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# Comparative Genotypic Analysis of *Beta* Casein Gene in Gir, Holstein Friesian, Jersey, and HF x Sahiwal by PCR-RFLP Method

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## Abstract

*The present study was undertaken with the aim of comparative genotypic analysis of the Beta Casein gene in zebu, exotic, and crossbred cattle. Caseins account for almost 80 % of milk proteins, and Beta Casein alone contributes to 45 % of it. Beta casein has 13 different variants, but the A1 and A2 variants are the most common. A1 Beta Casein is a significant risk factor in several human diseases like coronary heart disease, autism, and Type 1 diabetes. So in the present study, for comparative analysis total of 102 blood and semen samples were collected, which included 51 blood samples and 51 semen samples. From each cattle, both blood and semen samples were collected. Here total of 28 samples from Gir, 28 samples from HF, 26 samples from Jersey, and 20 samples from HF X Sahiwal cattle were collected. Extraction of Genomic DNA was done by the standard phenol-chloroform method. The genotyping of the Beta Casein gene was done by the PCR-RFLP method. All the breeds screened for polymorphism in beta casein gene showed three different genotypes – Homozygous A1A1 genotype, Heterozygous A1A2 genotype and Homozygous A2A2 genotype with respective frequencies 0 %, 7.14% and 92.86% in Gir cattle; 7.14%, 50% and 42.86% in HF cattle; 7.69%, 30.77 and 61.54% in Jersey cattle and 20%, 30% and 50% in HFCB cattle. The highest frequencies of the A2 allele were observed in the Gir breed (0.97). In opposite, in crossbreed (0.35) highest frequencies of the A1 allele were observed.*

**Keywords:** A1A2, Beta-casein, Crossbreed, Exotic Cattle, Indigenous Cattle, PCR-RFLP.

## Introduction

Dairy farming in India is one of the main pillar of the agriculture sector, which play a significant role in the economy of the country, as well as provides one of the basic requirements of nutrition. According to the 20th livestock Census, India accounts for 13.1 % of the global cattle population and 37.3% of the global livestock. There are three cattle breeds worldwide, which include Indigenous or Zebu, Exotic, and Crossbreed cattle. Indigenous breeds are particularly found in South Asian countries; on the other hand, exotic breeds are domesticated in European countries. Indigenous breeds and Exotic breeds can be differentiated by physical characteristics, as indigenous breeds are humped cattle while Exotic cattle are hump less cattle. Indigenous cattle breeds are adapted to harsh agro-climatic conditions because of their low metabolic rate at high temperatures (Pessoa *et al*., 2023). They are more resistant to tropical diseases and have a long breeding life of up to 10 to 12 years. Exotic breeds are less adapted to warm climates, but they can produce more milk as compared to indigenous breeds. To overcome these differences and particularly to increase milk production in Indigenous breeds, crossbreeding was done between Indigenous and Exotic breeds. Crossbreeding resulted in many mixed-breed cattle, which have variations in their genetic makeup. By cross-breeding, several economically important traits are improved in indigenous cattle breeds like milk yield, heat tolerance, and disease resistance. Cross-breeding resulted in a decrease of indigenous cattle breeds in India, and that is a very serious concern(Srinivas *et al*., 2019; Rehman *et al*., 2014).

Today, the consumption of milk is increasing worldwide as milk derivatives provide the main nutritional components like macro and micro nutrients, including proteins, vitamins, carbs, minerals, and immunoglobulin (Kamiński*et al*., 2023; Yadav *et al*., 2023). Bovine milk contains 3.5 % protein (Jayalalitha *et al*., 2022). Milk proteins are classified into two groups: whey and caseins. Whey constitutes about 20 % of the protein content of milk, and the remaining 80 % of the milk protein content consists of caseins (Ganguly *et al*., 2013*;* Shahlla *et al*., 2014). Caseins are classified into four groups: alpha casein S1, alpha casein S2, beta casein and kappa casein (Ben Farhat *et al*., 2023; Kumar *et al*., 2018; Pabitra *et al*., 2022). CSN1S1, CSN1S2, CSN2, and CSN3 genes encode beta casein, and all these protein-encoding genes are localised on chromosome 6 of cattle (Ayaz *et al*., 2023; Raja *et al*., 2021). Beta casein protein is a single polypeptide chain that contains 209 amino acid residues(Patel *et al*., 2020; Muhammed *et al*., 2012). There are 13 different variants of beta casein (Ganguly *et al*., 2013; Suchocki *et al*., 2012). The different variants of beta casein have amino acid changes at different positions but based on common substitution Pro67His, all variants are classified as A2 (A3, E, D, H2, I, J, K, L having proline at position 67) or A1 (B, C, F, G, H1 having histidine at position 67)(Mukesh *et al*., 2022; Rangel *et al*., 2017). A1 and A2 variants are classified into two groups based on Beta casomorphin-7 production. The A2 variant of Beta casein is considered as oldest variant, and other variants originated from it via mutations(Socol*et al*., 2021; Nuomin *et al*., 2022). The A1 allele is considered as the mutant protein where a C to A transition is observed, which produces histidine instead of proline. It is believed that this mutation arose about 5000 years ago and spread widely in various cattle breeds (Kovalyuk *et al*., 2021). Gastrointestinal proteolytic digestion of A1 milk with histidine at position 67 generates a 7 amino acid bioactive peptide ‘opioid’ called Beta casomorphin-7 (BCM-7) (Ardicli *et al*., 2023). While in A2 milk, proline at position 67 prevents the split and peptide of 9 amino acid residues, BCM-9 is released. Beta casomorphin-7 liberated by proteolytic digestion of A1 milk remains intact and enter the bloodstream. Beta casomorphin-7 have been suggested to show many immunological activities like chronic inflammatory responses such as allergy, mucin production, lymphocyte proliferation and skin reactions, heart disease, autism, Type-1 diabetes mellitus and sudden infant death syndrome (SIDF) (Mukesh *et al*., 2022; Barwar*et al*., 2023; Pandey *et al*., 2020). The genotypes of cattle producing A1 milk are A1A1 (homozygous) and A1A2 (heterozygous), the genotype of cattle producing A2 milk is A2A2 (homozygous)(Miluchová *et al*., 2014; Ladyka *et al*., 2023). For genetic improvement, adaptation to variable environmental conditions and evolution, genetic diversity is an essential element on the other hand, in order to define genotypes of cattle and their associations with productive, reproductive and economic traits, determination of gene polymorphism in farm animals breeding is also very essential.

Therefore, present work was done with main goal of comparative genotypic analysis of beta casein gene variants in Zebu, Exotic and HF x Sahiwal crossbred.

## Materials and Methods

### *Sample Collection*

In the present study, 51 semen samples and 51 blood samples were collected from bulls of Gir, Sahiwal, Jersey and

HF x Sahiwal cattle breeds. All samples were collected from the cattle farm of Amul Research and Development Association (ARDA)-Ode (Gujarat). The semen samples were collected as frozen straws. For blood samples, about 2-3 mL of blood was taken in an EDTA tube from the jugular vein using sterilized syringes. The sample set represented the following indigenous as well as exotic breeds: Gir (14 blood and 14 semen samples), Jersey (13 blood and 13 semen samples), Holstein Friesian (14 blood and 14 semen samples), and HF x Sahiwal (10 blood and 10 semen samples).

### *DNA Extraction*

Bovine Genomic DNA was extracted from 2-3 ml of whole blood and 0.25 ml of semen samples by using the standard phenol-chloroform method with slight modifications.

### *Amplification of the Beta Casein Gene by PCR*

121 bp fragment of the beta casein (CSN2) gene was amplified by standardized Polymerase Chain Reaction (PCR). Primers designed by McLachlan *et al*., 2006 were used for the PCR amplification. Sequence of forward primer 5' – CCT TCT TTC CAG GAT GAA CTC CAG G – 3' and sequence of reverse primer 5' – GAG TAA GAG GAG GGA TGT TTT GTG GGA GGC TCT – 3'. The amplification was performed in a total volume of 25 µL of PCR mixture. The composition of the PCR mixture is shown in Table 1.

**Table 1:** Composition of PCR reaction mix

| PCR Components | Volume |
|---|---|
| DNA Template (20 ng/µl) | 5 µl |
| Forward Primer (10 pmol/µl) | 1 µl |
| Reverse Primer (10 pmol/µl) | 1 µl |
| 2 X PCR Master Mix (1X) | 12.5 µl |
| Nuclease free water | 5.5 µl |
| **Total** | **25 µl** |

The following amplification parameters were applied: 95°C for 5 minutes, followed by 30 cycles: 95°C for 30 sec, 63°C for 30 seconds, 72°C for 30 seconds, and at the end reaction was completed by the final extension at 72°C for 5 minutes. PCR products were analyzed using 2 % gel electrophoresis. PCR products were loaded in 2% agarose gel prepared in 1X TBE buffer along with a 50 bp DNA ladder for comparison and electrophoresed at 120 V for 40 to 60 minutes. The gel was visualized under a UV-transilluminator, and the results were recorded.

### *PCR-RFLP*

The PCR products of 121 bp amplified by PCR amplification were digested with the restriction enzyme *Dde* I. The RFLP digestion mixture was incubated at 37°C overnight in a water bath. The composition of the restriction digestion mixture is shown in Table 2.

**Table 2:** Composition of restriction digestion mix

| RFLP Components | Volume |
|---|---|
| 1) Restriction enzyme | 0.3 µl |
| 2) Cutsmart buffer | 2.0 µl |
| 3) PCR product | 10.0 µl |
| 4) Nuclease free water | 7.7 µl |
| **Total** | **20 µl** |

Digested products were loaded in 3% agarose gel prepared in 1X TBE buffer along with 100 bp DNA ladder for comparison and electrophoresed at 120 V for 40 to 60 minutes. Then gel was visualized under UV-transilluminator and results were recorded.

## Results and Discussion

PCR amplified fragments were analyzed using 2 % gel electrophoresis and 121 bp PCR products were determined by comparing it with 50 bp DNA ladder. Electrophoretic analysis of PCR products obtained from blood Genomic DNA is shown in Figure 1.

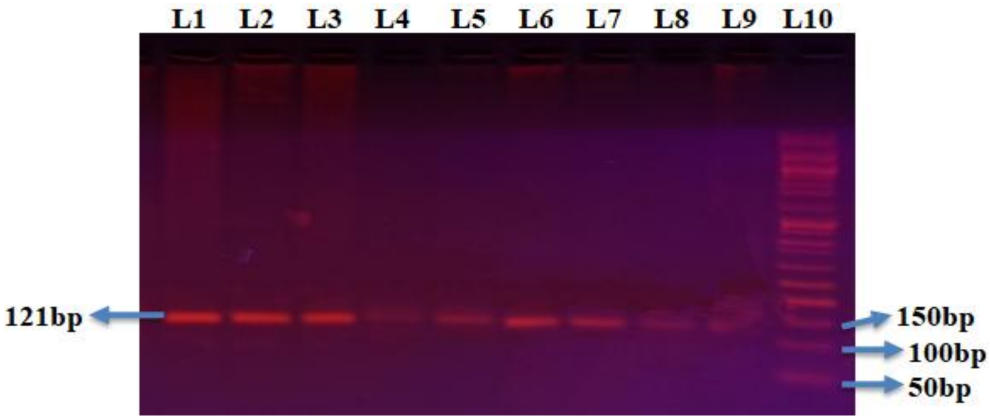


**Figure 1:** Representative results of PCR amplification of Beta Casein Gene, Lanes L1 to L9 – PCR products of 121 bp, Lane L10 – 50 bp DNA ladder

Fragments generated upon digestion of PCR products with *Dde* I were separated by 3 % agarose gel electrophoresis. 100 bp DNA ladder was added for determination of band pattern and genotypes. Restriction digestion and gel analysis revealed the presence of three different genotypes: A1A1, A1A2 and A2A2. A1A1 genotype yield one fragment of 121 bp, the A1A2 genotype yields three fragments of 121 bp, 86 bp and 35 bp where as A2A2 genotype yields two fragments of 86 bp and 35 bp.Electrophoretic analysis of PCR-RFLP products obtained by restriction digestion of PCR products is shown in Figure 2.

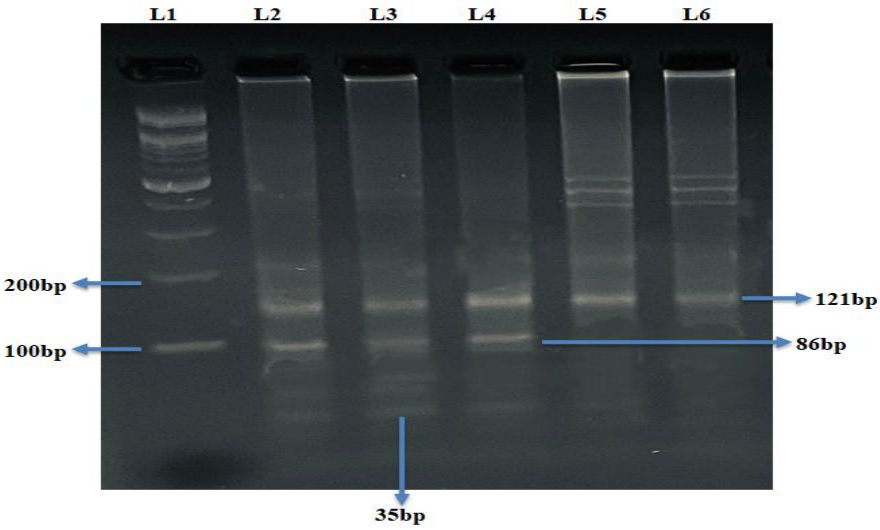


**Figure 2:** Representative results of PCR-RFLP analysis for CSN2 gene, Lane 1 – 100 bp DNA ladder; Lane 2, 3 & 4 – Genotype A1A2 (121 bp, 86 bp, 35 bp); Lane 5 & 6 – Genotype A1A1 (121 bp)

Cattle breeds included in this study showed all three genotypes. Genotyping of Beta casein variants in different cattle breeds revealed that Gir cattle breed showed the high genotypic frequencies for A2A2 genotype. Gir breed shown the highest genotypic frequency for A2A2 genotype (92.86%). Highest genotypic frequency for A1A2 heterozygous genotype was seen in HF breed (50%) followed by Jersey (30.77%) and HF x Sahiwal (30%). A1A1 homozygous genotype was highest in Cross breed (20%) followed by Jersey (7.69%) and HF (7.14%). Mean A1 and A2 allele frequency in Gir cattle breed was 0.030 and 0.97, respectively. Mean A1 and A2 allele frequency in exotic breeds was 0.27 and 0.73, respectively. Cross breed cattle showed 0.35 and 0.65 A1 and A2 allele frequencies, respectively. 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 was estimated as per the equation 2. Detailed genotype and breed wise allele frequencies are shown in table 3 and 4.

**Equation 1:** Genotype Frequency Equation

$$\text{Genotype Frequency} = \frac{\text{Total number of observed genotypes}}{\text{Total number of genotypes}}$$

**Equation 2:** Allelic Frequency Equation

$$\text{Allelic frequency of A1 allele} = \frac{2\text{F(A1A1)} + \text{F(A1A2)}}{2(\text{Total no of alleles})}$$

$$\text{Allelic frequency of A2 allele} = \frac{2\text{F(A2A2)} + \text{F(A1A2)}}{2(\text{Total no of alleles})}$$

**Table 3:** Genotype frequencies of CSN2 gene in different breeds of cattle

| Cattle breed | No. of Blood and Semen samples analyzed | Genotype frequency (%) | | |
|---|---|---|---|---|
| | | **A1A1** | **A1A2** | **A2A2** |
| Gir | 28 | 0 | 7.14 | 92.86 |
| HF | 28 | 7.14 | 50 | 42.86 |
| Jersey | 26 | 7.69 | 30.77 | 61.54 |
| 50%CBHF | 20 | 20 | 30 | 50 |

**Table 4:** Allelic frequency of A1 and A2 allele for CSN2 gene in different breeds of cattle

| Cattle Breed | Allelic Frequency | |
|---|---|---|
| | **A1 allele** | **A2 allele** |
| Gir | 0.03 | 0.97 |
| HF | 0.32 | 0.68 |
| Jersey | 0.23 | 0.77 |
| CBHF | 0.35 | 0.65 |
| **Mean Average** | **0.23** | **0.77** |

Our findings indicate the presence of three distinct genotypes—A1A1, A1A2, and A2A2—across all cattle breeds examined. The genotyping results showed that the Gir breed exhibited the highest frequency of the A2A2 genotype (92.86%), which is notably higher than that observed in other breeds. This high frequency of the A2A2 genotype in Gir cattle suggests a strong genetic predisposition or selective advantage for this variant within this breed. We observed the higher proportions of A1A1 genotype in Crossbreed (20%) compared to other breeds i.e. HF (7.14%) and Jersey (7.69%). None of the Gir cattle was found to be having A1A1 genotype. Half of the HF population being analyzed was found to be having A1A2 genotype, which was highest as compared to other breeds i.e. Gir (7.14%), Jersey (30.77%) and CBHF (30%). The prevalence of the A2A2 genotype in Gir cattle is consistent with studies suggesting that A2A2 variants may be more common in certain local breeds compared to exotic breeds. Similar line of research has been done by Mukesh *et al*., 2022 and found that A2 allele is predominant in native Indian cattle breeds, they reported the high allele frequencies for A2 allele (0.95) in Indian breeds. They also reported the higher percentage of heterozygous genotype A1A2 in crossbred and exotic cattle breeds and stated that there is need to screen sire lines to drift the herd towards A2. Pandey et al., 2019 also studied polymorphism of beta casein gene in Sahiwal and HF crossbred cattle and reported the high A2 allele frequency (0.85) in Sahiwal meanwhile in HF cross breed high A1 allele frequency (0.32) was seen as compared to Sahiwal breed. However, both the cattle breeds showed the high frequencies of A2 allele. Yadav *et al*., 2023 did the study on the status of A1/A2 beta casein gene in Gir and HF crossbred and calculated the respective gene frequency for A1 and A2 alleles, 0.00 and 1.00 in Gir breed and 0.30 and 0.70 in HF crossbred cattle.

## Conclusion

It may be concluded that indigenous Gir breed is having high potential of producing A2 milk as Gir cattle gave high genotypic frequencies of A2A2 Genotype 92.86 percent. Similarly, HF, Jersey and Cross breed cattle gave high genotypic frequencies for heterozygous A1A2 genotype, 50%, 30.77% and 30%, respectively, which leads to the production of A1 milk. Given the higher frequency of the A2A2 genotype in indigenous breeds, these breeds being the natural carrier of A2 allele can be used to meet the global demands of A2 cow milk. In future studies even if adverse effects of A1 milk are validated in humans then Indigenous cattle breeds can contribute in the task of drifting the herds towards A2 (Mukesh et al., 2022).

## Contribution by Authors

All the authors contributed equally to writing the manuscript. The final manuscript was read by all authors and consented to publication.

## Conflict of Interests

There is no conflict of interest.

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