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Original Contribution

GENETIC INSIGHTS INTO THE B-CASEIN (CSN2) LOCUS IN HOLSTEIN-FRIESIANS: A STEP TOWARDS A2 MILK PRODUCTION

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ABSTRACT

Milk plays a major role as protein source in human diet. Among the casein fractions, β-casein, encoded by the CSN2 gene, is characterized by the presence of several polymorphic variants. The most predominant types in dairy cattle are A1 and A2. Phylogenetic analyses confirm that the A2 variant is the original, while A1 is a result of a mutation that can lead to the release of a biologically active peptide (BCM-7) during digestion. The present study aims to assess the allele and genotype frequencies of the CSN2 locus in 30 Holstein-Friesian cows bred at the Academic Technological Complex of Trakia University – Stara Zagora, and to analyze the genetic equilibrium of the population according to the Hardy–Weinberg principle. The analysis was performed with the BovineSNP50 v3 Bead Chip and established the presence of 3 genotypes - A1A1, A1A2 and A2A2. The results exhibit an allele frequency of the A1 allele = 0.4 and of the A2 allele = 0.6, with a χ^2 test confirming that the population is in Hardy–Weinberg equilibrium ($\chi^2 = 0.37$, df = 1, $p > 0.05$). The witnessed high proportion of heterozygous individuals (A1A2) provides an opportunity for selection towards the A2A2 genotype. These results are fundamental for the development of breeding programs aiming the production of A2 β-casein milk, which has a growing consumer demand and potential health benefits.

Keywords: β-casein, CSN2 gene, Holstein-Frisian cattle, A2 milk

INTRODUCTION

Due to its high-quality protein, energy and biologically active substances, cow's milk is a valuable dietary component for humans at all stages of life (1). Nowadays more than 6 billion people globally consume milk and other dairy products on a daily basis, with a significant portion of it occurring in the developing countries (2). The growing demand for dairy products requires a sustained increase in milk production, with forecasts indicating a need for an annual growth of approximately 2% (3).

Dairy production is of key importance not only from a nutritional, but also from a socio-economic point of view, playing a significant role in the stability and competitiveness of the agricultural sector among the members of the European Union (4). Modern livestock breeding strategies aim high productivity and efficient use of the genetic potential of animals, which is particularly challenging in conditions of intensive production and increasing requirements for the quality of the final product (5).

In this context, molecular genetic markers are fundamental for the development of breeding programs aimed at improving the productive traits of cattle (6). The application of such techniques for assessment of animals' breeding values creates an opportunity for accelerated genetic progress and more precise management of the selection process (7). As a result, the global production of cow's milk and dairy products has made significant growth over the past few decades, which is primarily due to an improvement in individual's milk productivity, rather than an increase in the total number of reared animals (8).

About 80% of the total protein content of cow's milk is dominated by casein. It includes four main fractions – αS1-, αS2-, β- and κ-casein,

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encoded by the genes CSN1S1, CSN1S2, CSN2 and CSN3, respectively (9). The largest relative share is taken by the αS1-casein, followed by β-casein, κ-casein and αS2-casein (10). Polymorphisms of casein fractions in dairy cattle have been the subject of intensive research for decades, with a number of studies showing that they have a significant impact on the technological qualities of milk (11).

β-casein is of particular interest due to its high genetic variability and importance for the nutritional and biological properties of milk. The molecule consists of 209 amino acids and is characterized by the presence of numerous genetic variants arising as a result of point mutations (12). Historically, the oldest and primary variant of β-casein is the A2 type (13), which contains proline at position 67 of the polypeptide chain (14). A mutation in the locus replaced the proline amino acid with histidine, which established the A1 variant (15). The deviation is widespread among European cattle breeds, including the Holstein-Friesians (16).

The β-casein family in dairy cattle is mainly represented by the A1 and A2, while other genetic variants are found in very low frequencies (17). The significant structural difference between the A1 and A2 influences the way β-casein is processed during digestion (18). The presence of a proline at position 67 in the A2 variant hinders the proteolytic cleavage of the peptide bond in this region, while the A1 variant, containing a histidine, allows the release of the bioactive peptide β-casomorphin-7 (19). This peptide exhibits opioid-like activity and has been associated with potential adverse effects on human health, including metabolic, cardiovascular, and gastrointestinal disorders (20). Despite the ongoing debate about the health effects of the two variants, there is growing demand for the so-called “A2 milk”, produced from A2A2 genotype carriers (21). This further highlights the importance of the CSN2 gene, located in the casein gene cluster on the sixth chromosome in cattle (BTA6), as a selection marker in modern cattle breeding.

In addition, the β-casein locus is known to have a significant effect on the protein and fat content of milk, as well as on the total amount of milk produced (22). A study by Lin et al. (1989), demonstrates that cows with the A2A2 genotype produced 300 kg more milk in the first lactation compared with cows with the A1A1 genotype (23). Hence, the analysis of the allele and genotype frequencies of this locus can be used as indicator for the genetic structure of populations and creates prerequisites for its targeted use in modern breeding programs (24). The current experiment aims to determine these frequencies among Holstein-Friesian cattle bred at the Academic Technological Complex of Trakia University - Stara Zagora, as well as to assess the compliance of the studied population with the genetic equilibrium according to the Hardy–Weinberg principle.

MATERIALS AND METHODS

Experimental animals and sampling

The trial examined a total of 30 Holstein-Friesian cattle, reared at the Academic Technological Complex of Trakia University – Stara Zagora, Bulgaria. Animals were between 3 and 5 years of age and were randomly selected within the farm to avoid related individuals.

Tissue samples were collected from the ear cartilage via TSU gun and stored in special TSU tubes until DNA extraction. All sampling procedures followed the Veterinary medical act (VMA) and Regulation No. 20/12 on the minimal requirements for protection and welfare of experimental animals.

Genotyping

Molecular analyses of the CSN2 gene was performed using Illumina's BovineSNP50 v3 DNA Analysis BeadChip. The chip contains 53,218 informative single nucleotide polymorphisms (SNPs) that are evenly distributed across the bovine genome. Compared to the commercial 50K BeadChip, the v3 version provides nearly three times higher marker density, which allows increased accuracy and broader detection of alleles, including in the CSN2 gene. The mechanism relies on the hybridization of the DNA sample to specific oligonucleotide sites on the chip attached to microspheres (beads). After extraction and amplification of the DNA, the samples are labeled with fluorescent probes that bind specifically to different SNPs. Reading the fluorescence from the chip allows detection of the genotype in each SNP locus. Data is analyzed using a dedicated genotyping software that allows standardized approach for large scale genotype detection. All procedures were performed at Genetic Visions-ST, LLC (8137 Forsythia St, Suite 100, Middleton, WI 53562, USA). Results generated by Affymetrix and Illumina after July 30, 2019 are accredited to ISO17025:2017 (Cert 5436.01), which guarantees their compatibility with international standards for genetic analysis.

Allele and genotype frequency calculations

The witnessed allele and genotype frequencies of the CSN2 gene were processed according to the Hardy–Weinberg principle. This allows an assessment of the correspondence between the observed and expected genotype distributions under random distribution of alleles. Data was processed by the following equation:

$$p = \frac{2(A1A1) + (A1A2)}{2N}; \quad q = 1 - p$$

where p is the frequency of the A1 allele, q is the frequency of the A2 allele, N is the total number of investigated animals, "A1A1" is the number of animals homozygous for the A1 allele, "A1A2" is the number of heterozygous animals, and "A2A2" is the number of animals homozygous for the A2 allele.

This approach provides a quantitative assessment of the alleles and genotypes distribution in the population, as well as information on the presence of selection pressures that may affect the genetic stability and productivity of the herd.

RESULTS AND DISCUSSION

Results

The main objective of the present study was to assess the allele and genotype frequency distribution of the CSN2 gene in the studied population of Holstein-Friesian cows. The analysis is a prerequisite for the development marker assisted selection aiming to increase the frequency of the A2A2 genotype and the production of "A2 milk".

In the studied population of 30 animals, the following genotypes were observed: 4 cows (13.3%) were homozygous for the A1 allele (genotype A1A1), 16 cows (53.3%) were heterozygous carriers (genotype A1A2), and 10 cows (33.3%) were homozygous for the A2 allele (genotype A2A2). Based on these data, the calculated allele frequencies were: Allele A1: p = (2 × 4 + 16) / (2 × 30) = 24 / 60 = 0.4; Allele A2: q = (2 × 10 + 16) / (2 × 30) = 36 / 60 = 0.6.

The expected genotype frequencies according to the Hardy–Weinberg principle ($p^2 + 2pq + q^2 = 1$) were as follows: for genotype A1A1: $p^2 = 0.4^2$ x N = 0.16 × 30 ≈ 4.8 animals, for genotype A1A2: 2pq x N = (2 × 0.4 × 0.6) x 30 = 0.48 × 30 ≈ 14.4 animals and for genotype A2A2: q^2 x 30 = 0.6^2 x 30 = 0.36 × 30 ≈ 10.8 animals.

A χ^2 test was performed to statistically assess the deviations between the observed and expected genotype frequencies **(Table 1)**. The χ^2 value was calculated using the equation $\Sigma (O - E)^2 / E$, where: O is the observed number of animals and E is the expected number according to Hardy–Weinberg principle. The calculations for each genotype are as follows: for genotype A1A1: $(4 - 4.8)^2 / 4.8 = (-0.8)^2 / 4.8 \approx 0.13$; for genotype A1A2: $(16 - 14.4)^2 / 14.4 = (1.6)^2 / 14.4 \approx 0.18$ and for genotype A2A2: $(10 - 10.8)^2 / 10.8 = (-0.8)^2 / 10.8 \approx 0.06$. The total χ^2 value was = 0.13 + 0.18 + 0.06 = 0.37. With a degree of freedom of 1 (df = number of genotypes − number of alleles = 3 − 2 = 1) and a critical χ^2 value at $\alpha = 0.05$ equal to 3.84, the calculated χ^2 value is significantly below this threshold. The result indicates that the distribution of genotypes in the studied population does not differ significantly from the expected one and confirms that the population is in genetic equilibrium according to the Hardy–Weinberg rule with respect to the CSN2 locus. The insignificant deviation in the frequency of heterozygotes may be due to the limited sample size or the breeding practices on the farm. The results exhibit a predominance of the A2 allele, which allows its use as a selective marker for the production of milk with the preferred A2 β-casein type.

Table 1. *Chi-square (χ^2) test of the CSN2 gene in Holstein cattle reared at the Academic Technological Complex of Trakia University – Stara Zagora, Bulgaria (n=30).*

Genotype	Observed (O)	Expected (E)	$(O-E)^2/E$
A1A1	4	4.8	0.13
A1A2	16	14.4	0.18
A2A2	10	10.8	0.06
Σ			**0.37**

df = 1; χ^2 critical (0.05) = 3.84

DISCUSSION

The *Bos taurus* beta-casein gene (CSN2) consists of 9 exons and 8 introns (25). The coding sequence which encodes the protein is 675 bp long and translates into 224 amino acids (26).

An important polymorphism which significantly influences the milk production is g.8101C>A (p.His67Pro) (GenBank x14711:g.8101c>a) (27). This mutation leads to the production of two main proteins: A1 and A2. A1 variant has histidine at position 67 while A2 variant has proline at the same position (28). The mutation that leads to this change in the polypeptide sequence is a transversion C>A at codon 67 in exon 7 of the CSN2 gene where the codon CCT (which encodes the amino acid proline in A2 variant) is changed to CAT (which encodes the amino acid histidine in A1 variant) **(Figure 1)** (29).

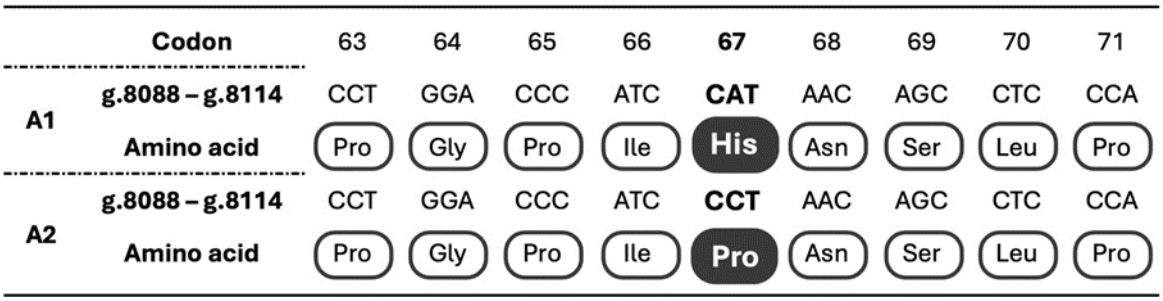

Figure 1. Single nucleotide polymorphism at position g.8101C>A (p.His67Pro) in the CSN2 gene, encoding Beta-casein variants in *Bos taurus*.

The results obtained demonstrate that the Holstein-Friesian cattle population reared at the Academic Technological Complex of Trakia University retains high genetic variability at the CSN2 locus. The observed high percentage of heterozygous individuals (A1A2) suggests that genetic diversity is well maintained and allows the development of MAS program targeting the A2 milk production. Our findings are on par with those reported by Kamiński et al. (2006), who documented allele frequencies of 0.402 for the A1 allele and 0.598 for the A2 allele (30). Cartuche-Macas et al. (2025) witnessed comparable allele frequencies (0.628 and 0.372 for A1 and A2, respectively) in Holstein-Friesian cattle raised in Ecuador (31). Truswell (2005) reported that the A1 allele was the most common allele found in dairy cows of northern European origin, such as Holstein-Friesian and Ayrshire. In contrast, the A2 allele is more frequently observed in the Guernsey and Jersey breeds (32). Bisutti et al. (2022) reported genotype frequencies of 0.43 for A1A2, 0.33 for A2A2 and 0.14 for A1A1 in 1133 Italian Holstein-Friesian cows (33). Sawicka-Zugaj et al. (2025) encountered opposite results to ours, with a predominance of the A1 allele (0.57) among the Polish Red cattle population (34). Similarly, the results of Cieslinska et al. (2019) also confirms the relatively low frequency of the A2 allele (0.37) (35). Such broad range of variation in allele frequencies between breeds implies that majority of European countries still do not carry out intensive selection of the CSN2 gene. The allele fluctuations between populations can be explained by local breeding practices, a limited number of bulls used and the lack of systematic genetic control over the distribution of alleles (36).

The insignificant deviations between the observed and expected genotype frequencies from our study, indicate that the CSN2 locus is not subject to strong selection pressure in the studied population and the distribution of genotypes corresponds to random mating. This provides a solid basis for the development of breeding programs aiming the increase of the A2A2 genotype frequency (37). The large share of A1A2 and A2A2 genotypes (53.3% and 33.3%, respectively) among the studied population highlights the potential for genetic improvement through the strategic use of bulls with the A2A2 genotype (38). Such an approach would contribute to the gradual conversion of the herd to an "A2 milk" β-casein type (39).

CONCLUSION

Our results support the concept that molecular genotyping and genomic testing provide valuable information for breeding decisions, especially when aiming to improve specific production traits such as β-casein structure. The present study shows that the Holstein-Friesian cattle population at ATC of Trakia University is in genetic equilibrium at the CSN2 locus and has a sufficiently high percentage of A2 allele carriers. Such results are key for the development of a breeding program aimed to increase the frequency of the A2A2 genotype carriers. The implementation of such strategies could have a significant economic impact and satisfy the growing consumer demand for milk with a specific β-casein composition.

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