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Research Article

Studies on the Association of PKLR Gene Polymorphism with Milk Production Traits in Cattle

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ABSTRACT

The present study aimed to investigate the association of PKLR (Pyruvate Kinase expressed in Liver and Red blood cells) gene polymorphism with milk production traits in thirty lactating Red Kandhari Cattle from the Marathwada region. The present investigation included PKLR gene polymorphism and its association with milk production traits, viz, Milk yield (Kg/day) and milk constituents, viz, Milk Fat per cent, SNF per cent, Protein per cent, Lactose per cent, Water per cent, and Salt per cent in Red Kandhari cattle. The isolated DNA of interest (region 3: g.15350898T>C for exon 10, which was CTTCGAG) from blood samples of Red Kandhari cattle was amplified (149 bp, 148 bp, and 71 bp PKLR gene) by PCR and the PCR products were analyzed by SSCP for PKLR gene polymorphism. The data from SSCP analysis were statistically analyzed using the HW-equilibrium method to detect gene and genotype frequencies. The milk yield and milk constituent data were compared with the reviewed literature. The results of the investigation of PKLR gene polymorphism in Red Kandhari cattle showed that all three primers showed the same pattern of these single-stranded DNA bands having the same migration patterns, confirming the monomorphism in all the samples of experimental animals. The known mutant alleles at PKLR gene were not observed in the present study. In Red Kandhari cows, the gene and genotypic frequencies of the PKLR gene were analyzed. The frequency of the C allele was observed to be zero, while the frequency of the T allele was 1. Consequently, the genotypic frequencies were as follows: 0.0 for CC, 0.0 for CT, and 1.0 for TT. The mean ± SEM values of milk yield (kg/day) of Red Kandhari cattle were observed to be 2.62±0.03. It is concluded that, In the present study, no polymorphism or association was observed with milk production traits in Red Kandhari cattle.

Keywords: *SSCP, Milk production traits, Red Kandhari cows, PKLR gene.*

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INTRODUCTION

The Maharashtra districts of Latur, Parbhani, Nanded, Hingoli, and Beed make up the breeding tract for the Red Kandhari cattle breed. Cow milk is known for its protein, lactose (milk sugar) and other micronutrients, which are very essential for a growing kid, a working person, a pregnant mother, a sick patient or an old person, as it is accepted nationwide due to its healthier composition. Ayurveda has long recognized the beneficial health effects of cow milk intake, i.e., stronger bones, brain growth, enhanced immunity and intelligence (Banerjee, 2018). Milk production traits have significant roles in dairy development and the related economy. The bovine milk production traits such as milk yield, fat content, protein content and somatic cell count (SCC) are the essential economic traits used to measure milk quality (Ma et al., 2021).

The PKLR gene is active (expressed) in the liver and red blood cells, supplying instructions for pyruvate kinase production. The peak of the QTL regions for milk fat percentage (QTL_ID: 104486) and protein percentage (QTL_ID: 104816, 104938) is located 0.02 Mb away from PKLR (chr.3: 15344765-15354042) (Nayeri et al., 2016). Therefore, this gene was evaluated as a potential candidate for dairy cow milk production features. The PKLR gene includes 21 SNPs, and substantial genetic relationships between these SNPs and dairy cow attributes related to milk production have been established (Du et al., 2022). The milk 4 production traits in dairy cattle may include eleven SNPs as probable causative mutations (Ma et al., 2021). The PKLR gene is 9.5 kb in size, has 12 exons, and is located on chromosome 1, specifically at the band 1q21. According to tissue-specific promoters, the gene encodes for the isoforms of the enzyme present in the liver (L) and erythrocytes (R). The two isoforms share ten exons, but exons 1 and 2 are exclusively transcribed to the PK-R and PK-L mRNAs, respectively. The PK-R cDNA encodes 574 amino acids across a length of 2060 bp. Two CAC boxes and four GATA motifs are located within 270 bp of the translational initiation codon in the R-type promoter region. The area from -120 to - 270 in erythroid cells acts as a potent enhancer, but the immediate 120 bp region exhibits basal promoter activity (Bianchi & Fermo, 2020). PKLR is involved in glycogen and lipid metabolism in liver tissues (Wang et al., 2000; Ahrens et al., 2013). The relationships between five dairy cattle milk production traits and the 21 SNPs in PKLR have been found to be significantly associated with milk production traits. In the first lactation, four SNPs were significantly correlated with milk yield, nineteen with fat yield, four with protein yield, and seventeen with protein percentage. (Du et al., 2022). In the second lactation, sixteen, twenty, and eighteen SNPs were significantly associated with milk yield, fat yield, and protein percentage ($p = 0.0436$), respectively (Du et al., 2022).

MATERIALS AND METHODS

The experiment was carried out on lactating Red Kandhari cows (n=30) (age ranged between 3-9 years) selected from the Livestock Farm Complex of the institute. All the selected animals were maintained in organized dairy farms or in field conditions under standard managemental practices, including regular vaccinations and deworming, provided with a standard diet and ad-libitum water. The fresh milk samples (50 mL) were collected in clean, sterilized screw-capped glass tubes during the morning milking time from each cow. The milk samples were used for quantification of components, *viz*, Milk Fat, SNF, Protein, lactose per cent, Water and Salt by using the Lactoscan milk analyzer.

Sample Collection

A total of 2 mL blood samples were collected from each of the experimental animals (n = 30) via jugular venipuncture into vacutainer tubes containing EDTA (0.5 mM, pH 8.0) as an anticoagulant. The samples were collected during the early morning hours to minimize physiological variation.

Genomic DNA Extraction and Quality Assessment

Genomic DNA was isolated from whole blood using the HiPura Blood Genomic DNA Miniprep Purification Kit (HiMedia Pvt. Ltd.) following the manufacturer's standard protocol.

The quantity and purity of DNA were assessed using a Nanodrop spectrophotometer, with OD_{260/280} values ranging between 1.76 to 1.80, indicating high purity. The integrity of genomic DNA was evaluated by 0.8% agarose gel electrophoresis (at 75 volts), stained with ethidium bromide, and visualized under a UV transilluminator. Only samples exhibiting minimal smearing and clear DNA bands were selected for further analysis.

Primer Preparation and Optimization

Working solutions of primers were prepared by diluting 5 µl of stock solution with 45 µl of nuclease-free distilled water.

Gradient PCR was conducted to optimize annealing temperature, with primers performing best at 57°C. Primer concentrations were maintained as per standard protocol.

Primers Used for PKLR Gene Amplification

The following primer sets were used for amplification of different regions of the **PKLR gene**:

- **Primer Set 1**
  - Forward: 5′-GATCCCAATACCCTTGCCCA-3′
  - Reverse: 5′-CACCAATAGCGGTGACCTCC-3′
- **Primer Set 2**
  - Forward: 5′-TCTGAGCCTTTAATCCCAGCTC-3′
  - Reverse: 5′-AGGTGACACTGTCCTTGGGA-3′
- **Primer Set 3**
  - Forward: 5′-TGTGACTGGAAGCTAGGAGAGA-3′
  - Reverse: 5′-AGACACTAACGAGGTGTGCC-3′

SSCP Protocol

PCR amplification of the PKLR gene was performed in a 25 µl reaction volume containing 12.5 µl HiChrome PCR Master Mix, 0.5 µl each of forward and reverse primers, 1 µl template DNA, and 10.5 µl nuclease-free water. Thermal cycling conditions included an initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 58–61°C for 30 sec, and extension at 72°C for 55 sec; followed by a final extension at 72°C for 10 min.

For SSCP analysis, PCR products were mixed with an equal volume of denaturing-loading buffer (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, and 20 mM EDTA, pH 8), denatured at 95°C for 7 min, and immediately chilled on ice. A 2 µl aliquot of each sample was loaded on a 12% non-denaturing polyacrylamide gel prepared with 7% glycerol in 1× TBE buffer. Electrophoresis was conducted in a vertical gel system at 16°C, using constant current (120 mA) and voltage (up to 200 V) for 3.5 to 4 hours. A pre-run at 18 mA for 30 minutes was conducted prior to sample loading. The gels were then visualized for band pattern analysis.

Gene Sequencing

Representative PCR products showing distinct SSCP banding patterns were selected for Sanger sequencing to confirm the nucleotide sequence of the amplified region. Prior to sequencing, PCR amplicons were purified using a commercial PCR purification kit (e.g., Qiagen PCR Purification Kit) following the manufacturer’s instructions.

Sequencing was carried out in both forward and reverse directions using the same primers as used for PCR amplification. The purified products were submitted to a

commercial sequencing facility (e.g., Eurofins Genomics, Bangalore, India). Chromatogram files (.ab1 format) were analyzed using BioEdit or Chromas software, and sequences were aligned and compared using BLAST against the bovine PKLR gene reference sequence.

Silver Staining of SSCP Gel:

Gels were stained using a modified protocol by Byun et al. (2009). Briefly, the gel was fixed in a solution of 10% ethanol, 0.5% glacial acetic acid, and 0.2% silver nitrate for 10–20 min, rinsed with distilled water, and developed using 3% sodium hydroxide and 0.1% formaldehyde until bands became visible. Gels were placed on a white lightbox and photographed immediately.

Documentation of SSCP Patterns:

SSCP banding patterns were observed visually under normal light and documented using a mobile **camera**. Although scanning was attempted, it was less effective for faint or thin bands compared to direct imaging on a white lightbox.

RESULTS AND DISCUSSION

The present study was carried out for the investigation of partial PKLR gene polymorphism and the association of PKLR genotype with milk production traits in lactating Red Kandhari cattle. The study was aimed for the analysis of milk composition and determination of PKLR gene polymorphism with milk production traits in Red Kandhari cattle. Under a UV transilluminator, each amplified PCR product was visible as a single band of the expected size (149 bp, 148 bp and 71 bp). The amplified PCR products (Fig. 1) were subjected to Single Strand Conformation Polymorphism (SSCP) analysis to screen for the presence of a mutant allele.

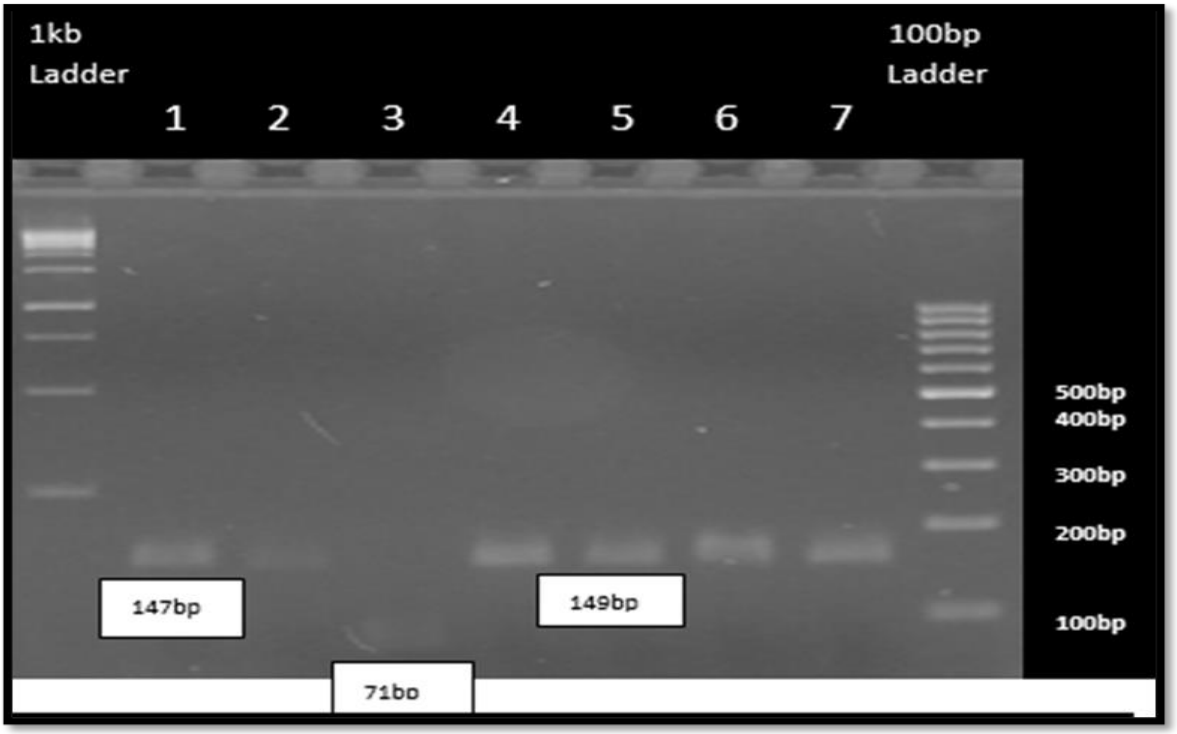

Fig.1: Agarose gel electrophoresis of PCR product of PKLR gene of Red Kandhari cattle. Digit 1 indicate sample of primer 2, Digit 3 indicate sample of primer 3 and digit 4,5,6 and 7 indicates the sample of primer 1.

Single Strand Conformation Polymorphism (SSCP) analysis

The amplified PCR products of different fragments were analyzed by Single Strand Conformation Polymorphism (SSCP) analysis. The experimental samples, specifically those reconstituted in a mixture of formamide dye and subjected to boiling, typically exhibited migration as a combination of double-stranded and single-stranded DNAs. Single-stranded

DNAs generally moved more slowly through polyacrylamide gels compared to their double-stranded counterparts (Maxam & Gilbert 1977, 1980; & Szalay et al., 1977). Detection of a single-stranded band occurred when the complementary strands of the DNA adopted conformations that could not be distinguished by Single-Strand Conformation Polymorphism (SSCP) analysis. In cases where the complementary strands folded into distinguishable conformations, two single-stranded DNA bands were identified. For a heterozygous alleles PCR product from a diploid organism, a minimum of four bands should be generated. Two bands should exhibit mobility identical to that of wild-type bands, and two should be characteristic of the specific mutation. However, in many instances, there might be more than two bands due to genetic diversity in the sample or because each of the DNA molecule's complementary strands folds into more than one conformation. The resulting pattern can be intricate and is not easily predictable based on DNA sequence, base composition, or fragment length. Nevertheless, a specific pattern often serves as a diagnostic indicator for a particular mutation (Sambrook & Russell, 2001).

The photographs showing monomorphic exons are depicted in different PCR-SSCP analysis of PKLR gene amplicons revealed same degree of genetic monomorphism with respect to each of the PKLR gene exons analyzed. PCR-SSCP analysis of amplicons of the exons-10 showed monomorphism in Red Kandhari lactating cattle and showed monomorphism.

Indicating the probable absence/lack of mutation/s suggesting a high degree of conservation of lack PKLR gene in the above exons in Red Kandhari lactating cattle. In the PCR-SSCP method, the products yielded only one type of pattern in all lactating Red Kandhari cattle (N=30) revealed a monomorphic pattern which was shown in Fig 4.3. The genotypic frequency for CC, CT and TT genotypes was observed as 0, 0 and 1, respectively. Similarly, the allelic frequencies for C and T alleles were 0 and 1 in lactating Red Kandhari cattle for the studied of PKLR gene (Table 1).

Table1. Gene and Genotypic frequencies of PKLR gene in Red Kandhari Cattle

CC Genotypes	Variant Number	Genotype Frequencies	Allele Frequencies	
CC	0	0	C	0
TT	30	1	T	1
CT	0	0		

Thus, only one genotype (TT) was noticed in the present study. Since all the lactating Red Kandhari cattle studied were homozygous (monomorphic) for the PKLR gene, the influence of the PKLR gene on milk yield could not be measured. In contrast with the present findings Du et al. (2022) reported PKLR gene polymorphic with milk production traits association in Chinese Holstein cows.

Only one pattern was observed within 30 animals (30 of each Red Kandhari cattle) screened for PKLR segment. Pattern I depicted two SSCP bands. Migration patterns of these single stranded DNA bands (conformers) of the same molecular weight were the same. There is no difference in their migration rates as shown in Fig 2 and Fig 3, it shows monomorphic pattern. All the three primers showed the same pattern of these single stranded DNA bands having same migration patterns confirm the monomorphism in all the samples of experimental animals.

The prime objective of the present research work was to study the PKLR gene polymorphism and its association with milk production traits in Red Kandhari cattle. The PCR-SSCP revealed the monomorphic nature of the PKLR gene. The known mutant alleles

at the PKLR gene were not observed in the present study. Hence, the association between the milk production traits and the PKLR gene polymorphism could not be established and shown in fig.4.

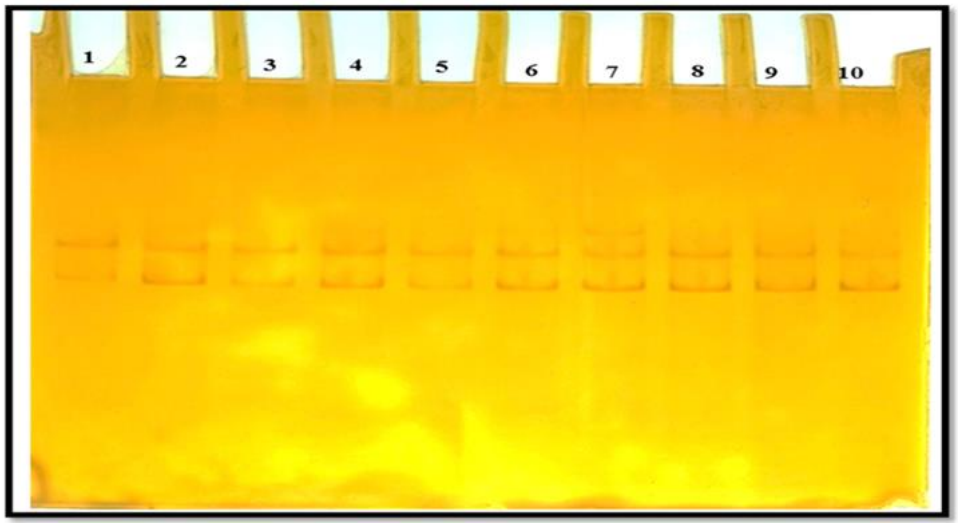

Fig. No.2. SSCP pattern of Primer 1 showing the pattern of migration rates with no difference

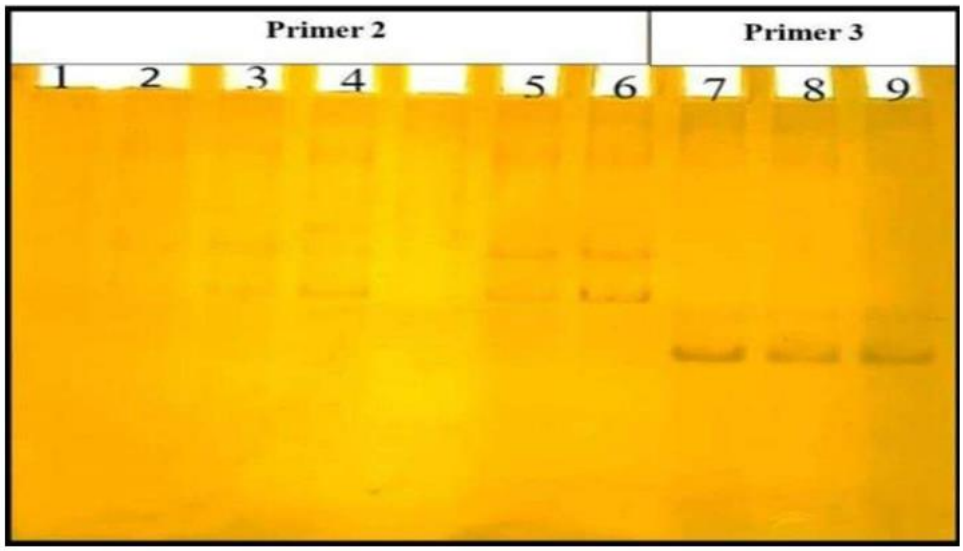

Fig. No. 3 SSCP pattern of Primer 2 and 3 of the samples 1-9 showing the pattern of migration rates with no difference

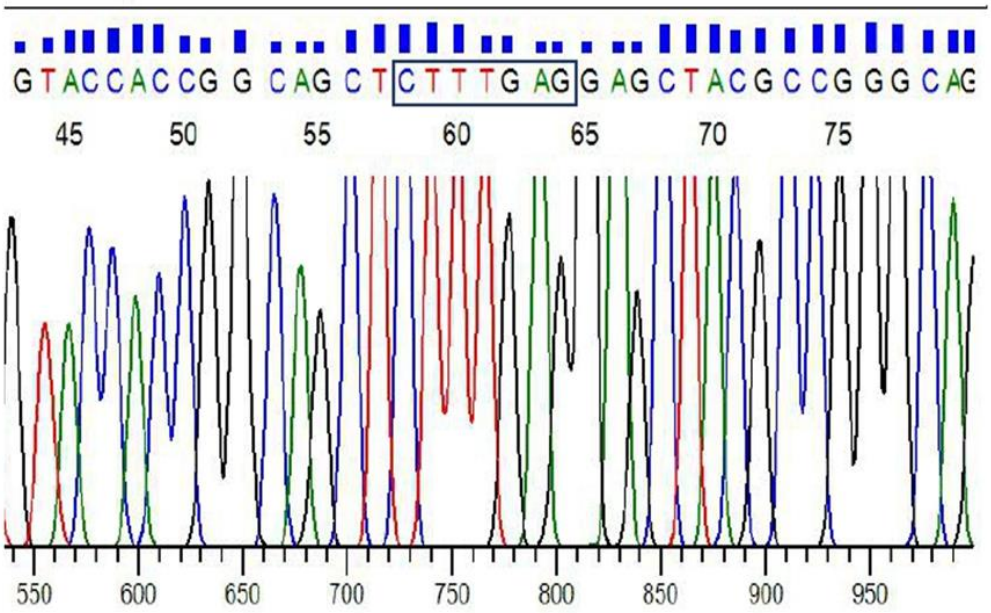

Fig. No. 4 Chromatogram of SNP region 3: g.15350898T>C of PKLR gene Sequencing of Sample 1

In contrast to the present study, Du et al. reported PKLR gene polymorphism and its association with milk production traits in Chinese Holstein cows. They observed that all the SNPs were significantly ($p \leq 0.0497$) associated with milk yield, milk fat and milk protein per cent. Also concluded that SNPs predicted to change the transcription factor binding site and mRNA secondary structure. This may affect the expression of PKLR, leading to changes in the milk production phenotypes. These SNPs might be the potential functional mutations for milk production traits in dairy cattle (Du et al., 2022). In the present study, no polymorphism was observed, so there is no association with milk production traits in Red Kandhari cattle.

Although no polymorphism was detected in the targeted region of the PKLR gene among the sampled Red Kandhari cattle, this finding may indicate a conserved nature of Exon 2 in this indigenous breed. The absence of variation could be due to breed-specific genetic stability or the limited sample size. As only a partial region was examined, future studies involving a larger and more genetically diverse population, along with full-gene sequencing, are recommended to comprehensively assess the potential association between PKLR gene variants and milk production traits.

CONCLUSION

The present study found that all Red Kandhari cattle are monomorphic for the PKLR (exon 10) gene studied, with a T allelic frequency of one. PKLR gene (exon 10) may not be regarded as the major gene associated with the milk production traits, especially milk fat per cent of the native Red Kandhari breed of cattle. This study suggests that further detailed investigation is needed to identify variations at other loci of the PKLR gene in Red Kandhari cattle and to study a larger sample size (Red Kandhari) with milk production traits.

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Author Contribution:

All authors have participated in critically revising of the entire manuscript and approval of the final manuscript.

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