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Original scientific article

GENOTYPIC ANALYSIS OF THE CSN2 GENE IN CATTLE: VALIDATION OF A1/A2 POLYMORPHISM BY SANGER SEQUENCING

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Milk β-casein variants, particularly A1 and A2, have gained attention due to their impact on digestibility and potential health effects. The *CSN2* gene, located on bovine chromosome 6, encodes β-casein, and a single nucleotide polymorphism (SNP) in exon 7 differentiates the A1 (histidine) and A2 (proline) alleles. The A1 variant is associated with the release of β-casomorphin-7 (BCM-7), which has been controversially linked to health concerns such as type 1 diabetes and cardiovascular disease, while A2 milk is considered easier to digest and more suitable for sensitive consumers. This study aimed to genotype selected cattle breeds for *CSN2* variants using PCR and Sanger sequencing. Blood samples were collected, and genomic DNA was extracted via the phenol–chloroform method. A 121 bp region of exon 7 was amplified using gene-specific primers, followed by electrophoresis and Sanger sequencing for SNP detection. Chromatogram analysis revealed three genotypes: A1A1, A1A2, and A2A2, with heterozygotes being most prevalent. Sanger sequencing proved to be a precise, reliable tool for confirming genotypes, especially in distinguishing heterozygous conditions and avoiding ambiguities common in conventional PCR methods. The allele distribution observed aligns with previous findings in both indigenous and exotic cattle breeds and supports the growing emphasis on A2 milk in breeding programs. Future prospects include expanding genotyping across larger herds, studying correlations between β-casein variants and milk production traits, and applying marker-assisted selection (MAS) to

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establish A2A2-dominant lines, which would enhance both dairy quality and market value for health-focused milk production.

Key words: β-casein, cattle genotyping, marker-assisted selection, milk quality, single nucleotide polymorphism (SNP)

INTRODUCTION

Milk is a vital component of the human diet, and its protein composition significantly influences its nutritional quality and digestibility. Among milk proteins, β-casein, encoded by the CSN2 gene located on bovine chromosome 6, plays a crucial role in determining milk functionality and health-related properties (FARRELL *et al*., 2004; RAMESHA *et al*., 2016). More than twelve genetic variants of β-casein have been identified, among which A1 and A2 are the most extensively studied due to their contrasting physiological effects (KAMINSKI *et al*., 2007; MISHRA *et al*., 2009).

The A1 and A2 variants differ due to a single nucleotide polymorphism (SNP) in exon 7 of the CSN2 gene, resulting in an amino acid substitution at position 67, where proline (A2) is replaced by histidine (A1) (JINSMAA & YOSHIKAWA, 1999). This substitution influences protein digestion, leading to the release of β-casomorphin-7 (BCM-7) from A1 β-casein, a bioactive peptide that has been controversially associated with various health issues such as cardiovascular diseases, type 1 diabetes, and neurological disorders (ELLIOTT *et al*., 1999; MCLACHLAN, 2001; WOODFORD, 2009). In contrast, A2 β-casein does not release BCM-7, making A2 milk more suitable for individuals with digestive sensitivity (KAMINSKi *et al*., 2007; RAMESHA *et al*., 2016).

Due to increasing consumer awareness and market demand for A2 milk, accurate genotyping of the CSN2 gene has become essential for dairy breeding and product labeling. Several molecular techniques such as PCR-RFLP, allele-specific PCR, and ARMS-PCR are commonly used for detecting A1/A2 polymorphism; however, these methods may sometimes produce ambiguous results or fail to detect rare mutations (NG-KWAI-HANG, 2003; PRIYADARSHINI & PRAKASH, 2018). Therefore, more precise and reliable approaches are required.

Sanger sequencing, first developed by SANGER *et al*. (1977), remains the gold standard for DNA sequence analysis due to its high accuracy and ability to detect single nucleotide variations at base resolution. It enables direct identification of SNPs and confirmation of heterozygous conditions, overcoming limitations of gel-based methods (KUMAR *et al*., 2019; YADAV *et al.*, 2023). This technique has been successfully applied in various studies to validate β-casein genotypes in cattle populations (SINGH *et al*., 2015; KUMAR *et al*., 2019).

Therefore, the present study was undertaken to determine the distribution of A1 and A2 alleles in selected cattle breeds, validate PCR-based genotyping results, and confirm SNP variation in the CSN2 gene using Sanger sequencing. The findings aim to support marker-assisted selection programs for improving milk quality and promoting A2 milk production.

MATERIALS AND METHODS

Materials and Reagents

The following materials and reagents were used:EDTA-coated vacutainer tubes, DNA extraction kit (HiPurA™ Bovine Genomic DNA Purification Kit, HiMedia) or the Phenol–

Chloroform–Isoamyl alcohol (PCI) method, Proteinase K, Sodium Dodecyl Sulfate (SDS), RNase A (optional), TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), Phenol:Chloroform:Isoamyl alcohol (25:24:1),3M Sodium Acetate (pH 5.2),70% and absolute ethanol,Nuclease-free water

Selection of cattle breeds for the Study:

The study included Gir, Holstein Friesian (HF), Kankrej, and HF × Jersey crossbred cattle. Indigenous breeds such as Gir and Kankrej are known to predominantly carry the A2 allele, whereas exotic breeds like Holstein Friesian often show a higher frequency of the A1 allele (MISHRA *et al*., 2007; GANGULY *et al*., 2013; RANGEL *et al*., 2017).

Sample Collection:

In the present study, blood samples were collected from a total of 200 unrelated cattle, including 95 Gir, 60 Holstein Friesian (HF) and Jersey, and 45 HF × Jersey crossbreeds, from farms located in Savarkundla, Gujarat. Data on milk yield and fat percentage were obtained from the farm owners. Blood samples were collected in vacutainers containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The collected samples were then transported to the laboratory under ice-cold conditions to maintain their integrity.

Genomic DNA Isolation

Genomic DNA was extracted using the conventional phenol–chloroform method, a widely used technique for obtaining high-quality DNA suitable for molecular analysis (SAMBROOK & RUSSELL, 2001). The protocol ensures efficient removal of proteins and contaminants, yielding DNA appropriate for PCR amplification and sequencing (SINGH *et al*., 2015).

Quality and Quantity Assessment

DNA concentration and purity were assessed using a Nanodrop spectrophotometer, with A260/A280 ratio used as a purity indicator. Integrity was verified by electrophoresis on a 0.8% agarose gel stained with ethidium bromide.

PCR Amplification of the CSN2 Gene

PCR amplification of the CSN2 gene was carried out targeting exon 7, which contains the A1/A2 polymorphic site. Similar amplification strategies have been widely used in previous studies for β-casein genotyping (PRIYADARSHINI & PRAKASH, 2018; KUMAR *et al*., 2019).

Primer	Sequence (5′ → 3′)	Product Size
Forward	CCTTCTTTCCAGGATGAACTCCAG	121 bp
Reverse	GAGTAAGAGGAGGGATGTTTTGTGGGAGGCTCT	

Thermal Cycling Conditions:

Step	Temperature	Time	Cycles
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 sec	30
Annealing	60°C	30 sec	30
Extension	72°C	30 sec	30
Final Extension	72°C	5 min	1
Hold	4°C	∞	—

Amplified products were resolved on a 2% agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator.

PCR–RFLP Analysis:

For genotyping, 10 µL of the amplified PCR product was subjected to digestion using 2 units of the restriction enzyme *DdeI* in a total reaction volume of 20 µL, followed by overnight incubation at 37°C. The resulting restriction fragments were separated by electrophoresis on a 3% agarose gel, stained with ethidium bromide, and visualized under ultraviolet (UV) light to detect specific polymorphisms in the CSN2 gene. This method has been extensively used for detecting CSN2 polymorphism due to its simplicity and cost-effectiveness (PRIYADARSHINI & PRAKASH, 2018).

Calculation of Gene and Genotype Frequency:

To evaluate the genotypic and allelic distribution of the CSN2 gene polymorphism, allele frequencies were estimated using the direct counting method in accordance with Hardy–Weinberg equilibrium principles. The Chi-square test was employed to assess any significant deviation between the observed and expected genotype frequencies. The expected frequencies were calculated using the Hardy–Weinberg equation, where p and q denote the frequencies of the respective alleles. This approach is fundamental in population genetics for determining whether the genetic structure of a population remains stable or is influenced by evolutionary forces.

Genotype frequency (%) = (Number of observed genotypes / Total number of genotypes) × 100

Allele frequencies were calculated as:
p = (2AA + AB) / 2N and q = (2BB + AB) / 2N, where N represents the total number of individuals.

Sanger Sequencing

Sanger sequencing was conducted using the chain termination method as described by SANGER *et al*. (1977). This method provides high accuracy in SNP detection and has been successfully used in cattle genotyping studies (KUMAR *et al*., 2019; YADAV *et al*., 2023).

Sequence alignment and chromatogram analysis were performed to confirm genotype identification. Confirmed PCR products were purified using a commercial PCR purification kit (e.g., Qiagen QIAquick PCR Purification Kit) to remove excess primers, dNTPs, and enzymes.
Sequencing Reaction:

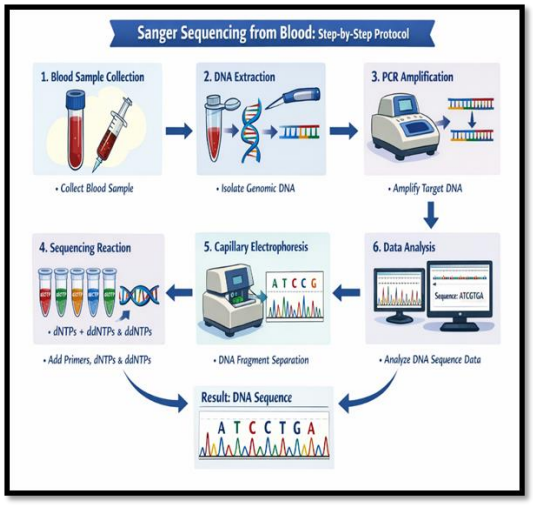
- Sequencing was carried out using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).
- Bidirectional sequencing was performed using the same forward and reverse primers.
- Electrophoresis and detection were conducted on an ABI 3130 or 3730 Genetic Analyzer, either in-house or through certified sequencing services.

Sequence Analysis

Raw sequencing files (.ab1 format) were analyzed using Chromas for chromatogram visualization and base calling. Sequences were aligned to the reference bovine *CSN2* gene (GenBank accession no. X14711) using standard alignment tools.

Genotypes were determined based on SNP presence at codon 67

Genotype	SNP Pattern	Amino Acid
A1A1	CAT (His)	Histidine
A2A2	CCT (Pro)	Proline
A1A2	CAT/CCT	Heterozygous



RESULTS

The extracted DNA was evaluated for quality using 1% agarose gel electrophoresis, which revealed distinct high molecular weight bands, indicating good DNA integrity suitable for subsequent PCR amplification. DNA purity was further confirmed using NanoDrop

spectrophotometry by measuring absorbance ratios at 260/280 nm. To investigate polymorphism in the β-casein (CSN2) gene, a 121 bp PCR-amplified fragment was subjected to digestion with the restriction enzyme DdeI. This enzyme specifically cleaves the A2 allele into two fragments of 86 bp and 35 bp, whereas the A1 allele remains undigested at 121 bp. Accordingly, the A1A1 genotype produced a single band of 121 bp, the A2A2 genotype yielded two bands of 86 bp and 35 bp, and the heterozygous A1A2 genotype exhibited all three bands (121 bp, 86 bp, and 35 bp). These distinct banding patterns enabled clear differentiation of genotypes across different cattle breeds, as illustrated in Figure 1.

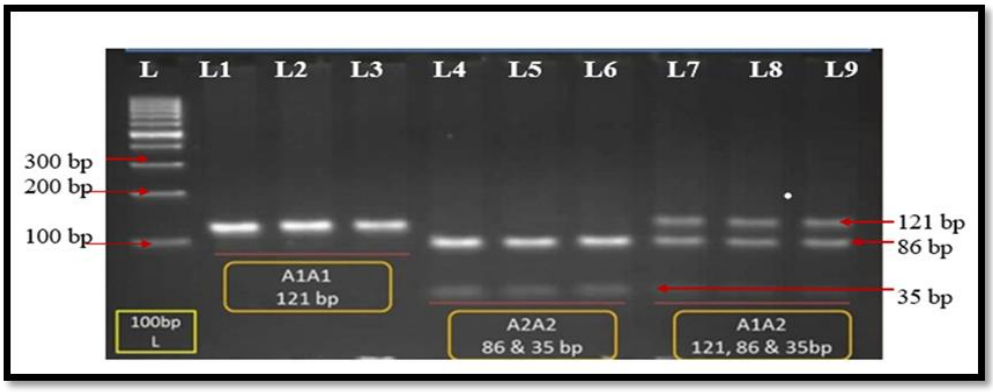


Figure 1. Agarose gel electrophoresis of PCR products.
Lane L: 100 bp DNA ladder; Lanes 1–3: Amplified *CSN2* exon 7 products (121 bp)in sample S1 to S3; Lane 4 to 6 : Sample S10 to S12 showed A2A2 genotype (86 and 35bp) and Lane 7 to 9 Showed A1A2 genotype in S21 to S23.

Table 1. Allele frequencies and result of Chi- square test for comparison of proportions to all different cattle breeds

Breed Name	Total No.	Genotype frequency	Total genotype	Total genotype frequency	Allele frequency		Chi square test	P value
					A1	A2		
Gir	95	A1A1 A2A2 A1A2	5 90 0	0.05 0.95 0.00	0.05	0.95	76.003	<0.0001 (df=1)
HF	60	A1A1 A2A2 A1A2	55 0 5	0.92 0.0 0.08	0.96	0.04	60.00	<0.0001 (df=1)
Hf× Jersey	45	A1A1 A2A2 A1A2	5 0 45	0.1 0.0 0.88	0.5	0.5	33.33	<0.0001 (df=1)

p value <0.05 Significant , df= Degrees of freedom

The distribution of genotypic and allelic frequencies of the CSN2 gene, along with the results of the chi-square test for deviation from Hardy–Weinberg equilibrium among different cattle breeds, are presented in Table 1.

Sanger Sequencing and Genotype Identification

Sequencing chromatograms were analyzed to determine the A1/A2 genotypes based on the **SNP at codon 67** (CCT → CAT). The SNP position was clearly identifiable in the chromatograms using both forward and reverse reads.

- **Homozygous A2A2** samples showed a clear **C peak** at the SNP site, indicating **proline (CCT)**.
- **Homozygous A1A1** samples showed a clear **A peak**, indicating **histidine (CAT)**.
- **Heterozygous A1A2** samples displayed **overlapping C and A peaks**, confirming the presence of both alleles at the SNP position (Figure 2).

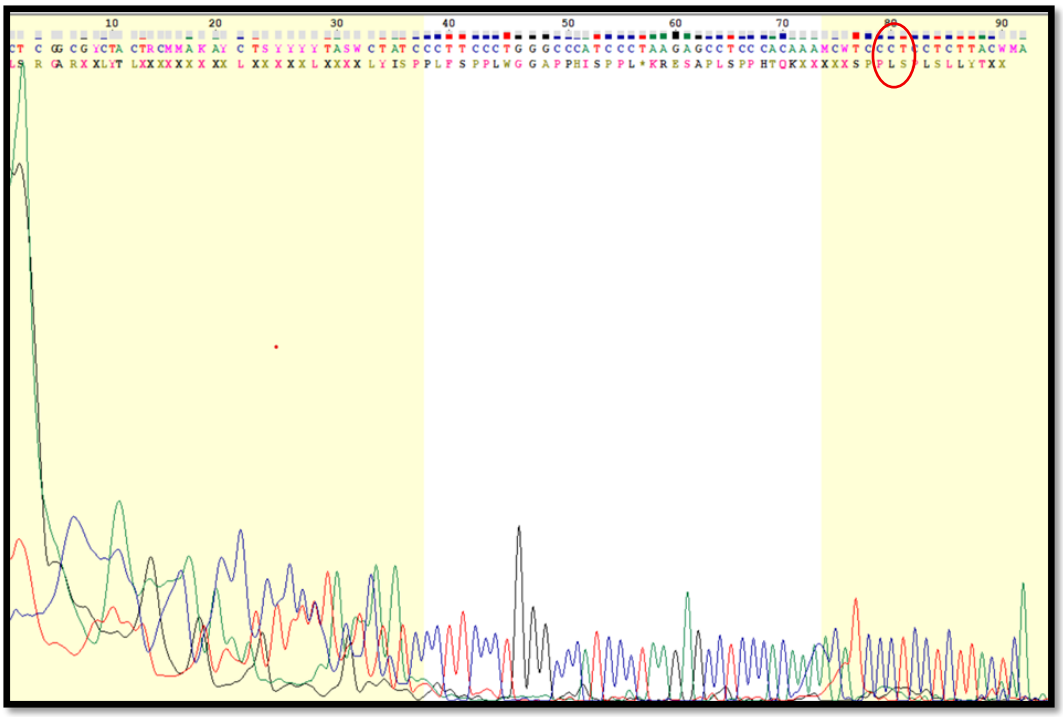


Figure 2. Representative sequencing chromatograms showing *CSN2* genotypes.
A2A2 genotype showing cytosine (C) at codon 67.

All sequences were aligned to the reference bovine *CSN2* gene (GenBank Accession No. X14711) to verify SNP position and genotype accuracy.

DISCUSSION

The present study revealed clear variation in β-casein genotypes among Gir, HF, and HF × Jersey cattle. Gir cattle showed a predominance of the A2 allele, with most animals exhibiting the A2A2 genotype and absence of the heterozygous A1A2 genotype, confirming that indigenous breeds largely possess the A2 variant. In contrast, HF cattle showed a high frequency of the A1 allele with predominance of the A1A1 genotype, indicating the dominance of A1 type in exotic breeds. The HF × Jersey crossbred population exhibited equal allele frequencies for A1 and A2, with a high proportion of heterozygous A1A2 genotype, reflecting genetic admixture and increased heterozygosity due to crossbreeding. The significant deviation from Hardy–Weinberg equilibrium ($P < 0.0001$) across all groups suggests the influence of selective breeding, non-random mating, and crossbreeding practices.

The current study also successfully identified CSN2 genotypes (A1A1, A1A2, A2A2) using PCR amplification followed by Sanger sequencing, confirming its reliability for accurate SNP detection at codon 67 of exon 7. The observed allele distribution is consistent with earlier reports, including KUMAR *et al*. (2019) and YADAV *et al.* (2023), who demonstrated the effectiveness of sequencing in validating β-casein genotypes and detecting heterozygosity. Studies from different regions (DREVYTSKA *et al*., 2025; AYTEKIN, 2024) also support the predominance of the A1 allele in exotic breeds and mixed genotype distribution in crossbred populations, while findings by RANGEL *et al*. (2017) highlight the natural predominance of the A2 allele in indigenous breeds such as Gir.

Overall, the findings emphasize the genetic and commercial importance of CSN2 polymorphism. The presence of A2A2 individuals supports the potential for marker-assisted selection to promote A2 milk production. Moreover, the study reinforces Sanger sequencing as a gold standard for genotyping accuracy, as also reported by MILUCHOVÁ *et al.* (2023). These results underline the importance of conserving indigenous germplasm while designing breeding strategies aimed at improving milk quality and meeting the increasing demand for A2 milk.

CONCLUSION

The present study demonstrates the reliability and precision of combining PCR-RFLP and Sanger sequencing for genotyping the CSN2 gene in cattle. While PCR-RFLP serves as a rapid and cost-effective screening method, Sanger sequencing provides high-resolution validation by accurately identifying the single nucleotide polymorphism at codon 67 of exon 7, enabling clear discrimination between A1A1, A1A2, and A2A2 genotypes. The observed distribution of β-casein variants revealed a predominance of the A2 allele in indigenous Gir cattle and a higher frequency of the A1 allele in Holstein Friesian populations, whereas crossbred animals exhibited increased heterozygosity, reflecting genetic admixture. The significant deviation from Hardy–Weinberg equilibrium indicates the influence of selective breeding and non-random mating practices. These findings underscore the genetic and commercial importance of CSN2 polymorphism and support the application of marker-assisted selection strategies to enhance the frequency of the A2 allele in dairy herds. Overall, this study reinforces Sanger sequencing as a gold standard for SNP validation and highlights its role in improving genotyping accuracy, thereby contributing to the development of sustainable breeding programs aimed at improving milk quality and meeting the growing demand for A2 milk.

ETHICS STATEMENT

All procedures involving animals were conducted in accordance with the guidelines set forth by the Canadian Association for Laboratory Animal Science (CALAS) and the Canadian Council on Animal Care (CCAC). Blood samples were collected exclusively by licensed veterinary doctors to ensure ethical and professional standards of animal care and use." Information regarding cattle was obtained from records maintained by the farm owners.

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GENOTIPSKA ANALIZA GENA CSN2 KOD GOVEDA: VALIDACIJA POLIMORFIZMA A1/A2 SANGEROVIM SEKVENCIRANJEM

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Izvod

Varijante b-kazeina mleka, posebno A1 i A2, privukle su pažnju zbog svog uticaja na svarljivost i potencijalnih zdravstvenih efekata. Gen CSN2, koji se nalazi na hromozomu 6, kodira b-kazein, a polimorfizam jednog nukleotida (SNP) u eksonu 7 razlikuje alele A1 (histidin) i A2 (prolin). Varijanta A1 je povezana sa oslobađanjem b-kazomorfina-7 (BCM-7), koji je kontroverzno povezan sa zdravstvenim problemima kao što su dijabetes tipa 1 i kardiovaskularne bolesti, dok se mleko A2 smatra lakšim za varenje i pogodnijim za osetljive potrošače. Cilj ove studije bila je genotipizacija odabranih rasa goveda za CSN2 varijantom korišćenjem PCR i Sangerovog sekvenciranja. Prikupljeni su uzorci krvi, a genomska DNK je ekstrahovana metodom fenol-hloroform. Region od 121 bp eksona 7 je amplifikovan korišćenjem gen-specifičnih prajmera, nakon čega je usledila elektroforeza i Sangerovo sekvenciranje za detekciju SNP-a. Analiza hromatograma otkrila je tri genotipa: A1A1, A1A2 i A2A2, pri čemu su heterozigoti najzastupljeniji. Sangerovo sekvenciranje se pokazalo kao precizan i pouzdan alat za potvrđivanje genotipova, posebno u razlikovanju heterozigotnih stanja i izbegavanju dvosmislenosti uobičajenih u konvencionalnim PCR metodama. Primećena distribucija alela poklapa se sa prethodnim nalazima kod autohtonih i egzotičnih rasa goveda i podržava sve veći naglasak na A2 mleku u programima uzgoja. Buduća ispitivanja uključuju genotipizaciju većih stada, proučavanje korelacija između varijanti b-kazeina i osobina proizvodnje mleka i primenu selekcije uz pomoć markera (MAS) za uspostavljanje A2A2-dominantnih linija, što bi poboljšalo i kvalitet mleka i tržišnu vrednost za proizvodnju mleka usmerenu na zdravlje.

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