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Research Article

Identification of Single Nucleotide Polymorphism (SNP) in 5'-UTR of *CYP11B1* Gene in Sahiwal and Tharparkar Cattle Breeds of Pakistan

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ABSTRACT

One of the major goals of animal genetics is to understand the role of common genetic variants in disease susceptibility as well as in production traits. Sahiwal and Tharparkar cattle could be considered as global animal genetic resources as the two breeds are best in Pakistan due to high milk-producing ability, resistance against tropical diseases, and heat tolerance. The *CYP11B1* gene codes for a mitochondrial enzyme is known as steroid 11-beta-hydroxylase. In cattle, it catalyzes the conversion of 11-deoxycortisol to cortisol and 11-deoxycorticosterone to corticosterone. The present study was aimed to identify the single-nucleotide polymorphisms in *CYP11B1* gene in Sahiwal and Tharparkar cattle breeds of Pakistan. We collected 27 Sahiwal breed samples from RCC Jhang and 49 Tharparkar cattle samples from Government livestock farms Umerkot (Location 1).Sequence analysis of *CYP11B1* gene's exon 1 has shown total 22 polymorphic sites in both breeds14 SNPs were found in Tharparkar cattle and 8 in Sahiwal cattle. Moreover 7 non-synonymous mutations were in Tharparkar breed 5 were identified in Sahiwal breed. In this study SNP based phylogenetic tree of Tharparkar and Sahiwal cattle was constructed, and the results have shown promising findings Tharparkar cattle from Bos taurus as well as Sahiwal cattle breed. Sequences of Tharparkar cattle have also shown divergence within breed as the sequences have formed 3 different clades at different positions. This polymorphism study could be an effective genetic tool for the development of trait-specific DNA markers for Pakistani cattle breeds, and therefore help the animal breeders and scientist to identify domestic livestock diversity that allows different livestock species to adapt to future environmental changes and avoid inbreeding.

Keywords: *CYP11B1*, SNPs, Sahiwal cattle, Tharparkar Cattle, Candidate-Gene Approach, Milk producing traits.

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INTRODUCTION

Over the last 50 years, genetic improvement has been credited with contributing to over 56% of the increase in average milk yield. Genomic selection is an important emerging approach carrying tremendous potential for features that are important to the economy. At the genomic level, many polygenic characteristics, including milk fat, protein, and yield, have been described, and crucial selection signatures have been found. Milk provides nutrients in the form of proteins, lipids (fat), and lactose, as well as a valuable delivery system for other essential components, including vitamins, minerals, bioactive lipids, cholesterol. According to Shahzad (2022), the milk fat percentage, which is the primary source of energy, varies greatly between species (Shahzad, 2022).

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The overall fat content of milk differs among species based on factors including feeding, milk yield and stage of lactation (Rahman *et al.*, 2017). It has been recorded that rhinoceros' milk contains 0.2% fat, while whale milk can contain up to 50% fat. According to Babawale, cholesterol is the main sterol in whole milk and has concentrations ranging from 10–30 mg/dL (0.25–0.77 mM), making it one of the most significant components of milk fat content.

In dry areas, ruminants are a major source of meat and dairy products. According to the Asian Agriculture Sector, cattle farming can be an essential source of income for farmers and a major supplier of meat and milk. Cattle contribute over 35% of the total milk production in Asian countries. Red Sindhi, Sahiwal and Tharparkar are the most popular native cattle breeds in Asia (Rahman *et al.*, 2017). In the last five years, the number of cattle in Asia has grown significantly, to approximately 5.7 million animals. Due to abundant rainfall in the Mediterranean region, cattle species are raised on transhumant systems that include grazing grasses, small bushes and plants that grow around August and June (Ubach *et al.*, 2023). According to Joy *et al.* (2020), cattle can produce a significant amount of meat and milk in desert environments due to their adaptability to difficult conditions, including high temperatures and a lack of nutrition (Joy *et al.*, 2020). Sahiwal and Tharparkar are economically important indicine cattle that are well adapted to the surrounding environment with desired milk production quality (Dash *et al.*, 2022).

Insights into the genomic regions under putative selection, may reveal the molecular mechanisms affecting the quantitative and other important traits (Illa *et al.*, 2021; Gutierrez-Reinoso *et al.*, 2021) . Consequently, there is a great interest in understanding the genomic architecture of growth and milk traits of these animals in order to improve milk characteristics. To enhance the selection of breeding animals that can genuinely improve crucial traits, improvement programs based on genetic information ought to be established (Berghof *et al.*, 2019; Xu *et al.*, 2020). Researchers have conducted genotyping of molecular markers in various cattle breeds to explore the correlation between potential genes and milk performance parameters. *PRL, LEP, IGF1, DGAT1, STAT5, CSN1S2, GHR,* and other highly significant candidate genes that have been linked to milk and growth traits had already been studies in the last few decades (Abousoliman *et al.*, 2020; Gavran *et al.*, 2021).

The regulation of the genes, encoding the enzymes needed for lipid production helps animals in maintaining lipid homeostasis. Most extensively studied are the CYP system that activates a wide range of genes i.e., *CYP11B1* for lipid metabolism (Cantiello *et al.*, 2022; Lu *et al.*, 2022). The conversion of 11 deoxy-cortisol to cortisol and 11 deoxy-corticosterone to corticosterone hormones in cattle is catalyzed by steroid 11-beta-hydroxylase (*CYP11B1*) (Mukangwa *et al.*, 2020). Steroid hormones are considered significant bio-regulators; cortisol is the primary hormone associated with both lipogenesis and lipolysis (Yang *et al.*, 2021; Kamgang *et al.*, 2023; Rahimi *et al.*, 2020). The location of the bovine *CYP11B1* gene is close to marker ILSTS039 on the chromosomal region BTA14q14 (DN Das and Mundhe, 2019; Amweg *et al.*, 2017; Mukangwa *et al.*, 2020). According to Mukangwa *et al.* (2020) and Kaupe *et al.* (2007), this marker is associated with both milk yield and milk component yields. Cattle's growth performance and milk quality are associated with polymorphisms of *CYP11B1* (Kaupe *et al.*, 2007).

Single nucleotide polymorphism (SNP) identification may be a useful method for comprehending and explaining the physiological basis of profitable traits (Ghoreishifar *et al.*, 2020; Fang and Pausch, 2019; Vineeth *et al.*, 2020). More than 2.2 million putative SNPs were identified from the sequenced genome of cattle (Stothard *et al.*, 2011; Rosse *et al.*, 2017). These can be used to look into population structure, kinship, individual identification, and parentage inference (Gamarra *et al.*, 2020). As of now, the primary focus of the genetic investigation of cattle breeds has been on evaluating potential genes related to milk traits. Therefore, the objective of the current study is to find segregating polymorphisms of a potential candidate gene for milk production traits in Sahiwal and Tharparkar breeds.

MATERIALS AND METHODS

Sample Collection

Samples associated with data were collected from the Government livestock farms Umerkot (Location 1) and the Research center for Conservation of Sahiwal Cattle Jhang (location 2). Phenotypic data as well as blood samples were collected from 27 animals of the Tharparkar breed from location 1 and 49 samples of Sahiwal breed from location 2. Prior to DNA extraction, blood samples had been stored at -20 °C.

Extraction of DNA

DNA was extracted through a commercial Thermo Scientific Extraction Kit, and 1.5% agarose gel was used to analyze DNA samples.

Primer Design

Primer sequences required for the amplification of *CYP11B1* were obtained from literature and the bovine genomic

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sequences via Bovine Genome Project (Baylor College of Medicine (DN Das and Mundhe, 2019), and were confirmed via using NCBI and BLAST.

PCR Amplification

A polymerase chain reaction was subsequently performed by using extracted samples in order to amplify particular areas of the candidate gene and identify single nucleotide polymorphisms (SNPs) (DN Das and Mundhe, 2019; Kaupe *et al.*, 2007). The PCR test was carried out in accordance with the manufacturer's instructions (Thermo Scientific) using the appropriate primer sets listed in table 1 in a total volume of 50µL roduction traits in Sahiwal and Tharparkar breeds.

Table 1: Oligonucleotide Sequence of *CYP11B1* gene

Gene		Primer Sequence	Amplicon Size(bp)	Region
CYP11B1	Forward	5'ATACTGGAGGGGGAGGAGG-3.'	568 bp	Putative Exon 1
	Reverse	5'-GGACAGAACGTGAGGGTGTT-3'		

PCR Amplification was done by adjusting the proportions of $MgCl_2$, dNTPs (deoxy-nucleotide triphosphate) and Taq Polymerase, the table 2 list the general composition of PCR reaction mixture.

Table 2: PCR Reaction Mixture Composition

Sr. No.	Recipe Ingredients	Quantity (µl)
1	DNA (25ng/ µl)	3x2
2	Forward-Primer (100pmol/ µl)	1x2
3	Reverse-Primer (100pmol/ µl)	1x2
4	Magnesium chloride	2.5x2
5	DNTPs	2.5x2
6	10x Taq. Buffer	2.5x2
7	Taq.(Thermus aquaticus) DNA Polymerase	0.3x2
8	Sterilized H_2O (ampule)	12.2x2
Total Volume		50

Optimizing the reaction conditions for the amplification of molecules was done by setting initial denaturing at 95 ºC for 4 minutes in the first stage. The second stage included 35 cycles, with each cycle lasting 30sec at 94 ºC for denaturation process, 45sec for primer annealing step at 65 ºC and 60sec for extension step at 72 ºC. The third phase involved a last extension lasting 10 minutes at 72 ºC, and the fourth involved keeping the final temperature at 4 ºC until the thermocycler's PCR products were collected. Resultant amplification products were validated on a 2% agarose gel under UV-light.

Sequence Analysis

Amplified samples of the *CYP11B1* gene were sequenced and obtained in raw form. To view sequences, Finch TV was used. BLAST was then performed in order to obtain reference sequences (Erasmus, 2021). CLC sequence viewer '8' was used to perform multiple sequence alignment of samples and for SNP identification. Moreover Phylogenetic tree was also made through CLC sequence viewer '8'.

RESULTS

The PCR product of *CYP11B1* (Exon 1) showed amplification at 568bp of selected animals shown in Figure 1. The image clearly depicted the good amplification of DNA samples at optimized protocol in lane 1, 2, 3, 5, 7 and 8. While the samples in lane 4 and 6 were not fit for further processing, so these samples were not proceeded further. The amplified products with good results were utilized for sequencing.

The DNA sequencing method was used for identification of SNPs in *CYP11B1* gene in Tharparkar and Sahiwal cattle breeds of Pakistan. Multiple sequence alignment of samples with reference sequences has shown SNPs in different positions shown in Figure 2. Sequence analysis of the *CYP11B1* gene revealed total of 22 variations in two cattle breeds (Tharparkar and Sahiwal), out of which 14 SNPs were observed in Tharparkar cattle and 8 SNPs were

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observed in Sahiwal cattle out of them, 7 were non-synonymous mutations in Tharparkar breed and 5 non-synonymous mutations observed in Sahiwal breed (Table 3).

A chromatogram showing allele variants at the same positions, and multiple sequence alignment of Sahiwal and Tharparkar within breed (Figure 3).

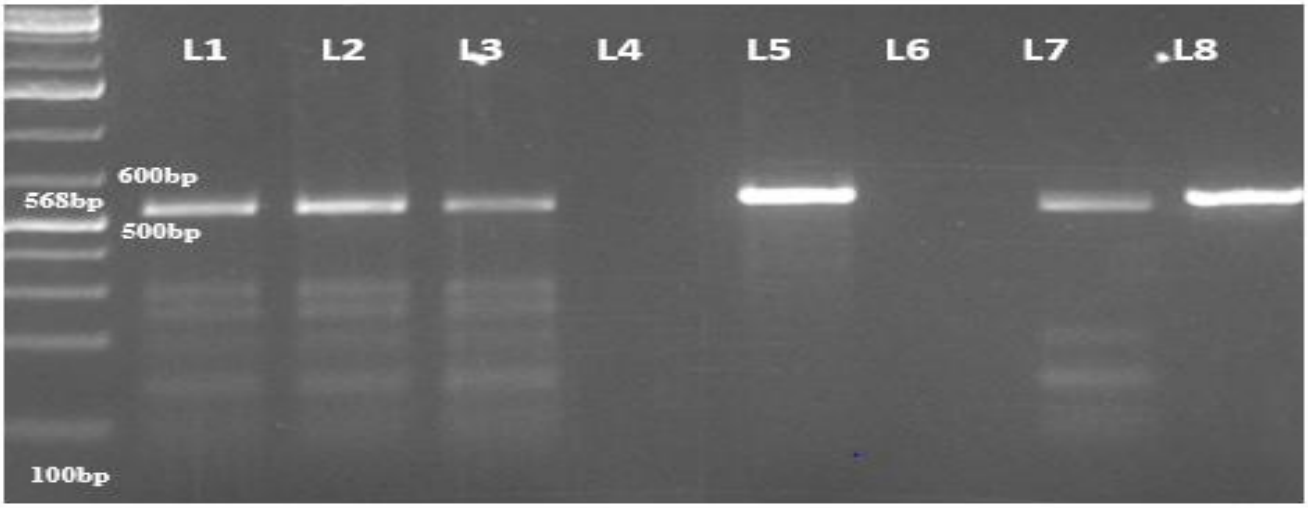

Figure 1: Amplified PCR products of putative exon I of *CYP11B1* gene (568bp) on 2 % agarose gel. Lane 0 from left DNA ladder (Thermo, MBI, Fermentas), Lane 1-8 selected animal samples

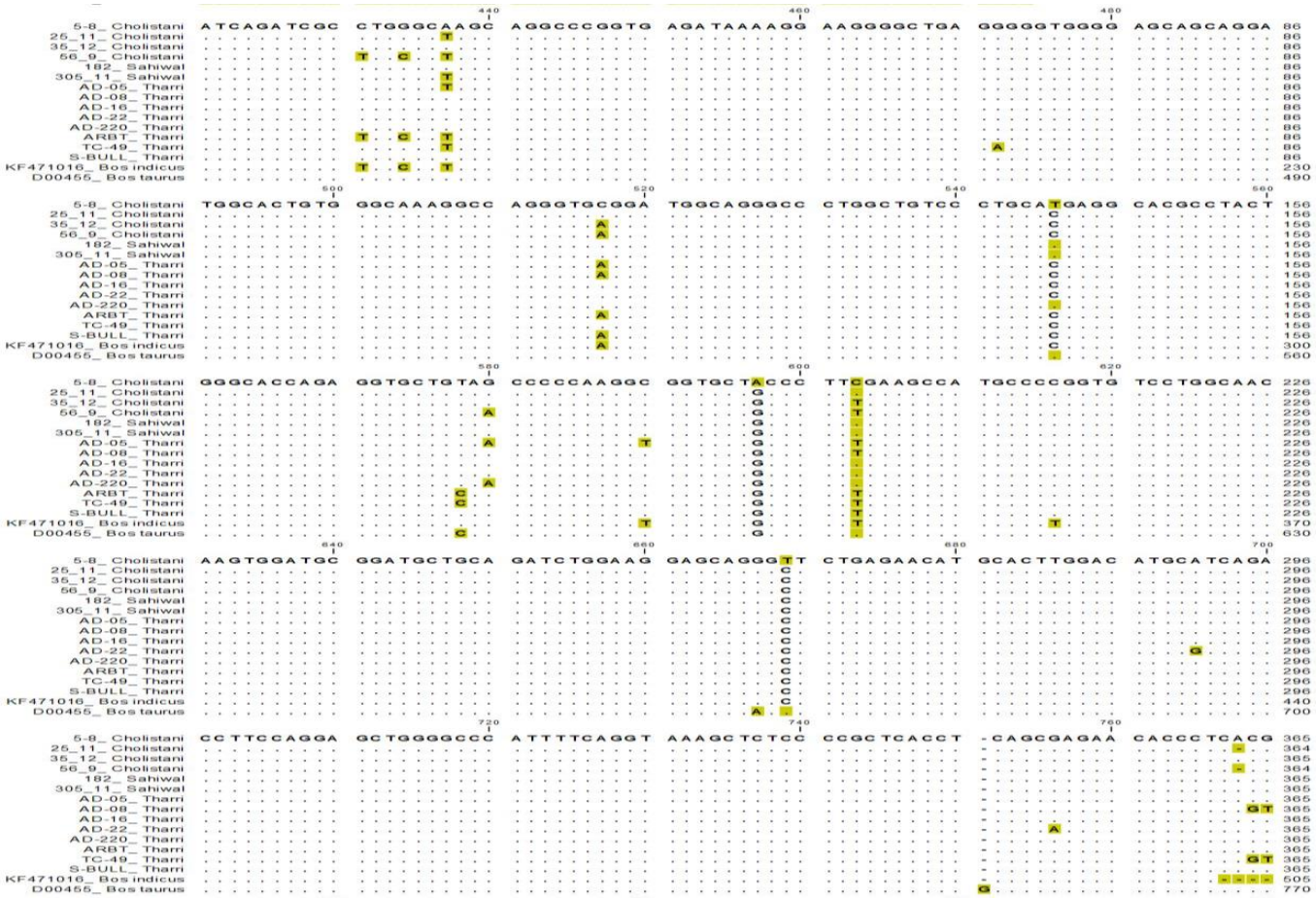

Figure 2: Multiple sequence alignment of Sahiwal, Tharparkar, and Cholistani cattle breeds with reference sequences (Accession no KF471016 and D00455) showing variations. *Dots (.) shows the similarity of sequences. Gaps (-) shows missing nucleotide at that position in the sequence.

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Table 3: Summary of SNPs in *CYP11B1* gene identified in two Indigenous cattle breeds of Pakistan.

Gene Locus	Breed	Chromosome	Single Nucleotide Polymorphism	
			Total no of mutations	Non-Synonymous mutations
CYP11B1	Tharparkar	14	14	07
	Sahiwal	14	08	05

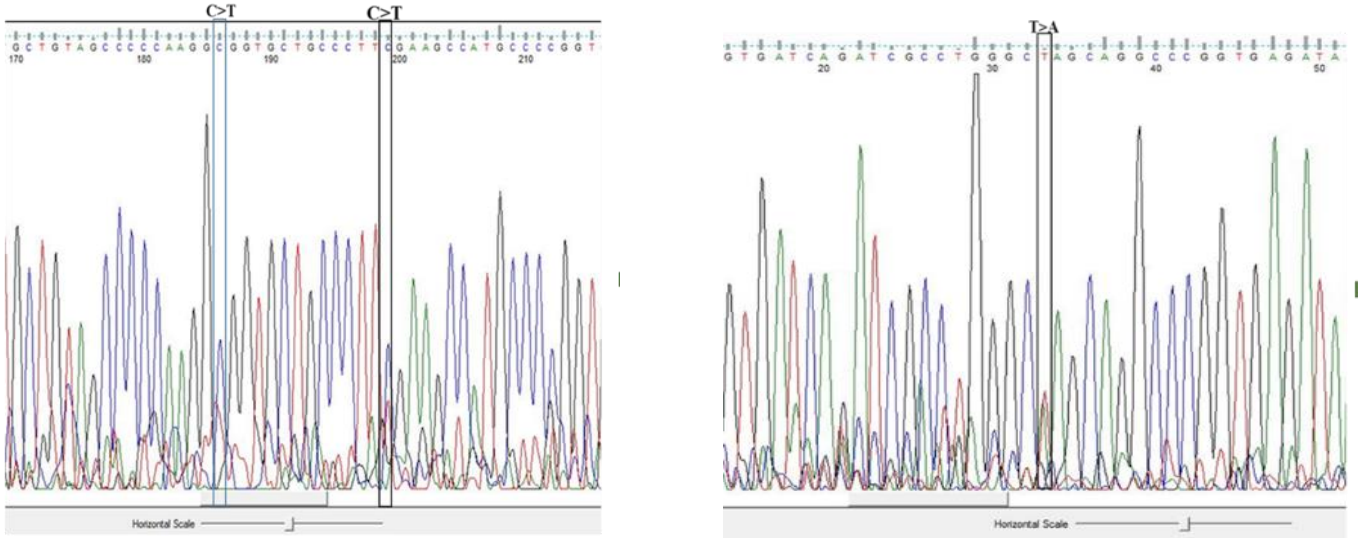

Figure 3. Multiple sequence alignment of (a) Tharparkar and (b) Sahiwal cattle within breed indicating presence of different alleles at same position.

SNP-based Phylogenetic tree revealed that Tharparkar cattle breed is more closely related to the *Bos indicus* (Accession number. KF471016/ *Bos indicus*) and the Sahiwal is forming a clade with the *Bos Taurus* (D00361/ *Bos taurus* and D00455/ *Bos taurus*). SNP based phylogenetic tree also showing the variations within cattle breeds as showing in Figure 4. Tharparkar cattle formed clades at 3 positions in tree indicating the heterogeneity within breed. This genetic analysis of Tharparkar cattle has revealed the existence of three genetically distinct groups or lineages within the breed. This genetic heterogeneity suggests that different genetic lineages or subpopulations exist within the Tharparkar cattle breed, which may have evolved or been influenced by various factors over time. Sahiwal samples also fall in the same clade except one. The out-group used in this tree construction was *Ovis aries* (Accession no. L28716/ *Ovis aries*).

DISCUSSION

Development in animal genetics facilitated the identification of genetic variations associated with health and reproduction traits. The identification and utilization of DNA markers for the production of desirable traits has ended up resulting in improved cattle health and productivity. The quantitative trait loci (QTL) associated with the commercially significant features of dairy and meat cattle have been the subject of significant studies. The majority of the identified QTLs on BTA14 in dairy cattle are thought to be associated with variables related to milk production, such as milk yield, fat yield (%), protein content (%), and protein yield. There are currently several hundred genes known to be involved in milk production, and research on *DGAT1*, *PRL*, *PPARGC1A*, and *CYP11B1* in cow's milk has been reported (Javed *et al.*, 2013; DN Das and Mundhe, 2019). Keeping in view the above facts a research plan was made to conduct a study on genetic characterization of *CYP11B1* gene in local Sahiwal and Tharparkar breed and to find out potential SNPs for future marker assisted selection.

The single nucleotide polymorphisms in the *CYP11B1* gene of the Pakistani cattle breeds Tharparkar and Sahiwal were found using a DNA sequencing method. Sequence analysis of the *CYP11B1* gene revealed total of 22 variations in two cattle breeds (Tharparkar and Sahiwal). These variations are illustrated in Figure 2, and they are A>T, G>C, G>A, and C>A at different places. Of these variations, 14 SNPs were found in Tharparkar cattle while 8 in Sahiwal cattle. According to previous study results, the G>A mutation in *CYP11B1* in dairy cattle was linked to an increase in milk fat percentage (Javed *et al.*, 2013; DN Das and Mundhe, 2019). In 2018, Manzoor and colleagues also revealed a high level of sequence variation across the *CYP11B1* locus, i.e. A>G, G>A, G>A, and A>G at loci

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1310397, 1310450, 1310462, 1310487, and 1310519 (Das *et al.*, 2019). Identification of SNPs in the *CYP11B1* gene in Tharparkar and Sahiwal cattle is a remarkable discovery that could have profound impact on cattle genetics and breeding programmes in Pakistan. The significant variation within a breed is also evident in the chromatogram of sequences as illustrated above in the chromatogram Figure 3. These results suggest that there may have been crossbreeding in the Tharparkar cattle animals from where samples were collected, and there is very little probability of crossbreeding in the samples taken from the Sahiwal breed.

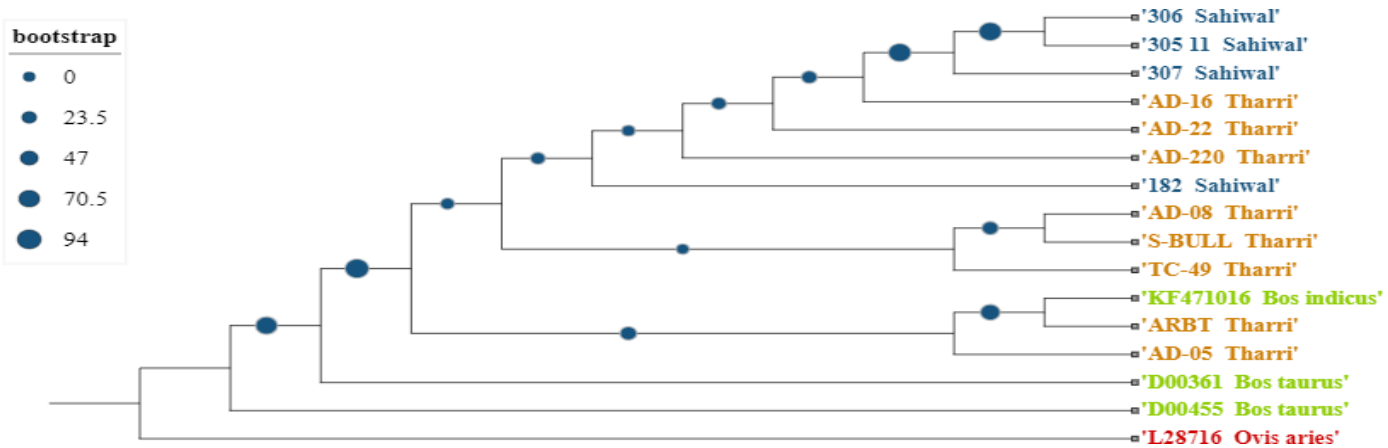

Figure 4: SNP Based Phylogenetic analysis using putative exon I of *CYP11B1* gene. Tree was constructed using UPGMA method. Genbank accession numbers used were (Accession no KF471016/ *Bos indicus,* D00361/ *Bos taurus* and D00455/*Bos taurus*) as reference sequences and (Accession no. L28716/ *Ovis aries*) as out- group. * Green color indicates reference sequences; red color indicates outgroup and brown color indicates the targeted samples. * Circles indicate bootstrap value.

The fundamentals of molecular phylogenetics are the analysis of mutations at different locations in sequences and the development of hypotheses about the evolutionary relatedness of biomolecules. SNP-based phylogenetic tree of cattle samples revealed that the Tharparkar cattle breed is more closely related to the (*Bos indicus)* and Sahiwal is making a clade with the (*Bos Taurus)*.The work of Kumar also supports the results of the present study (Kumar and Chopra, 2015) . In current study SNP based phylogenetic tree of Sahiwal and Tharparkar cattle revealed promising findings as Tharparkar cattle has shown divergence from *Bos taurus* as well as Sahiwal cattle breed. Moreover, sequences of Tharparkar cattle have also shown divergence within breed as the sequences have formed 3 different clades in different positions shown in figure 4. Multiple sequence alignment and chromatogram of the sequences also shows the great variation within breed illustrated in figure 3. These findings indicate the fact that the samples collected from Tharparkar cattle breed might have crossbreeding. In contrast, Sahiwal cattle has much less variation within breed as only one sample has shown two variations when compared to other Sahiwal samples in phylogenetic tree as samples forming same clade except one. This genetic divergence can be harnessed for selective breeding and conservation efforts, leading to more effective and sustainable management of the indigenous breeds. Furthermore, it underscores the importance of considering local and regional contexts in cattle breeding and conservation programs (Wambura *et al.*, 2000; Ghildiyal *et al.*, 2023).

When estimating a gene's overall contribution to a particular trait of interest, any difference in the regulatory regions of the genes is crucial; not just SNPs in the intronic or exonic regions of genes play a particular function in deciding how much these genes can produce. As a result, this offers another encouraging avenue for future studies to investigate candidate genes and explore new biological markers for marker-assisted selection.

CONCLUSION

The identification of genomic regions and potential genes related with milk fat content aids in better understanding the physiology behind milk production aspects and enables breeders to enhance milk fat ratio through genetic selection. In this study eight polymorphic sites were identified in Sahiwal cattle breed and fourteen SNPs identified in Tharparkar cattle breed. According to this study, Tharparkar cattle showed more variation within the breed as compared to Sahiwal cattle breed which led to an analysis of the purity of the breed. These SNPs influenced some of

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the associated milk production traits and breeding values that need to be further examined by combining sequence analysis with the PCR-RFLP genotyping method to identify genotypes appropriate for milk traits and perform an association study with phenotypic traits. A larger number of animals need to be studied further to identify genetic markers and their associations with economically significant traits for marker-assisted selection. Moreover, animal breeding should be in line with genetic approaches for better identification of the genes that have associations with milk fat content and confirm these findings before widespread application.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this research.

COMPETING OF INTEREST

The authors declare no competing interests.

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