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Study of Beta-Casein Gene Polymorphism In Gir Cattle From Gujarat State

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Bovine milk and dairy products are vital components of traditional diets globally, with milk proteins comprising approximately 80% casein and 20% whey. Among the caseins, ß-casein is a major hydrophobic protein with two common genetic variants, A1 and A2. The difference between these variants lies in a single amino acid substitution at position 67, where histidine (CCT) in A1 is replaced by proline (CAT) in A2. This study aimed to investigate the ß-casein gene polymorphism in 200 Gir cattle from Shri Krishna Farm, Savarkundla using the PCR-RFLP technique. Genomic DNA was extracted using the Phenol-Chloroform-Isoamyl Alcohol method, and its quality and quantity were assessed through agarose gel electrophoresis and Nanodrop spectrophotometry. PCR amplification yielded a 121 bp fragment, which was then subjected to restriction digestion using the DdeI enzyme. The analysis revealed two genotypes—A1A2 and A2A2—with respective genotypic frequencies of 0.125 and 0.875, while the A1A1 genotype was not observed. The allele frequencies were 0.06 for A1 and 0.94 for A2, confirming the predominance of the A2 allele in Gir cattle and suggesting their potential for producing A2 milk, which is considered healthier.

Keywords: Allele Frequency; Beta-Casein; Genotype; Milk; PCR-RFLP.

Bovine milk and its derivatives are integral to diets worldwide, with the casein protein family (á, â, ê) constituting approximately 80% of milk proteins. Among these, â-casein accounts for about 30–35% of total casein and is notably more water-soluble, particularly at lower temperatures, compared to other caseins. A single nucleotide polymorphism (SNP) in exon 7 of the CSN2 gene CCT '! CAT results in a histidine-to-proline substitution, rather than proline-to-histidine, giving rise to the two primary â-casein alleles: A2 (Prov w) and A1 (Hisv w). The A1 variant has been associated with enzymatic cleavage during digestion, yielding â-casomorphin-7 (BCM-7), an opioid peptide that may influence gut inflammation, glucose metabolism, and cardiovascular risk, whereas the A2 variant is considered safer and has been linked to better digestibility, lower inflammatory response, and beneficial lipid profiles (Mukesh et al., 2022; Cartuche-Macas et al., 2025; Sawicka-Zugaj et al., 2025).

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In India, indigenous zebu *(Bos indicus*) breeds predominantly carry the A2 allelemaking them promising candidates for health-oriented dairy breeding programs. For example, Gir, Sahiwal, Tharparkar, and Rathi cattle often exhibit near fixation for the A2 allele or very low A1 frequencies (Yadav et al., 2023; Paradkar et al., 2023). However, crossbreeding with taurine or exotic lines such as Holstein Friesian and Jersey tends to introduce higher frequencies of A1 alleles, as observed in Frieswal and HF crosses (Kumar et al., 2019; Adesh et al., 2019). Therefore, molecular genotyping of â-casein is essential for marker-assisted selection (MAS) aimed at expanding A2 allele presence while controlling A1 introgression.

Gir cattle, indigenous to the Gir forest region in Gujarat, are renowned for their adaptability, disease resistance, and relatively high milk fat content, making them strategically important for A2 milk production. In this study, we analyzed the A1/A2 allele and genotype frequencies in a Gir cattle herd (n = 200) using PCR–RFLP, validated Hardy–Weinberg equilibrium, and compared the frequencies with crossbred Holstein Friesian populations. Our findings confirmed a high prevalence of the A2 allele in Gir cattle, consistent with previous reports, and emphasize the need for conservation and selective breeding strategies to maintain and enhance A2 allele-rich herds in India (Mukesh et al., 2022; Yadav et al., 2023; Paradkar et al., 2023; Cartuche-Macas et al., 2025).

MATERIALS AND METHODS

Experimental materials

A total of 200 blood samples were collected from Gir cattle *(Bos indicus)* maintained at Shree Krishna Dairy Farm, Savarkundla, Amreli district, Gujarat, India. The Gir breed, native to the Saurashtra region, is renowned for its high milk yield and disease resistance, making it one of the most important indigenous breeds for A2 milk production (Singh et al., 2020). Blood samples (3 mL each) were collected aseptically from the jugular vein using sterile 5 mL vacutainer tubes containing anticoagulant (heparin). All animals were apparently healthy, unrelated, and selected randomly to avoid sampling bias. Samples were transported on ice and stored at –20°C until further processing (Kumar et al., 2018; Yadav et al., 2021).

Genomic DNA Isolation

Genomic DNA was extracted using the phenol–chloroform–isoamyl alcohol (PCI) method, following the protocol of Patel et al. (2017) with slight modifications. Briefly, 3 mL of heparinised blood was mixed with an equal volume of Solution I (10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl, , and 20 µL Nonidet P40), vortexed, and incubated at –20°C for 15 minutes to lyse red blood cells. The lysate was centrifuged at 10,000 rpm for 10 min at 4°C to pellet nuclei. The pellet was resuspended in 800 µL Solution II (10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl, , 0.5 M NaCl, 2 mM EDTA, and 0.5% SDS) along with 5 µL Proteinase K (20 mg/mL) and incubated at 60°C overnight for complete protein digestion.

The lysate was treated with 500 µL equilibrated phenol followed by 200 µL PCI mixture (25:24:1) to remove proteins. DNA was precipitated with 2 volumes of chilled ethanol and washed with 70% ethanol to remove impurities. The DNA pellet was air-dried and resuspended in nuclease-free water.

The quality of the extracted DNA was verified by 1% agarose gel electrophoresis, while purity and concentration were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) by measuring the A260/A280 ratio. Ratios between 1.7–2.0 were considered acceptable (Khan et al., 2019).

Polymerase Chain Reaction (PCR)

The â-casein gene (CSN2) was targeted for genotyping of A1/A2 alleles. A pair of bovine-specific oligonucleotide primers reported by McLachlan et al. (2006) was used to amplify a 121 bp fragment of exon 7 of the CSN2 gene.

PCR amplification was carried out in a 25 µL reaction volume containing 100 ng genomic DNA, 10 pmol of each primer, 12.5 µL of 2× PCR Master Mix (Thermo Scientific), and nuclease-free water. The amplification was performed in a Veriti™ Thermal Cycler (Applied Biosystems, USA) using the following conditions:

Amplified PCR products were confirmed by electrophoresis on 2% agarose gel stained with ethidium bromide and visualized under a UV transilluminator (Gel Doc™, Bio-Rad).

Restriction Fragment Length Polymorphism (PCR-RFLP) and Electrophoresis

For genotyping of A1/A2 alleles, 10 µL

PCR products were digested with 4 units of *Dde*I restriction enzyme (New England Biolabs, USA) in a final volume of 20 µL. The digestion reaction was carried out overnight at 37°C in a water bath.

The digested products were separated on a 3% agarose gel containing 1× TBE buffer at 120 V for 60–70 minutes. A 50 bp DNA ladder (Thermo Scientific) was used as a molecular size marker. The gel was visualized under a UV transilluminator and photographed for further analysis.

Gene and Genotype Frequency Calculation

Allele frequencies were calculated using the direct counting method, following the principles of Hardy-Weinberg equilibrium (HWE) (Hardy, 1908). The Chi-square test was applied to evaluate deviations between observed and expected genotype frequencies. The allele frequencies were determined using the formulas Genotype frequency for A1A2, A1A1 and A2A2 genotype for beta casein gene was estimated as per the equation 1. Allelic frequency of A1 and A2 alleles for beta casein (CSN2) gene gene was estimated as per the equation

Equation 1: Genotype Frequency = Total number of observed genotypes / Total number of genotypes (Table 3).

Table 1. Primer detail

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

Table 2. Thermal cycler conditons

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

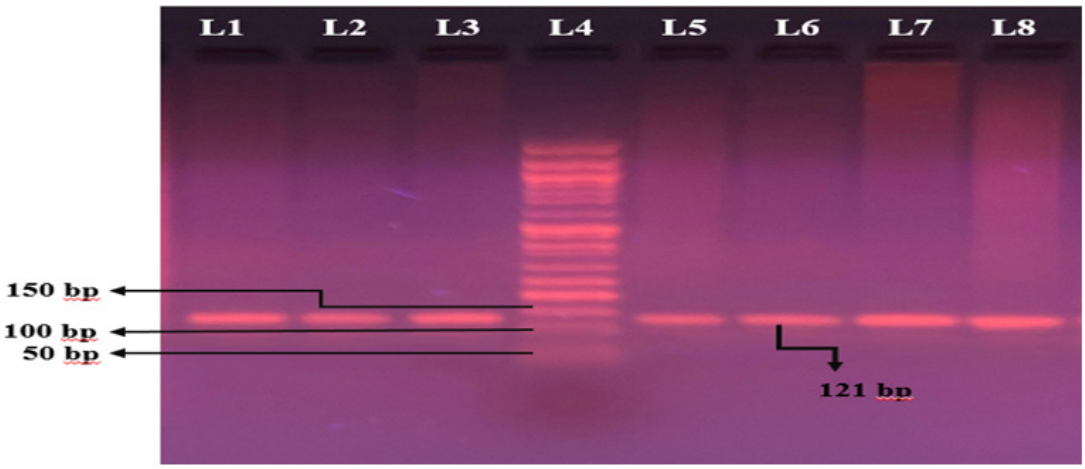

Fig. 1. PCR products of the CSN2 gene were resolved on a 2% agarose gel. Lanes 1–3 (S37–S39) and lanes 5–8 (S40–S43) showed amplification products of approximately 121 bp. Lane 4 contained a 50 bp DNA ladder, which served as a molecular size marker. The bands in all sample lanes corresponded well to the expected product size of 121 bp, indicating successful amplification of the target CSN2 gene fragment

Equation 2: Allelic Frequency Equation Allelic frequency of A1 allele = 2F(A1A1) + F(A1A2))/ 2(Total number of alleles) (Table 4).
Allelic frequency of A2 allele = 2F(A2A2) + F(A1A2))/ 2(Total number of alleles)

where A1A1, A1A2, and A2A2 represent genotype counts, and N is the total sample size. This analysis helps assess the genetic stability of the population and the influence of evolutionary forces on genetic variation (Oliveira et al., 2021).

RESULTS AND DISCUSSION

Genomic DNA extracted from 200 Gir cattle was evaluated for quality and quantity. Electrophoretic assessment on a 1% agarose gel revealed high molecular weight, sharp DNA bands for all samples, indicating excellent integrity suitable for downstream molecular analyses such as PCR and RFLP. DNA purity, measured using a Nanodrop spectrophotometer (Nanodrop 1000, Thermo Scientific, USA), showed A260/ A280 ratios ranging from 1.8 to 2.0, confirming acceptable purity for molecular applications. DNA concentrations varied between 30–250 ng/iL, providing sufficient template for PCR amplification.

PCR amplification of the â-casein gene produced the expected 121 bp fragment in all 200 samples, as confirmed on a 2% agarose gel. These PCR products were then digested with the *Dde*I restriction enzyme to identify A1/A2 polymorphisms.

Table 3. Genotypic frequency of A1 and A2 allele of CSN2 gene

Total no. of animals	Genotype frequency			Total
	A1A1	A2A2	A1A2	
200	00	0.125	0.875	1.0

Table 4. Allelic frequency of A1 and A2 allele of CSN2 gene

Allelic frequency		p-value
A1	A2	
0.06	0.94	<0.0001(P<0.05)

(p<0.05 significant)

The predominance of the A2 allele observed in this study (A2 = 0.94; A1 = 0.06) aligns with findings from Mukesh et al. (2022), who reported an A2 allele frequency of 0.95 in indigenous breeds such as Sahiwal, Tharparkar, and Gir. Similarly, Yadav et al. (2023) documented complete fixation of the A2 allele (frequency = 1.0) in Gir cattle. The slight variation observed

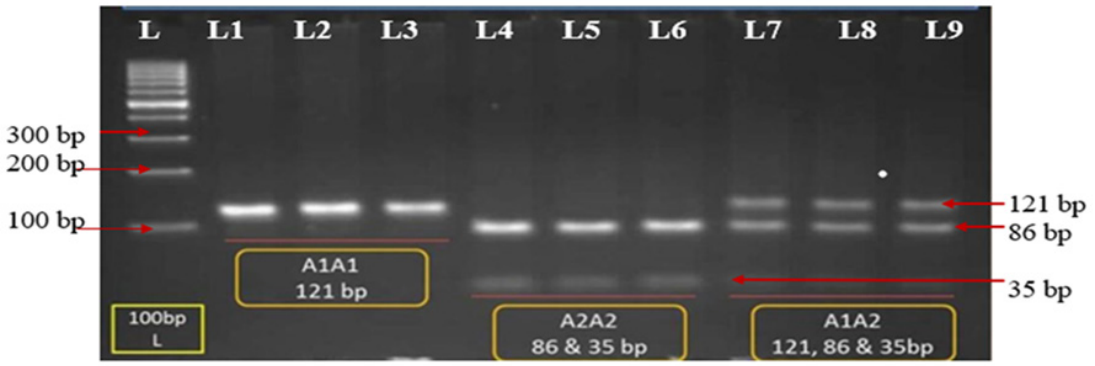

Fig. 2. PCR-RFLP pattern of the CSN2 gene on 3% agarose gel. Lanes 1-3 represented the A1A1 genotype, as shown at 121 bp. Lanes 4 to 6 represented the A2A2 genotype, showing bands at 86 bp and 35 bp. Lanes 7 to 9 represented the A1A2genotype, showing 121, 86, and 35 bp. Lane L represented the Ladder, 100 bp, respectively.

in the current study may be attributed to regional or herd-level differences, smaller sample sizes, or introgression from crossbreeding practices.

Pandey et al. (2019) observed an A2 allele frequency of 0.85 in Sahiwal cattle, while significantly higher A1 allele frequency (0.32) was reported in Holstein Friesian crossbreds. This aligns with the present study's findings, where HF crossbreds showed A1 = 0.29 and A2 = 0.71, confirming that exotic germplasm contributes to increased A1 allele incidence.

At the global level, Karthickeyan et al. (2020) reported high A2 allele frequencies in Ongole and Deoni breeds, whereas Kaminski et al. (2007) reported elevated A1 allele prevalence in European dairy breeds, especially Holstein Friesians. These results highlight a general trend where indigenous breeds conserve the A2 allele, while European breeds selected for higher milk yield tend to carry more A1 alleles.

From a nutritional perspective, the predominance of the A2 allele is significant. Previous studies (Jianqin et al., 2016) have suggested that A2 milk is easier to digest and may reduce gastrointestinal discomfort, in addition to potentially lowering risks of type 1 diabetes, ischemic heart disease, and autism. Therefore, maintaining high A2 allele frequencies in indigenous breeds not only supports genetic diversity but also has public health benefits.

The absence of the A1A1 genotype in Gir cattle further underscores the breed's suitability for selective breeding programs aimed at expanding A2 milk production. Similar observations were made by Joshi et al. (2021) in Red Sindhi and Rathi cattle, reinforcing the idea that most Indian zebu breeds are naturally predisposed toward A2 milk production. However, the presence of A1A2 heterozygotes (12.5%) indicates a risk of A1 allele propagation if crossbreeding with exotic breeds is not carefully managed.

Overall, this study supports the hypothesis that zebu cattle act as reservoirs of the A2 allele. Conservation of indigenous breeds such as Gir, Sahiwal, and Tharparkar, along with well-structured breeding policies that limit introgression of A1 alleles from exotic germplasm, will be crucial for ensuring sustainable A2 milk production in India and beyond.

CONCLUSION

This study establishes the predominance of the A2 allele in Gir cattle (A2 = 0.94; A1 = 0.06), confirming their genetic potential as a sustainable source of A2 milk. The findings, statistically significant ($p < 0.05$), highlight the role of indigenous zebu cattle in safeguarding A2 milk production, which is associated with better digestibility and reduced risk of certain health disorders compared to A1 milk. Conservation and promotion of A2 allele-rich breeds like Gir, Sahiwal, and Tharparkar will be crucial for enhancing human health, nutritional security, and long-term dairy sustainability.

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Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

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 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.

Clinical Trial Registration

This research does not involve any clinical trials.

Permission to reproduce material from other sources

Not Applicable

Author contributions

Shailja Jitendrabhai Vyas - Methodology; software; writing – review and editing; Anjali Kulkarni - Validation, Investigation original draff.

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