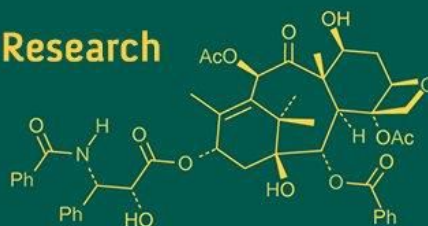
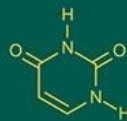
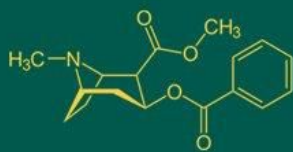


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Genetic polymorphism of the osteopontin gene and milk composition traits in Red Kandhari cattle

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Abstract

Osteopontin (OPN) is an important multifunctional glycoprotein involved in milk production, bone metabolism, and immune regulation in cattle, making it a potential candidate gene for production trait studies. The present investigation aimed to detect polymorphism in the intron-4 region of the bovine OPN gene and to evaluate its association with milk composition traits in Red Kandhari cattle. A total of 30 lactating Red Kandhari cows were genotyped using the *BsrI*/PCR-RFLP technique. A 290 bp fragment of the OPN intron-4 region was amplified through touchdown PCR using annealing temperatures ranging from 63 °C to 53 °C, followed by restriction digestion with *BsrI* enzyme. PCR-RFLP analysis revealed two genotypes, TT (290 bp) and CT (290, 200, and 90 bp), while the CC genotype was absent in the studied population. The observed frequencies of TT and CT genotypes were 0.97 and 0.03, respectively, with corresponding allele frequencies of 0.983 for T and 0.017 for C, indicating predominance of the T allele. Cows carrying the TT genotype exhibited mean fat, SNF, protein, and lactose contents of 4.27 ± 0.05 , 8.43 ± 0.04 , 3.38 ± 0.03 , and 4.40 ± 0.03 per cent, respectively. The single CT genotype cow showed slightly higher values for these traits. Due to limited genotype variability, statistical association between OPN intron-4 polymorphism and milk composition traits could not be established. The study confirms polymorphism at the OPN intron-4 locus in Red Kandhari cattle and highlights the need for large-scale molecular screening to elucidate the functional role of OPN in indigenous cattle.

Keywords: Osteopontin (intron -4) gene, PCR-RFLP, Milk traits, Red Kandhari cattle

Introduction

India has emerged as the world's leading milk producer, recording 254.19 million tonnes of milk in 2022-23, a ~35.4% increase over 2018-19 (Anonymous, 2024-25). Dairy farming supports the livelihoods of millions of rural households; however, this impressive national output masks a critical challenge. Despite having the largest cattle population, milk yield per animal in India remains low, especially in indigenous cattle, which produce on average 4.03 kg of milk per day, compared with 11.36 kg in exotic and 8.32 kg in crossbred cows. Improving productivity in indigenous cattle is therefore essential to meet the growing demand for milk while preserving adaptability and sustainability.

Conventional breeding approaches have contributed to genetic progress, but their effectiveness is limited by the late expression and moderate heritability of milk production traits. In recent years, molecular breeding strategies, such as marker-assisted and genomic selection, have provided powerful tools for accelerating genetic improvement. Among the available molecular markers, single nucleotide polymorphisms (SNPs) are particularly valuable for identifying genetic variations associated with economically important traits. Several candidate genes influencing milk yield and composition have been reported, with the osteopontin (OPN) gene receiving considerable attention.

The bovine OPN gene, also referred to as ETA-1 or secreted phosphoprotein-1 (SPP-1), is located on chromosome 6 and consists of seven exons (Sodek *et al.*, 2000) [27]. Osteopontin is a multifunctional phosphoglycoprotein expressed in various tissues and body fluids, including milk. Its expression is notably elevated during lactation, and it plays a key role in mammary gland development, cellular differentiation, and immune regulation, all of which are closely linked to milk production (Nagatomo *et al.*, 2004) [17]. Previous studies have demonstrated significant associations between OPN gene polymorphisms and milk yield,

milk composition, somatic cell count, and lactation persistency in exotic and crossbred cattle populations (Leonard *et al.*, 2005; Khatib *et al.*, 2007; Alain *et al.*, 2009; Boleckova *et al.*, 2012; De Mello *et al.*, 2012; Kowalewska-Luczak and Kulig, 2013^a, 2013^b; Salehi *et al.*, 2015; Manzoor *et al.*, 2016; Bissonnette, 2018) [15, 10, 1, 5, 7, 11, 12, 24, 16, 4]. In contrast, similar information for Indian indigenous breeds remains limited, restricting the effective use of molecular tools in their improvement.

Red Kandhari cattle, an important indigenous breed of the Marathwada region, are primarily distributed in the Latur, Nanded, and Parbhani districts of Maharashtra. Locally known as *Lal Kandhari*, this breed is valued for its hardiness, adaptability to harsh climatic conditions, disease resistance, and high breeding efficiency. Although traditionally valued as draught animals, Red Kandhari cows produce 400-600 kg of milk per lactation, with an average fat content of 4.6% (NBAGR, 2020) [18], indicating clear potential for genetic enhancement of milk traits.

The National Cattle Breeding Policy emphasizes genetic enhancement of indigenous cattle through selective breeding and modern biotechnological interventions. Early and accurate identification of elite germplasm carrying favourable alleles is essential for accelerating genetic progress. Given the economic and adaptive importance of Red Kandhari cattle and the proven role of the OPN gene in milk production traits, the present study was undertaken to explore the genetic variability of the OPN (intron-4) gene and its association with milk traits in this indigenous breed.

Materials and Methods

Animal selection and sample collection

The study was conducted at the Genetic Investigation Laboratory (GIL), Department of Animal Genetics and Breeding, Mumbai Veterinary College, Mumbai. A total of 30 lactating Red Kandhari cows maintained at a private herd in Sayala Khating village, Parbhani district, Maharashtra were selected for the study. Blood samples (4-6 ml) were collected aseptically in EDTA vacutainers and stored at 4°C until analysis. Milk samples (10 ml) were collected after complete milking and analyzed on the same day for fat, protein, SNF, and lactose using a Lactoscan milk analyser.

Genomic DNA extraction and quality assessment

Genomic DNA was isolated from blood samples using the phenol-chloroform-isoamyl alcohol method described by Sambrook and Russell (2006) [23]. The concentration and purity of DNA were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, USA), while DNA integrity was verified by electrophoresis on 0.8% agarose gel. DNA samples with an OD260/280 ratio of 1.8-2.0 were used for further analysis and diluted to a working concentration of 50-100 ng/μl.

PCR amplification of the Osteopontin gene

Polymorphism analysis was performed for the intron-4 region of the Osteopontin (OPN) gene. A 290 bp fragment was amplified using primers (F 5'-GCAAATCAGAAGTGTGATAGAC-3' and R 5'-CCAAGCCAAACGTATGAGTT-3') reported by Leonard

et al. (2005) [15]. PCR was performed in a 25 μl reaction volume containing PCR master mix (Takara Bio), primers, genomic DNA, and nuclease-free water, with a negative control included. Amplification was carried out using an initial denaturation at 95°C for 5 min, followed by 32 cycles of denaturation at 94°C for 45 s, touchdown annealing from 63°C to 53°C (−2°C per cycle) for 45 s, extension at 72°C for 45 s, and a final extension at 72°C for 7 min. PCR products were resolved on 1.8% agarose gel.

PCR-RFLP analysis

Genotyping of the OPN intron-4 region was performed using the *Bsr*I restriction enzyme to detect polymorphism. PCR products were digested with *Bsr*I at 65°C for 4.5 hours, followed by electrophoresis on 2.5% agarose gel. Restriction patterns were visualized under UV illumination and documented using a gel documentation system. Genotypes were determined based on fragment size comparison with a 100 bp DNA ladder.

Estimation of genotype and allele frequencies

Genotype and allele frequencies of the Osteopontin gene were calculated using standard population genetics methods based on observed genotype counts.

Results

The present study was conducted to evaluate polymorphism in the intron-4 region of the Osteopontin (OPN) gene in Red Kandhari cattle and to assess its association with milk composition traits. PCR amplification of the target region was successfully optimized using a touchdown annealing protocol ranging from 63 to 53 °C. The optimized PCR conditions resulted in clear and specific amplification with minimal non-specific products. All genomic DNA samples yielded a uniform PCR amplicon of 290 bp (Fig. 1) corresponding to the intron-4 region of the OPN gene. The consistency of amplification across all samples indicated the reliability and reproducibility of the assay.

PCR-RFLP analysis of the amplified products using the *Bsr*I restriction enzyme revealed two genotypes, TT and CT. The TT genotype was characterized by a single undigested fragment of 290 bp, whereas the CT genotype produced three fragments of 290 bp, 200 bp, and 90 bp (Fig. 2). Among the 30 Red Kandhari cows analyzed, 29 animals (0.97) exhibited the TT genotype, while only one animal (0.03) showed the CT genotype. The CC genotype was not detected in the studied population. Allelic frequency analysis showed a predominance of the T allele (0.983) over the C allele (0.017), indicating near fixation of the T allele at the OPN intron-4 locus in Red Kandhari cattle.

Milk composition traits were recorded for all animals included in the study. Cows with the TT genotype showed mean fat, SNF, protein, and lactose percentages of 4.27 ± 0.05 , 8.43 ± 0.04 , 3.38 ± 0.03 , and 4.40 ± 0.03 , respectively. The single animal with the CT genotype exhibited slightly higher values for these traits; however, due to the presence of only one CT genotype animal, statistical association analysis between OPN intron-4 genotypes and milk composition traits could not be performed.

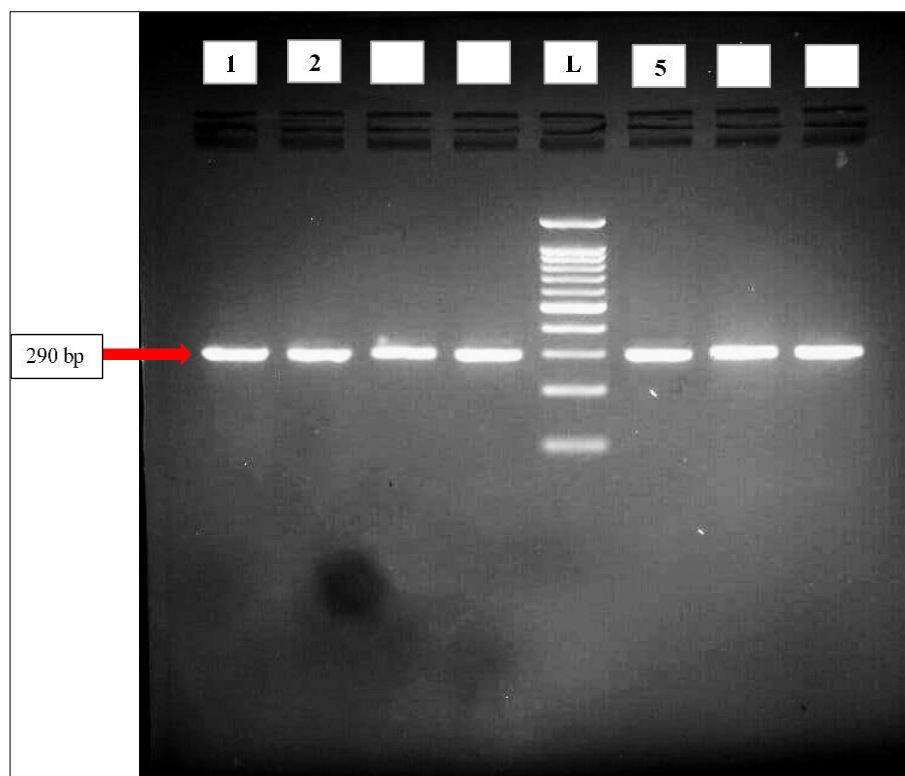


Fig 1: 290 bp PCR amplicon of OPN (intron 4) gene in Red Kandhari Cow. Lane 1-7 290 bp PCR product ; Lane L- 100 bp DNA marker

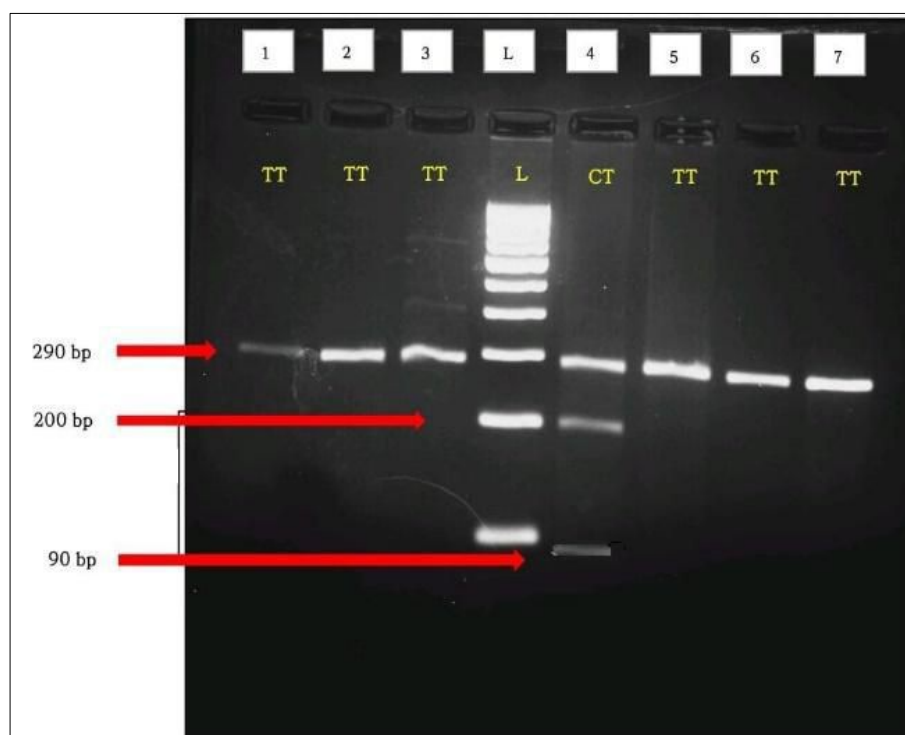


Fig 2: *Bsr* I restriction digestion of 290 bp OPN (intron-4) gene fragment, Lane 1-3 and 5-7: TT Genotype ; Lane 4 : CT Genotype; Lane L- 100 bp DNA Ladder

Discussion

The successful amplification of a 290 bp fragment corresponding to the intron-4 region of the OPN gene under optimized PCR conditions demonstrates the efficiency of the touchdown annealing strategy employed in the present study. Similar annealing profiles and amplicon sizes for OPN intron-4 have been reported earlier in various indigenous and exotic cattle breeds, confirming the methodological consistency and reliability of the assay

(Leonard *et al.*, 2005; Sharma *et al.*, 2019; Kate *et al.*, 2023; Shingane, 2024) [15, 25, 8, 26].

The predominance of the TT genotype and the high frequency of the T allele observed in Red Kandhari cattle are in agreement with findings reported in several Indian indigenous breeds, including Gir, Gaolao, Dangi, Sahiwal, and Haryana cattle (Sharma *et al.*, 2019; Shingane, 2024; Kate *et al.*, 2023; Karwa, 2023) [25, 26, 8, 9], as well as East Anatolian Red cattle (Oztabak *et al.*, 2008) [19]. In contrast, studies in exotic and crossbred cattle populations have

consistently reported the presence of all three genotypes (TT, CT, and CC) with comparatively balanced allele frequencies (Leonard *et al.*, 2005; Khatib *et al.*, 2007; De Mello *et al.*, 2011; Boleckova *et al.*, 2012) ^[15, 10, 6, 5]. The absence of the CC genotype and near fixation of the T allele in Red Kandhari cattle may be attributed to breed-specific evolutionary forces such as natural selection, adaptation to local agro-climatic conditions, restricted gene flow, or genetic drift.

The association between OPN intron-4 polymorphism and milk composition traits could not be statistically evaluated in the present study due to extremely low genotype variability, particularly the occurrence of only one CT genotype animal. Similar limitations have been reported earlier in Dangi cattle, where the presence of only a single CT genotype animal precluded meaningful association analysis (Karwa, 2023) ^[9]. Although the CT genotype cow exhibited slightly higher milk composition values, these observations remain indicative rather than conclusive.

Previous studies investigating the association of OPN intron-4 polymorphism with milk production traits have yielded contradictory results. Several researchers reported significant associations of the C allele with milk fat and protein percentages in Holstein, Jersey, and Czech Fleckvieh cattle (Leonard *et al.*, 2005; Khatib *et al.*, 2007; Boleckova *et al.*, 2012; Kowalewska-Luczak and Kulig, 2013^a) ^[15, 10, 5, 11]. Conversely, other studies identified the T allele as favourable for milk fat percentage or milk yield (Pasandideh *et al.*, 2015; De Mello *et al.*, 2012) ^[20, 7]. In contrast, numerous investigations reported no significant association between OPN intron-4 polymorphism and milk production traits in both indigenous and exotic cattle breeds (Salehi *et al.*, 2015; Zakizadeh *et al.*, 2015; Kulaj *et al.*, 2019; Lali *et al.*, 2020; Shingane, 2024) ^[28, 13, 14, 26]. These inconsistencies across studies may be attributed to differences in breed composition, sample size, production systems, statistical models, and linkage disequilibrium patterns between the investigated polymorphism and causative variants.

Beyond intron-4, polymorphisms in other regions of the OPN gene have demonstrated functional relevance for economically important traits. Associations with milk protein content, lactation persistency, mammary gland health, and metabolic disorders have been reported in both cattle and buffalo populations, indicating that the influence of OPN polymorphisms on production traits is locus-specific and breed-specific (Manzoor *et al.*, 2016; Bissonnette, 2018; Raschia *et al.*, 2018; Raza *et al.*, 2021; Bauer *et al.*, 2023) ^[16, 4, 21, 22, 3].

Conclusion

The present study confirmed the presence of polymorphism at the OPN intron-4 *BsrI* locus in Red Kandhari cattle, characterized by a predominance of the TT genotype and T allele and absence of the CC genotype. However, due to limited genotype variability, the association between OPN intron-4 polymorphism and milk composition traits could not be established. These findings emphasize the need for large-scale studies across the entire breeding tract of Red Kandhari cattle to identify additional polymorphisms and to better elucidate the role of the OPN gene in milk production and adaptation of indigenous cattle.

Conflict of interest: Authors declare no Conflict of Interests for this article.

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