this trait. 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 beta-casein genotypes.

In terms of breeding, the Simmental breed has a high chance of homozygous herds' accelerated formation due to the significantly higher frequency of heterozygotes (51%).

Table 1. Frequency of CSN2 (rs43703011) genotypes and alleles in different Ukrainian cattle breeds

Breed	Distribution*	Genotypes,% Number of animals, pcs			Allele frequency	
		A1/A1	A1/A2	A2/A2	A1	A2
Lebedyn cattle	A	14.6	31.7	53.7	0.30	0.70
	T	12	26	44		
Ukrainian brown dairy breed	A	6.8	43.2	50.0	0.28	0.72
	T	3	19	22		
Simmental cattle	A	7.3	51.2	41.5	0.33	0.67
	T	3	21	17		
Ukrainian black speckled milky	A	38.5	34.6	26.9	0.56	0.44
	T	10	9	7		

* A – actual number of genotypes; T – theoretical number of genotypes

Curik I, Havranek J, Samarzija D, 1997. Milk protein polymorphism and genetic structure of Croatian Simmental cattle. In: Milk protein polymorphism. Proceedings of the IDF Seminar held in Palmerston North, New Zealand. Int Dairy Fed: 93–99.

According to gene frequency, creating homozygous A2/A2 herds for beta-casein is quite possible in a few generations, especially among Brown and Simmental breeds and directed selection of homozygous bulls. Apriori, using sexed sperm and embryos will significantly accelerate this process among breeding stock.

Author contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Drevytska T., Pavlenko Y., Skliarenko Y., Lahuta T. The first draft of the manuscript was written by Ladyka V., Dosenko V., Sklyarenko Y. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Consent for publication

Not applicable.

Conflict of interest

The authors declare no competing interests

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