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Mrunal Chaudhari
 Department of Animal
 Genetics and Breeding,
 Mumbai Veterinary College,
 MAFSU, Nagpur,
 Maharashtra, India

Mahadeo Sawane
 Department of Animal
 Genetics and Breeding,
 Mumbai Veterinary College,
 MAFSU, Nagpur,
 Maharashtra, India

Sajid Ali
 Department of Animal
 Genetics and Breeding, College
 of Veterinary and Animal
 Science, MAFSU, Nagpur,
 Maharashtra, India

Mahesh Chopade
 Department of Animal
 Genetics and Breeding, College
 of Veterinary and Animal
 Science, MAFSU, Nagpur,
 Maharashtra, India

Vikrant Pawar
 Department of Animal
 Genetics and Breeding,
 Mumbai Veterinary College,
 MAFSU, Nagpur,
 Maharashtra, India

Corresponding Author:
Vikrant Pawar
 Department of Animal
 Genetics and Breeding,
 Mumbai Veterinary College,
 MAFSU, Nagpur,
 Maharashtra, India

PCR-RFLP analysis of lactoferrin (Intron-6) gene in Red Kandhari cattle

Mrunal Chaudhari, Mahadeo Sawane, Sajid Ali, Mahesh Chopade and Vikrant Pawar

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Abstract

The present study aimed to investigate the association between bovine lactoferrin (LTF) intron-6 genotypes and milk traits in Red Kandhari cows. Blood and milk samples were collected from 30 lactating cows in Sayala Khating village, Parbhani district, Maharashtra. Genomic DNA was extracted and subjected to PCR amplification of a 301 bp intron 6 fragment, followed by EcoRI-based PCR-RFLP analysis. All animals were found monomorphic, with fixation of the 'A' allele and genotype frequency of AA = 1.00. The mean values ($\pm$ SE) for milk composition were: fat $4.28 \pm 0.05\%$, solid-not-fat (SNF) $8.44 \pm 0.04\%$, protein $3.39 \pm 0.03\%$, and lactose $4.41 \pm 0.03\%$. Association studies could not be established due to the monomorphic pattern of LTF intron-6 region. The findings underscore the need for expanding sample sizes and incorporating advanced genomic approaches to better understand the genetic architecture underlying milk traits in indigenous cattle breeds like Red Kandhari.

Keywords: Lactoferrin (intron-6) gene, PCR-RFLP, Red Kandhari cattle, milk traits

Introduction

Milk production in dairy cattle is a complex trait influenced by both genetic and environmental factors. Conventional genetic improvement has primarily relied on phenotypic selection and progeny testing. However, the sequencing of the bovine genome in 2007 revealed approximately two million single nucleotide polymorphisms (SNPs), many of which serve as functional markers for production and health traits. Among candidate genes, the lactoferrin (LTF) gene has been identified as a key regulator of milk quality and mammary gland health (Ogorevc *et al.*, 2009) [8]. LTF encodes an iron-binding glycoprotein involved in antimicrobial defence, immune modulation, and maintenance of udder health. It's concentration in milk is inversely associated with somatic cell count, making it a reliable biomarker for mastitis resistance.

The bovine LTF gene is located on chromosome 22q24, spans 23-35 kbp, and comprises 17 exons and 16 introns, with introns 4 and 6 reported to be highly polymorphic and associated with milk traits in exotic and crossbred cattle. Beyond its primary role in iron transport and antimicrobial defence, variations in the LTF gene have been linked to milk production parameters and somatic cell counts. Such associations underpin the utility of LTF as both a marker for milk quality and a candidate for improving udder health through genetic selection (Sharma *et al.*, 2015) [15].

Red Kandhari cattle, an indigenous breed of Maharashtra, India, represent an important genetic resource well adapted to local agro-climatic conditions. Traditionally valued as draft animals, these cattle produce modest milk yields (1.5-4.0 kg/day) with relatively high fat content (average 4.57%). Their disease resistance, adaptability to harsh climates, and ability to sustain on low-quality fodder highlight their role in sustainable livestock production. Nonetheless, the breed's productivity has declined due to weak selection pressure and indiscriminate crossbreeding with exotic breeds, leading to genetic dilution. Recognizing this, the Government of India has launched conservation and improvement programs of indigenous cattle such as the Rashtriya Gokul Mission. Genomic selection, utilizing molecular markers like SNPs and functional genes such as LTF, offers a promising strategy for enhancing milk traits while preserving breed integrity. In this context, the present study was undertaken to investigate polymorphism in intron-6 of the LTF gene in Red Kandhari

cattle using PCR-RFLP and to assess its potential association with milk traits.

Materials and Methods

Sample Collection and DNA Extraction

The study was conducted on 30 lactating Red Kandhari cows from Sayala Khating village, Parbhani district, Maharashtra. For each animal, both milk and blood samples were collected following standard ethical protocols. Blood samples were subjected to genomic DNA extraction using the conventional Phenol: Chloroform: Isoamyl alcohol (P:C:I) method. DNA concentration ranged from 46.6 ng/μl to 152.7 ng/μl, with an optical density (OD) ratio between 1.8 and 2.0, indicating high purity subjected for downstream molecular analyses.

PCR amplification of LTF Intron 6

The 301 bp intron 6 region of the bovine lactoferrin gene was amplified using primers F: 5'-GCC TCA TGA CAA CTC CCA CAC-3' and R: 5'-CAG GTT GAC ACA TCG GTT GAC-3' described by Seyfert and Kuhn (1994) [13]. PCR reactions were optimized for 25 μl volume containing TaKaRa 2X PCR Master Mix, specific primers (10 pmole each), template DNA, and nuclease-free water. The thermal cycling profile included an initial denaturation at 95 °C for 5 minutes; 34 cycles of denaturation at 94 °C for 1 minute, annealing at 62 °C for 1 minute, extension at 72 °C for 1 minute; and a final extension at 72 °C for 10 minutes. Amplified products (301 bp) were visualized using 1.7% agarose gel electrophoresis with ethidium bromide staining, and bands were compared to a 100 bp DNA ladder.

Restriction Fragment Length Polymorphism (RFLP) Analysis

For polymorphism analysis, 10 μl of PCR product was digested with *EcoRI* (1 μl) restriction enzyme in a 20 μl reaction containing 2 μl buffer and 7 μl nuclease-free water, incubated at 37 °C for 18 h. Digested fragments were separated on 2.5% agarose gel and visualized under UV light.

Milk Trait Analysis

Milk samples were analysed for key compositional traits, including fat, solid-not-fat (SNF), protein, and lactose content, using standard biochemical methods. Mean values and standard errors were calculated across the sampled population.

Data Analysis and Association Testing

Allele and genotype frequencies were calculated based on RFLP band patterns. Due to the monomorphic nature of the locus, association analysis between LTF genotypes and milk traits could not be conducted. Nevertheless, descriptive statistics for milk composition traits were reported.

Results and Discussion

Genomic DNA extracted from Red Kandhari cows exhibited high purity and integrity, as confirmed by spectrophotometric analysis and agarose gel electrophoresis. PCR amplification of the LTF intron 6 region consistently yielded the expected 301 bp band at 62 °C annealing temperature in all samples, indicating successful primer design and reaction optimization (Plate 1). A 301 bp LTF intron 6 fragment has likewise been amplified in several

cattle breeds, confirming the wide applicability of this primer pair (Seyfert and Kuhn, 1994; Wojdak-Maksymiec *et al.*, 2006; Sender *et al.*, 2006; Gursel *et al.*, 2013; Patel *et al.*, 2015; Sharma *et al.*, 2020; Lavanya, 2022) [13, 18, 12, 2, 9, 16, 5].

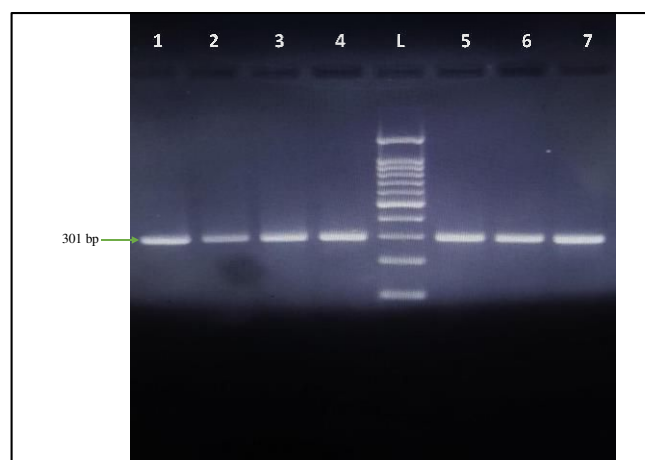


Plate 1: Agarose gel (1.7%) electrophoresis of bovine lactoferrin (intron 6) gene PCR amplicon (301 bp) in Red Kandhari Cow. Lane-1-4 and 5-7, Lane L-100 bp DNA ladder

Restriction digestion with *EcoRI* revealed a monomorphic pattern across all 30 sampled Red Kandhari cows. All individuals displayed the 'AA' genotype (301 bp), corresponding to fixation of the 'A' allele at this locus (Plate 2). Animals with genotype 'AB' (301 bp, 201 bp, and 100 bp) and 'BB' (201 bp, and 100 bp) were not observed in present study. No alternative alleles or genotypes were detected, precluding the calculation of heterozygosity or assessment of Hardy-Weinberg equilibrium. The absence of polymorphism in the LTF intron 6 region among studied Red Kandhari cows may reflect breed-specific evolutionary histories, selection pressures, and demographic events.

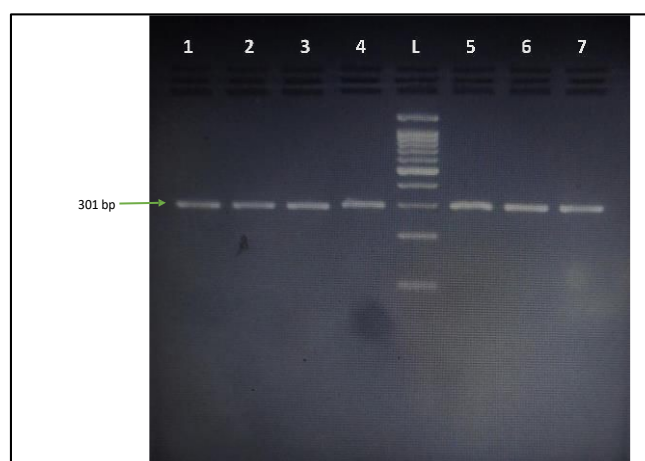


Plate 2: Agarose gel (2.5%) electrophoresis of *EcoRI*/PCR-RFLP products in Red Kandhari cow. Lane 1-4 and 6-8: 'AA' genotype (301 bp) Lane L: 100 bp DNA ladder

Similar findings were reported by Saran *et al.* (2023) [11], who reported fixation of the 'A' allele (frequency = 1.0) at the LTF intron 6 locus in Rathi and Sahiwal cattle using *EcoRI*/PCR-RFLP. In the same study, Tharparkar cattle exhibited a high frequency of the AA genotype (0.966) with rare occurrence of AB (0.034), whereas Kankrej cattle showed relatively lower AA frequency (0.694) and higher B

allele frequency (0.1667). Similarly, Dhond (2023) ^[1] in Dangi cows reported near fixation of the A allele (0.95) with genotype frequencies of 0.90 (AA) and 0.10 (AB).

Comparable results were obtained in other indigenous and crossbred populations, where polymorphism was limited to AA and AB genotypes with predominance of the A allele. In Holstein Friesian (HF) and HF crossbreds, AA and AB genotype frequencies have been reported in the range of 0.606-0.77 and 0.23-0.393, respectively (Maletic *et al.*, 2013; Hemati *et al.*, 2014; Nanaei *et al.*, 2016; Zinnatov *et al.*, 2020; Lavanya, 2022) ^[6, 3, 7, 19, 5]. In purebred Gir cattle, Lavanya (2022) ^[5] observed AA (0.767) and AB (0.233), while in Deoni cows Shivshankar *et al.* (2022) ^[17] reported AA (0.71) and AB (0.29), with the B allele occurring at a low frequency (0.15). Notably, the BB genotype was absent across these studies, reinforcing the predominance of the A allele in *Bos indicus* breeds.

Contrastingly, all three genotypes (AA, AB, BB) have been reported in European and other exotic breeds. Seyfert and Kuhn (1994) ^[13] in German Black Pied cattle, Sender *et al.* (2006) ^[12] in Holstein, Wojdak-Maksymiec *et al.* (2006) ^[18] in Polish Black-and-White, and Sharifzadeh and Doosti (2011) ^[14] in Iranian Holstein populations reported substantial frequencies of the BB genotype, ranging from 0.30 to 0.59. Similarly, studies on South Anatolian Red, East Anatolian Red (Gursel *et al.*, 2013) ^[2], Holstein (Rodriguez *et al.*, 2013) ^[10], and Jersey crossbreds (Sharma *et al.*, 2020) ^[16] confirmed the occurrence of all three genotypes. These findings suggest that while the A allele is predominant in indigenous zebu breeds, European breeds and their derivatives retain higher levels of polymorphism, including the BB genotype.

Descriptive analysis of milk composition revealed mean values ($\pm$ SE) of $4.28 \pm 0.05\%$ for fat, $8.44 \pm 0.04\%$ for solid-not-fat (SNF), $3.39 \pm 0.03\%$ for protein, and $4.41 \pm 0.03\%$ for lactose. These values were broadly comparable to the findings of Ghafoor *et al.* (1980), who reported $4.57 \pm 0.03\%$ fat, $8.62 \pm 0.01\%$ SNF, $3.17 \pm 0.03\%$ protein, and $4.24 \pm 0.04\%$ lactose in Red Kandhari cows. These findings reaffirm the breed's characteristic profile of high milk fat and moderate protein content.

In the present study, all 30 Red Kandhari cows were found to carry the 'AA' genotype, indicating a monomorphic nature of the LTF (intron 6) gene in this population. Consequently, association analysis between LTF genotypes and milk traits could not be performed. In various studies non-significant associations between bovine LTF (intron 6) polymorphism and milk composition traits have been reported in Gir, HF crossbreds (Lavanya, 2022) ^[5], and Dangi cattle (Dhond, 2023) ^[1]. Nanaei *et al.* (2016) ^[7] observed no significant effect of LTF genotypes on 305-day milk yield, protein percentage, pregnancy length, or milking days in Holstein Friesian cattle, although AB cows had higher fat percentage and somatic cell score (SCS) than AA. Contrastingly, several studies have highlighted the role of LTF polymorphism in milk traits and udder health. For instance, Wojdak-Maksymiec *et al.* (2006) ^[18] and Rodriguez *et al.* (2013) ^[10] reported significant associations between LTF genotypes, SCC, and milk protein in Holstein cows. Similar associations with milk yield and composition were noted in HF and Ayvengo line cattle (Zinnatov *et al.*, 2020; Khalil *et al.*, 2021) ^[19, 4]. Furthermore, Sharifzadeh and Doosti (2011) ^[14], Hemati *et al.* (2014) ^[3], Patel *et al.* (2015) ^[9], and Saran *et al.* (2023) ^[11] documented significant

relationships between LTF genotypes and SCC/mastitis resistance in different cattle populations. Shivshankar *et al.* (2022) ^[17] further suggested that the 'AA' genotype/A allele of the LTF gene may confer mastitis resistance in Deoni cows.

The utility of a genetic marker in selection programs hinges on its polymorphism and functional relevance to the trait of interest. The fixation of the 'A' allele at the LTF intron 6 locus in Red Kandhari cattle suggests that this marker is currently uninformative for within-breed selection. However, markers that are polymorphic in other regions of the genome or unscreened animals in the breeding tract may still be valuable for introgression strategies aimed at improving milk traits.

Conclusion

The present study demonstrated a monomorphic pattern of the bovine lactoferrin (LTF) intron 6 gene in Red Kandhari cattle, with complete fixation of the 'A' allele. Consequently, no association could be established between LTF genotypes and milk composition traits, despite recording consistent values for fat, SNF, protein, and lactose. These findings highlight the genetic uniformity of this locus in the breed and emphasize the necessity of evaluating larger populations and exploring additional genomic regions or markers. Such approaches will be crucial for uncovering potential genetic variability and strengthening breeding strategies aimed at improving milk traits while conserving the unique genetic identity of Red Kandhari cattle.

Conflict of Interests

Authors declare no Conflict of Interests for this article.

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