



GENOTYPIC UNIFORMITY OF ACYL-COA SYNTHETASE LONG-CHAIN FAMILY MEMBER 1 GENE AND MILK QUALITY PARAMETERS IN INDIGENOUS RED KANDHARI COWS

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ABSTRACT

The present study explored polymorphism in *ACSL1* gene, a candidate gene involved in fatty acid metabolism and milk fat synthesis, in thirty lactating Red Kandhari cattle. Two reported SNP regions, g.35446C>T and g.35941G>A, were targeted for genotyping. The polymerase chain reaction for SNP g.35446C>T, and g.35941G>A yielded PCR amplicon of 148 and 142 bp, respectively. SSCP analysis revealed no mobility variation, and subsequent Sanger sequencing confirmed monomorphic patterns with homozygous CC and GG genotypes, respectively. These findings indicated that the selected *ACSL1* regions are conserved in this indigenous breed. Although milk trait data—including fat percentage, protein percentage, lactose, solid-not-fat, somatic cell score, and milk yield—were collected, the absence of polymorphism precluded genotype-phenotype association analysis. Documenting monomorphic loci is scientifically relevant as it establishes a genetic baseline for Red Kandhari cattle and contributes to the broader understanding of breed-specific genetic diversity. This study represents the first report of *ACSL1* gene monomorphism in Red Kandhari cattle. The lack of polymorphism limited association analysis with milk traits and the results underscore the importance of expanding genetic studies to include additional SNPs, larger populations, and comparative analyses across breeds.

Keywords: Genetic association, milk, monomorphic, polymerase chain reaction, Red Kandhari

INTRODUCTION

The acyl-CoA synthetase long-chain family member 1 (*ACSL1*) gene encodes a key enzyme responsible for the activation of long-chain fatty acids, an essential step in lipid metabolism. High levels of *ACSL1* expression are observed in metabolically active tissues such as liver, adipose tissue, skeletal muscle, and mammary gland (Muioio *et al.*, 2000; Widmann *et al.*, 2011), reflecting its pivotal role in energy homeostasis and milk fat synthesis. Transcriptomic studies in dairy cattle have revealed that *ACSL1*, along with other lipid metabolism-related genes such as acetyl-CoA carboxylase alpha (*ACACA*), glycerol-3-phosphate acyltransferase, mitochondrial (*GPAM*), fatty acid synthase *FASN*, lipin 1 (*LPIN1*), and acyl-CoA synthetase long-chain family member 6 (*ACSL6*), is significantly up-regulated during lactation (Bionaz and Looor, 2008; Singh *et al.*, 2023), underscoring its importance in milk production traits.

In cattle (*Bos taurus*), *ACSL1* gene spans 64,883 bp, comprises 23 exons and 22 introns, and is located on chromosome 27 (Bionaz and Loor, 2008). The *ACSL1* protein is localized to the outer mitochondrial membrane (Soupene and Kuypers, 2008) and plays a significant role in triglyceride, phospholipid, and cholesterol ester biosynthesis as well as fatty acid oxidation (Soupene *et al.*, 2010; Ellis *et al.*, 2010). In skeletal muscle, *ACSL1* is found in vesicles containing glucose transporter 4 (GLUT4), and its deficiency impairs glucose homeostasis (Grevengoed *et al.*, 2015). Functionally, bovine *ACSL1* catalyses the conversion of long-chain fatty acids, ATP, and coenzyme A into long-chain fatty acyl-CoA, a critical step in fatty acid metabolism (Bernard *et al.*, 2005). Notably, in buffalo mammary tissue, *ACSL1* exhibits the highest expression levels (Liang *et al.*, 2020). Quantitative trait locus (QTL) studies have also linked *ACSL1* to variations in the relative concentrations of unsaturated fatty acids (UFAs), omega-3 fatty acids, polyunsaturated fatty acids, and docosahexaenoic acid (DHA) (Widmann *et al.*, 2011).

Genetic variation within *ACSL1* is associated with differences in milk fat content in various livestock species. For example, in yaks, specific *ACSL1* gene mutations reportedly enhance milk fat yield (Zhao *et al.*, 2019). Despite its functional importance, the genetic polymorphism of *ACSL1* remains largely unexplored in indigenous Indian cattle breeds. 'Red Kandhari', a hardy draught and dual-purpose breed native to Maharashtra (India), which is valued for its adaptability and milk quality. Investigating *ACSL1* polymorphisms in this breed could provide valuable insights into its role in milk fat synthesis and facilitate marker-assisted selection for genetic improvement. Therefore, the present study was aimed to characterize the genetic polymorphism of *ACSL1* gene in Red Kandhari cattle and explore its potential association with milk production traits.

MATERIALS AND METHODS

Experimental animals

Thirty apparently healthy Red Kandhari lactating cows were randomly selected from the Livestock Farm Complex, College of Veterinary and Animal Sciences, Parbhani (India). They were maintained in organized dairy farms under standard management practices, including regular vaccinations and deworming, and provided with a standard diet and *ad-libitum* water at the farm. Prior approval from the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary & Animal Sciences, Parbhani (India) was obtained for the work vide No. IAEC/110/23 dated 15/12/2023.

Collection of samples

Milk: The fresh milk samples (50 mL) were taken during morning milking period from apparently healthy Red Kandhari lactating cows ($n = 30$). Milk samples were collected in clean, sterilized screw-capped glass tubes. The potassium permanganate solution was used to wash each cow's udder before milking. The milk samples were immediately transported to the laboratory in ice packs. The milk yield, protein, fat content, and somatic cell score were measured in the milk samples.

Blood: The blood samples (2 mL) were collected from all the cows in sterile vacuum tubes (vacutainers) containing EDTA (0.5 mM, pH 8.0) as the anticoagulant by jugular venipuncture. Immediately after collection, the blood samples were transported to the laboratory in ice packs. Whole blood samples (2 mL) were kept at -20°C for the isolation of DNA to determine *ACSL1* gene.

Sample processing and analysis

The Lactosure Eco-S solar-powered milk analyzer (Scientific Technology Solutions, India) was used to measure fat and protein contents in milk samples. The somatic cell count (SCC) which includes leucocytes (75%), i.e., neutrophils, macrophages, lymphocytes, erythrocytes, and epithelial cells (25%), is a valuable predictor of intra-mammary infection. The measurement of somatic cells in milk,

known as somatic cell count, was determined as per Schalm *et al.* (1971). The SCS was calculated as per the formula given by Cítek *et al.* (2022):

$$\text{Somatic cell score (SCS)} = \log_2 (\text{SCC}/100,000) + 3$$

Wherein SCC is the somatic cell count (cells mL⁻¹ milk); The division by 100,000 standardizes the scale; The log₂ transformation normalizes the distribution. Adding 3 ensures that an SCC of 100,000 cells mL⁻¹ corresponds to an SCS of 3.

Isolation, quantification and quality check of blood genomic DNA

HI Pura blood genomic DNA Miniprep Purification kit (HI Media Pvt Ltd.) was used to isolate genomic DNA as per the manufacturer's instructions. The genomic DNA underwent a quality and quantity assessment following its separation from blood samples. A high-quality DNA sample is crucial to achieve the desired outcome. The concentration and purity of acquired DNA was measured at 260/280 nm absorbance using a Nano Drop™ Lite spectrophotometer (Thermo Scientific, India).

Primers for *ACSL1* gene determination

Oligonucleotide primers specific to the milk-associated SNP regions of *ACSL1* gene were designed using NCBI Primer-BLAST (Ye *et al.*, 2012). The primer sequence used were: V1, 5'TGACGAGCCCCTGGTGTATT 3' (20 bp), V2, 5'TTGAAGAAAGACCCCTCTGTCTG 3' (22 bp), V3, 5'CTTCATCTCTGACGGTGGCA 3' (20 bp), and V4, 5'AGCATTTTCCCCCTGGGTTT 3' (20 bp). The targeted genomic region, previously reported in Chinese Holstein cattle based on NGS data (Liang, *et al.*, 2020), was identified and adapted for amplification in the Red Kandhari cattle genome.

Agarose gel electrophoresis

Gel electrophoresis was performed using 1.5% agarose in 1 X TAE buffer (pH 8.0). Agarose (1.5 g) was dissolved in 1 X TAE buffer, reaching a final volume of approximately 100 mL, and then heated for 90 sec in a microwave oven. Subsequently, 5 µL 100 mL⁻¹ gel solution was mixed with ethidium bromide. The melted agarose was then added to a sealed gel casting tray along with the comb placed in it. Upon solidification, the gel was moved to an electrophoresis tank that was filled with 1X TAE buffer. The buffer was kept at least 1 cm above the gel. The wells were carefully charged with 5 µL DNA mixed with 1 µL 6X gel loading dye. The electrophoresis was run for 55 min at 75V. Agarose gel electrophoresis was used to assess the PCR-amplified product of *ACSL1* gene. High-quality DNA was then utilized for further analysis using the PCR-SSCP method (Sambrook and Russell, 2001).

Single-stranded conformational polymorphism

A straightforward and effective method for identifying even minute changes in PCR-amplified product is single-strand conformation polymorphism (SSCP) approach (Sambrook and Russell, 2001). The basic concept is that minute alterations to a nucleic acid influence the migration of a single-stranded DNA fragment, leading to detectable variations in mobility on a non-denaturing polyacrylamide gel. Acrylamide monomers, ammonium persulphate (APS), N, N-methylene bis-acrylamide (Bis), and N, N, N', N'-tetramethylene diamine (TEMED) are the components used to synthesize the matrix in non-denaturing PAGE. Free radicals are produced when ammonium persulphate (APS) is dissolved in water. The single-stranded conformational polymorphism was done as per the Yadav and Kale (2009).

Gene sequencing

The representative PCR product's distinct SSCP patterns were separated and subjected to further analysis using direct sequencing (Barcode Biosciences Pvt. Ltd., Bangalore, India). Direct sequencing was done using the forward and reverse allele-specific PCR primer sets that were developed explicitly for PCR-SSCP test. To collect representative sequences by direct sequencing, at least three distinct animal samples representing each distinct PCR-SSCP pattern were provided.

Statistical analysis

The experimental data on milk composition was presented as mean ± SEM, while for computation of

genotype frequency and gene frequency the Hardy Weinberg equilibrium formula (Mahe and Grosclaude, 1993) was used obtained by direct counting.

$$\text{Genotype frequency} = \frac{\text{Number of animals of particular genotypes}}{\text{Number of animals of all genotypes}}$$

Gene frequency (p) = Genotypic frequency of homozygous genotype for that allele (P) + $\frac{1}{2}$ genotypic frequency of heterozygous genotype (H)

Where; p = gene/allele frequency of allele of interest, P = genotype frequency of homozygous genotype for that allele, and H = genotype frequency of heterozygous genotype.

RESULTS AND DISCUSSION

Standardization of polymerase chain reaction (PCR)

The PCR reactions were carried out in a 25 μ L volume containing 1 μ L template DNA. The PCR conditions used with V1-V2 primers for amplification of SNP region g.35446C>T were:

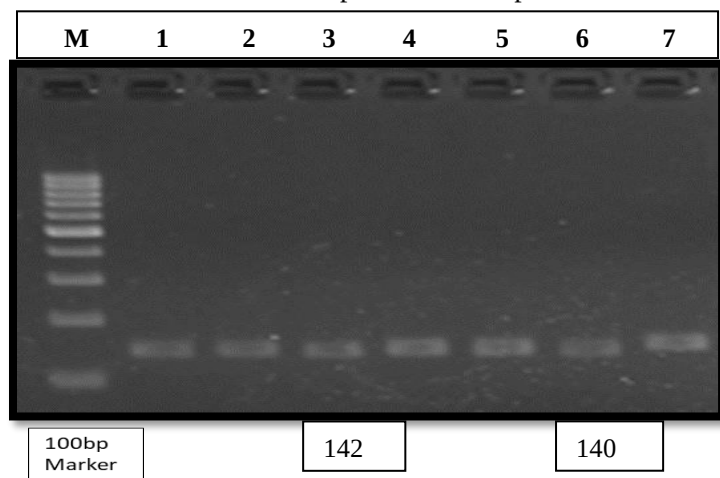


Fig. 1: Agarose gel electrophoresis of PCR product of *ACSL1* gene of lactating Red Kandhari cow. “M” is 100 bp DNA size marker, digits 1 to 5 are different samples displaying band of 142 bp, digit 6 is the sample showing band of 140 bp, and digit 7 is the sample showing band of 148 bp

Initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 60.2°C for 30 sec, and extension at 72°C for 2 min; and final extension at 72°C for 10 min withhold at 4°C. After PCR crisp single band with no smearing pattern was obtained (Fig. 1).

For SNP region amplification of g.35941G>A region, the conditions used with V3-V4 primers were:

Initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 59.1°C for 30 sec, and extension at 72°C for 2 min; and final extension at 72°C for 10 min withhold at 4°C.

Agarose gel electrophoresis

The polymorphic site was considered for amplification of SNP region g.35446C>T. All amplified PCR products gave a single compact band of the expected size of 148 bp (Fig. 1). With SNP region g.35941G>A, all amplified PCR products gave a single compact band of the expected size of 142 bp.

Single strand conformation polymorphism (SSCP) analysis

SSCP analysis was performed on amplified PCR products to detect mobility shifts in different fragments of *ACSL1* gene with SNP regions g.35446C>T and g.35941G>A in lactating Red Kandhari cows. The band patterns/variants were characterized by the number of bands, and mobility shifts were identified for different fragments of *ACSL1* gene with both SNP regions.

Distribution of PCR-SSCP patterns

In present study, two single-stranded DNA bands found in thirty Red Kandhari cattle showed similar migration patterns, indicating monomorphism in the samples. The photographs showing monomorphic introns 2 are depicted in different PCR-SSCP analyses of *ACSL1* gene with SNP region

g.35446C>T (Fig. 2) and SNP region g.35941G>A (Fig. 3) amplicons. PCR-SSCP analysis of amplicons of introns showed monomorphism, indicating the probable absence/lack of mutation(s), thus

RK21 RK22 RK23 RK24 RK25 RK26 RK27 RK28 RK29 RK30

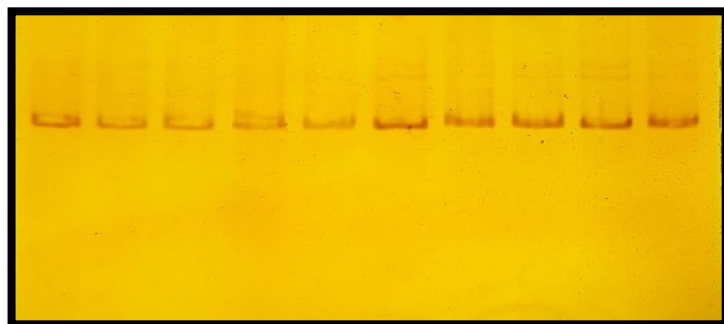


Fig. 2: PCR-SSCP pattern of intron 2 of *ACSL1* gene with SNP region g.35446C>T amplicon amplified with V1-V2 primers

RK10 RK11 RK12 RK13 RK14 RK15 RK16 RK17 RK18 RK19

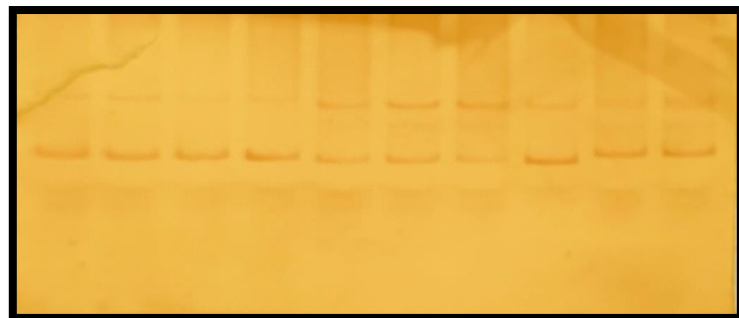


Fig. 3: PCR-SSCP pattern of intron 2 of *ACSL1* gene with SNP region g.35941G>A amplicon amplified with V3-V4 primers

suggesting monomorphism in lactating Red Kandhari cows.

SSCP is an old method used in polymorphism study. The technique is based on the principle that the conformation of single-stranded DNA depends on the nucleotide sequence present in it. The conformation of single strand changes with the presence of SNP in the region. When run after formamide treatment, the single strands show different mobility patterns if SNP mutation is present in different samples. The study revealed that for g.35446C>T, and g.35941G>A, the mobility shift did not change, indicating that the intended regions are monomorphic. Our conclusion of monomorphic nature is consistent with the previously reported SSCP studies (Sadan, 2021).

PCR-SSCP and sequencing were used to analyse the relationship between polymorphism and milk production attributes in genetic

variation within the 3'UTR of buffalo mammary-derived growth inhibitor (MDGI) gene. The findings demonstrated significant differences in the frequency distribution of SSCP mutations among Mehsana buffaloes (Dubey *et al.*, 2017). The bovine growth hormone (GH) gene sequence variants were examined using SSCP analysis, which demonstrated a favourable correlation with the characteristics related to milk production in dairy cattle. Using SSCP method, the bovine STAT5A gene was analysed by using SSCP method for mutations, which may be responsible for quantitative variations in milk yield and composition. The results found one new SNP in cows (Brym *et al.*, 2004). SSNPs of lactoferrin gene were found in Murrah (*Bubalus bubalis*) buffaloes through PCR-SSCP studies (Singh *et al.*, 2020).

Determination of *ACSL1* gene with SNP's

The genotype at SNP locus was first confirmed by direct sequencing of the corresponding PCR amplicon (NCBI sequence Id: PZ824036, PZ824037, PZ824038, PZ824039, PZ824040 and PZ824041) denoted with seq1 to Seq 6, respectively (Fig. 4a). The sequencing results revealed a homozygous CC genotype at the polymorphic locus. Based on Hardy-Weinberg equilibrium, the various allelic combinations of gene were assessed for genotypic and allele frequencies derived from gel electrophoresis data. Genotyping of *ACSL1* gene at SNP region g.35446C>T in 30 lactating Red Kandhari cows (samples RK1 to RK30) (Fig. 1) revealed a PCR fragment size of 148 bp, with all individuals exhibiting CC genotype.

Genotypic analysis of *ACSL1* gene at SNP region g.35446C>T in 30 Red Kandhari cows revealed a monomorphic pattern, with all individuals exhibiting CC genotype and no occurrence of CT or TT

CLUSTAL 0(1.2.4) multiple sequence alignment

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SeqA1      GTTTCAGAGGGGCTTACACGTGTCCAGTAAGCATCGGCCTG 42
SeqA2      GTTTCAGAGGGGCTTACACGTGTCCAGTAAGCATCGGCCTG 42
SeqA3      GTTTCAGAGGGGCTTACACGTGTCCAGTAAGCATCGGCCTG 42
SeqA4      GTTTCAGAGGGGCTTACACGTGTCCAGTAAGCATCGGCCTG 42
SeqA5      GTTTCAGAGGGGCTTACACGTGTCCAGTAAGCATCGGCCTG 42
SeqA6      GTTTCAGAGGGGCTTACACGTGTCCAGTAAGCATCGGCCTG 42
SeqSNP1     -----AGTAAGCATCGGC--- 13
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CLUSTAL 0(1.2.4) multiple sequence alignment

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SeqB1      CCCACCCCTCCAGCCCATCTCCCTCCTGCCCATGTGAGAAACAAATAGAAATAAG
SeqB2      CCCACCCCTCCAGCCCATCTCCCTCCTGCCCATGTGAGAAACAAATAGAAATAAG
SeqB3      CCCACCCCTCCAGCCCATCTCCCTCCTGCCCATGTGAGAAACAAATAGAAATAAG
SeqB4      CCCACCCCTCCAGCCCATCTCCCTCCTGCCCATGTGAGAAACAAATAGAAATAAG
SeqB5      CCCACCCCTCCAGCCCATCTCCCTCCTGCCCATGTGAGAAACAAATAGAAATAAG
SeqB6      CCCACCCCTCCAGCCCATCTCCCTCCTGCCCATGTGAGAAACAAATAGAAATAAG
SeqB7      CCCACCCCTCCAGCCCATCTCCCTCCTGCCCATGTGAGAAACAAATAGAAATAAG
SeqB8      CCCACCCCTCCAGCCCATCTCCCTCCTGCCCATGTGAGAAACAAATAGAAATAAG
SeqB9      CCCACCCCTCCAGCCCATCTCCCTCCTGCCCATGTGAGAAACAAATAGAAATAAG
SeqSNPSite -----CCTGCCCATGTG-----
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Fig. 4: Multiple sequence alignment (MSA) of SNP region with sequences; MSA of SNP region g.35446C>T (left hand side) and SNP region of g.35941G>A amplicon amplified with sequences

SeqB 9, respectively, (Fig. 4b), with all individuals showing a PCR fragment size of 142 bp and possessing the GG genotype. No GA or AA genotypes were detected. This resulted in genotypic frequencies of 1.0 (GG), 0.0 (GA), and 0.0 (AA), and allele frequencies of 1.0 for G allele and 0.0 for A allele, indicating complete homozygosity and fixation of G allele in the population. The consistent presence of G allele at 100% frequency suggests its possible link to specific milk composition traits in Red Kandhari cattle. This too appears the first report on *ACSL1* gene genotyping in this breed.

DNA sequencing analysis

The presently studied monomorphic sites were analysed with DNA sequencing to ensure that the intended region depicting non-varying mobility on SSCP is indeed monomorphic, so we selected random samples for DNA sequencing for g.35446C>T and g.35941G>A. Based on previous SSCP results, we expectedly did not find any SNP variations in those sites in all thirty animals. So, DNA sequencing data conforms to SSCP data, which shows monomorphism for both regions under study (Fig. 4a). The DNA sequence analysis of *ACSL1* gene with SNP region g.35941G>A amplicons showed homozygous genotype of GG (Fig. 4b).

Milk production traits

The mean ($\pm$ SEM) values of milk composition parameters in lactating Red Kandhari cattle were as follows: fat, $4.65 \pm 0.20\%$; solids-not-fat (SNF), $8.24 \pm 0.07\%$; protein, $3.06 \pm 0.02\%$; lactose, $4.43 \pm 0.04\%$; water, $87.09 \pm 0.23\%$; and somatic cell score, 4.089 ± 0.09 . The average daily milk yield was 2.47 ± 0.03 kg. The somatic cell counts $< 100,000$ indicated that cows do not have udder infection. In present study the somatic cell score was in normal range. The study on Red Kandhari cows revealed a mean daily milk yield of 2.47 ± 0.03 kg, which falls within the range of 1.5-4.0 kg as reported by Pundir and Singh (2008) for this breed, and closely matched their recorded peak yields

genotypes. Consequently, the genotypic frequencies were 1.0 (CC), 0.0 (CT), and 0.0 (TT), while the allele frequencies were 1.0 for C allele and 0.0 for T allele, consistent with Hardy-Weinberg equilibrium expectations for a fixed allele. The complete homozygosity for C allele suggests its fixation in the studied population, which may be related to the specific milk composition traits in Red Kandhari cattle. A thorough perusal of literature has revealed that this is the first report describing *ACSL1* gene genotyping in Red Kandhari cows.

Determination of *ACSL1* gene with SNP region g.35941G>A variants

Genotyping of *ACSL1* gene at SNP region g.35941G>A in 30 lactating Red Kandhari cows revealed complete genetic uniformity (NCBI sequence Id: PZ824042, PZ824043, PZ824044, PZ824045, PZ824046, PZ824047, PZ824048, PZ824049, and PZ824050 denoted with seq1 to

of 2.0-4.0 kg in breeding tract. The observed milk fat and SNF contents were consistent with Ghafoor (1987), while the yield values were comparable to 2.27 ± 0.35 kg as reported by Siddiqui *et al.* (2000), who also noted insignificant effect of parity or calving season on peak yield. The composition values for fat, protein, lactose, and SNF were in close line with Tirfie (2023), who emphasized the influence of genetic and non-genetic factors such as breed, age, health, lactation stage, diet, season, and milking method. Notably, the milk fat percentage in Red Kandhari cows was higher than that reported for crossbred cows (Chandrakar *et al.*, 2017), revealing the breed's potential for producing high-fat milk.

Association of SSCP variants on somatic cell count and milk production traits

The *ACSL1* gene is 64,883 bp located on chromosome 27, with 19 intronic and 20 exonic regions. It helps in triglyceride synthesis, and triglycerides are the major part of the composition of fatty acids. The expression of *ACSL1* is higher in buffalo mammary tissues and the gene is involved in milk production traits. So, it has been reported to be a key gene that can be studied for polymorphism and association studies (Liang *et al.*, 2020). In polymorphism study in Chinese Holstein cows, Liang *et al.* (2020) found six SNP regions associated with milk parameters which were 5'UTR-g.20523C>G, g.35446C>T, g.35651G>A, g.35827C>T, g.35941G>A, and g.51472C>T. The SNP g.35827C>T and SNP g.35941G>A regions were in linkage disequilibrium.

In present study *ACSL1* gene polymorphism was observed for the first time on indigenous 'Red Kandhari' breed. Considering the SNP regions mentioned above, we selected SNP g.35446C>T and SNP g.35941G>A. With the selection of g.35941G>A, one can have an idea of an additional SNP region g.35827C>T, as both are linked. In Chinese Holstein cattle for SNP g.35446C>T, the reported genotype frequency was 0.780 for CC genotype, 0.182 for CT genotype, and 0.037 for TT genotype. In case of g.35941G>A, the genotype frequency for GG genotype was 0.542; while for GA genotype 0.369, and for AA genotype 0.089. In both SNP g.35446C>T and g.35941G>A, the homozygous alleles CC and GG have higher genotype frequencies than other genotypes. In Red Kandhari cattle only one genotype for SNP g.35446C>T and g.35941G>A was observed with CC and GG, respectively. The reason for the absence of another genotype could be the higher CC and GG genotypes in natural cattle populations, as also observed in Chinese Holstein cattle by Liang *et al.* (2020).

In the reported study on Chinese Holstein cows, the SNP g.35446C>T, the CC genotype has significantly lower protein content and test day milk yield than TT genotype. With SNP g.35827C>T, the genotype CC had all studied parameters such as test day milk yield, fat content, and protein content higher than TT genotype (Liang *et al.*, 2020). The g.35827C>T and g.35941G>A are in linkage disequilibrium, so we expect similar milk parameter variations for g.35941G>A as in g.35827C>T.

In present study milk samples from Red Kandhari cattle were analysed for fat, protein, milk yield, and somatic cell score. the samples from Red Kandhari cattle were analysed for their association with milk parameters to study polymorphism of g.35446C>T and g.35941G>A. In present study, both genomic regions were found monomorphic for g.35446C>T, g.35941G>A with CC and GG genotypes, respectively in all thirty cattle. We found that considering the monomorphic nature of SNP, the association of milk characters with statistical tests is not possible. Based on a previously reported study on Chinese Holstein cows, we can say that g.35446C>T, CC genotype may have the implications of lower test day milk yield and lower protein content, with higher fat and higher somatic cell score. In case of g.35941G>A, there could be lower test day milk yield, fat and protein contents with higher somatic cell scores in GG genotype (Liang *et al.*, 2020).

The current study found a monomorphic nature for both regions under study. Considering the current facts, the study suggests including a larger population size to detect possible intended mutations at those sites. Also, including the additional SNP sites of *ACSL1* may give an idea of its association with milk production traits. Future work should employ whole-gene resequencing and genome-wide association studies to identify informative markers linked to milk production and composition. Such efforts will enhance the utility of molecular genetics in breeding programs aimed at improving dairy traits in indigenous cattle.

Conclusions: The present study revealed that at *ACSL1* gene intron 2, SNP sites g.35446C>T and g.35941G>A, all thirty unrelated red Kandhari cattle exhibited complete homozygosity for CC and GG genotypes, respectively. Both genomic regions studied were monomorphic in nature, with no allelic variation detected in the studied population. These findings suggest a fixation of the respective alleles within this breed. To gain a more comprehensive understanding of the genetic variability of *ACSL1* and its potential association with milk production traits, future research should investigate additional SNPs within this gene using a larger and more diverse sample set.

Animal ethics: The study was carried out on thirty unrelated Red Kandhari cattle after seeking proper approval from the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary and Animal Sciences, Parbhani (India) vide No IAEC/110/23 dated 15/12/2023.

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Competing interest statement: The authors declare that they have no conflict of interest.

Author contributions statement: VBS performed the major experiments. VBS, PYV and MJS helped in genomic DNA isolation, VBS, MFS, SAA and LAP helped in blood collection and PCR standardization. PBG planned, supervised the experiments and done bioinformatics work. Everyone contributed to writing the manuscript.

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