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Full Length Article

Genetic Exploration of Casein Gene Family Associated with Milk Quality in Pakistani Buffalo Breeds

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Abstract

Casein proteins of milk are the key factors in defining the nutritional quality and processing characteristics of milk and the genetic variation in casein genes is known to affect such characteristics in dairy species. Nevertheless, there is a lack of complete data on the diversity of indigenous Pakistani buffalo breeds in terms of casein gene. The objectives of the current study were to describe genetic diversity in the key casein genes of Nili-Ravi, Kundi, and Azakheli buffalo and evaluate the possible structural and functional consequences of the identified variants. Blood samples were used to extract genomic DNA, amplified using conventional PCR and sequenced using Sanger sequencing of regions of interest. The bioinformatic pipelines were used to analyze sequence data to determine genetic variants, conduct phylogenetic analysis, and predict the structural effects of missense mutations. The number of variants that have been identified in the three casein genes (*CSN2*, *CSN3* and *CSN1S1*) was 2254, and *CSN1S1* had the most polymorphic variants. The breed-specific analysis also indicated a significant genetic differentiation, with the Kundi buffalo having the highest count of variants. There was a specific nonsynonymous mutation in *CSN3* (c.467C>T) in Nili-Ravi buffalo that caused a p.T156I amino acid substitution, which was a predicted localized structural and physicochemical change of κ-casein. Phylogeny also favored breed-level separation. The results indicate a significant amount of diversity of casein genes in Pakistani buffalo which could serve as a source of important molecular evidence in further association studies, breed improvement efforts, and conservation of native buffalo genetic resources.

Keywords: Caseins; Genetic polymorphism; Bubalus bubalis; Milk Proteins; Phylogeny

Introduction

Milk is a multifaceted biological liquid in which the nutritional and technological properties are regulated to a large extent because of the protein structure. Caseins are almost 80% of the total protein content of milk and are the most important proteins in terms of their functions related to the transport of calcium, the formation of micelles and dairy processing aspects like coagulation, curd firmness and cheese yield (Nayik *et al*. 2024). Therefore, genetic diversity in casein encoding genes has been widely studied as a factor that determines the quality of milk and industry performance in dairy animals.

Casein gene family is a closely related cluster of genes, and these genes include *CSN1S1* (αs1-casein), *CSN2* (β-casein), *CSN3* (κ-casein), each contributing distinct functional properties to milk. The αs1- and *β*-caseins constitute the major structural components of casein micelles, whereas κ-casein plays a critical stabilizing role by preventing uncontrolled aggregation and regulating enzymatic coagulation during dairy processing (Rehman *et al*. 2021). Single Nucleotide Polymorphism (SNP) of these genes can alter the structure of the proteins, intermolecular interactions, and finally milk processing behavior.

Extensive polymorphism has been described in the casein genes of cattle, and milk production, protein content, coagulation traits, and cheese-making efficiency have been reported to have many variants (Czerniawska-Piątkowska *et al*. 2023; Demirel and Çak 2025). The *CSN3* gene, the κ-casein, variants are closely associated with technological characteristics like the rennet coagulation time and curd

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firmness. This protein has also been noted to be functionally sensitive as single amino acid replacements have been reported to have quantifiable impact on the stability of the micellar structure and performance in its processing (Sharaz and Rabindrakumar 2024).

The buffalo (*Bubalus bubalis*) milk is different as it contains higher fat, protein, and total solids content, and it is, therefore, particularly useful in producing cheese and fermented milk products. Buffalo plays a significant role in the world milk production especially in south Asia (Liao *et al*. 2025). Pakistan is one of the top producers of buffalo milk, and the country has native breeds of buffalo that include Nili-Ravi, Kundi and Azakheli which are the mainstay of the national dairy industry. Although it is an economically significant aspect, the molecular mechanism of the milk protein diversity in the Pakistani buffalo breeds is under-characterized (Anas *et al*. 2023).

Previous studies in buffalo have also shown moderate yet functionally significant polymorphism of casein genes with alleles of breed-specificity that influence the next-generation milk composition and technological characteristics (Khan *et al*. 2023). Nevertheless, the majority of the literature is devoted to the Mediterranean, Murrah or Chinese populations of buffalos, but there is still a knowledge gap on the topic of South Asian and Pakistani buffalo breeds. Since the breeding history, management practices and environmental adaptations of Pakistani buffalo differ, chances are there that they have distinct genetic variations that may be of interest to milk quality and milk processing (Debaky *et al*. 2019).

The recent developments in molecular genetics and bioinformatics have made it possible to exactly characterize and be able to interpret genetic variants in functions. Targeted Sanger sequencing is a powerful method to identify polymorphisms in candidate genes, whereas the computational pipelines are used to identify the variants in the candidate genes, phylogenetic analysis, and protein structure modeling (Pauciullo *et al*. 2025). Particularly, mechanistic understanding of how amino acid substitutions affect side-chain chemistry, local interactions, and protein functionality is given using structural modeling of missense mutations to infer functions beyond the variation they cause at the sequence level (Panahi *et al*. 2024).

Simultaneously, phylogenetic analysis of milk protein genes provides useful data concerning relationship genetically, on differentiation of different breeds, and evolutionary history. Since the genes of casein are prone to both a functional constraint and a selective pressure due to the dairy production, they are informative markers of the population genetics and domestication history of buffalo (Parveen *et al*. 2023; Qin *et al*. 2025). A combination of genetic diversity analysis with phylogenetic reconstruction thus offers an all-encompassing framework of both the functional and evolutionary phenomena of milk protein variation.

Since there are very few molecular data of Pakistani buffalo, this study sought to provide a systematic characterization of genetic variation of the *CSN1S1*, *CSN2* and *CSN3* genes in Nili-Ravi, Kundi and Azakheli breeds. The particular aims were to determine gene- and breed-specific variants, find functionally relevant nonsynonymous mutations, provide an estimate of their structural effects by protein modeling, and derive phylogenetic information by using sequences of the casein genes. It is expected that the findings will aid further appreciation of the diversity of milk proteins in Pakistani buffalo and that they will form a molecular basis of future association studies, genetic enhancement and conservation.

Materials and Methods

Sample collection

Blood sample collection of different buffalo breeds was conducted from different regions of Pakistan. Nili-Ravi, Azakheli and Kundi buffalo samples were collected from Punjab, KPK and Sind regions of Pakistan, respectively Physical parameters and health status were recorded at the time of sampling. Sampling period was August 2024 to December 2024.

DNA amplification and sequencing

Genomic DNA was extracted using standard Organic (Phenol-Chloroform) method. Primers for all three genes were designed: *CSN1S1*: F: CAGGTACCCCAAGAAAAGCC and R: CACTGCTCCACATGTTCCTG with product size 559bp, *CSN2*: F: TAAAATCCACCCCTTTGCCC and R: ACATCAGAAGTTAAACAGCACAG with product size 526 bp, *CSN3*: F: TGTGCTGAGTAGGTATCCTAGTTATGG R: GCGTTGTCTTCTTTGATGTCTCCTT with product size 453 bp. For PCR amplification reactions, the final volume of PCR was 2 5 μL containing extracted genomic DNA 2 μL, 1 μL of each primer, 8 μL ddH_2O and 13 μL Dream Taq Green Master Mix (Thermo Scientific, USA). The cycling conditions included initial denaturation at 94°C/5 min, followed by 35 cycles of amplification which included 94°C/30 sec, the optimum temperature for annealing/30 sec and 72°C/30 sec and final extension at 72°C/10 min. Subsequently, the PCR products were visualized on 0.8% agarose gel after horizontal gel electrophoresis followed by staining with Ethidium Bromide. The unpurified PCR product was then sanger sequenced.

Bioinformatic analysis

Sanger sequencing files (ab1 format) for the *CSN1S1*, *CSN2*, and *CSN3* genes were collected from various breeds of *Bubalus bubalis*, including Azakheli, Kundi and Nili-Ravi. Each ab1 file represents a pooled DNA sample from 10 individual buffaloes from each breed to enhance genetic diversity representation.

Raw ab1 files were processed using custom Python code with the Biopython library, namely abifpy to decode the raw ab1 files and extract sequence data and Phred quality scores. The analysis of sequence quality was based on the average Phred score per read, and the reads with a score below the cutoff (*e.g*., Q20) were of low quality. Moreover, base quality at the ends of the sequences was removed to maintain the fidelity of the data to further analysis; a quality summary was obtained and stored in an ab1 processing summary.csv file.

Gene of interest reference sequences were retrieved from the NCBI Nucleotide database with accession numbers: *CSN1S1*: JN786874.1 to *CSN1S1*, *CSN2*: NM_001290879.1 to *CSN2* and *CSN3*: XM_006071122.2 to *CSN3*. These sources helped in the alignment and variant calling.

Each gene was aligned using Clustal W by multiple sequence alignment (MSA) and comparison of sample sequences to NCBI reference sequences using a Python script and the ClustalW interface of MEGA12. The SNPs were discovered based on a comparison of the aligned sequences with references, and a CSV table was recorded containing the differences in the identified variants.csv.

Gene *CSN1*, *CSN2* and *CSN3* phylogenetic trees were built with the use of the Neighbor-Joining algorithm using the distance matrices of the alignments. MEGA12 was used to create these trees and displayed as PNG images.

SWISS-MODEL was used to perform protein modelling to determine the alteration of amino acid due to missense mutation.

Results

Variant Summary

When the sequences of the samples were compared with the corresponding reference sequences, 2254 genetic variants were detected in the sequence of the three genes, namely *CSN1*, *CSN2* and *CSN3*. The variability of these variants in the three casein genes is summarized in Table 1. Table 2 shows the distribution of variants among the various breeds of buffalo (Azakheli, Kundi and Nili-Ravi).

Comparison of common and distinct variants showed that 4 variants were shared in all 3 breeds identified, indicating the possible existence of conserved polymorphic sites. On the other hand, 221 of these variants were found to be exclusive to one breed, which may have breed-specific genetic markers that can be useful in breed identification or trait association research (Fig. 1 and 2).

Identified specific mutation

Exon 5 CSN3 gene c.467 C>T missense mutation was discovered in buffalo samples of Nili-Ravi. The mutation results in the replacement of the Threonine (T) by Isoleucine (I) at 156 i-e p. T156I (Table 3; Fig. 3 and 4).

Table 1: Number of variants per gene

Gene	Count
CSN1S1	1013
CSN2	688
CSN3	553

Table 2: Number of variants per breed

Breed	Count
Kundi	834
Nili-Ravi	614
Azakheli	594

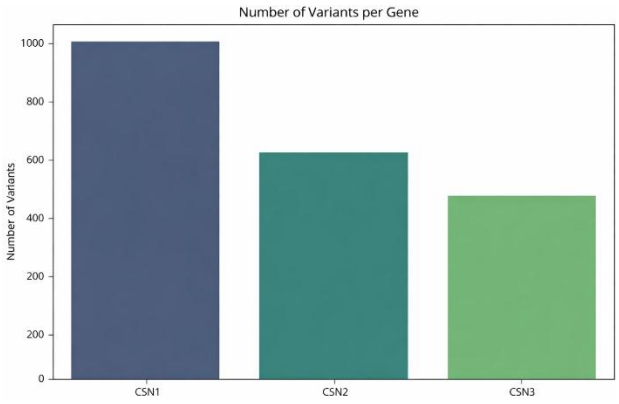

Fig. 1: Visually represents the number of variants identified for each gene, showing that *CSN1S1* exhibits the highest number of variations

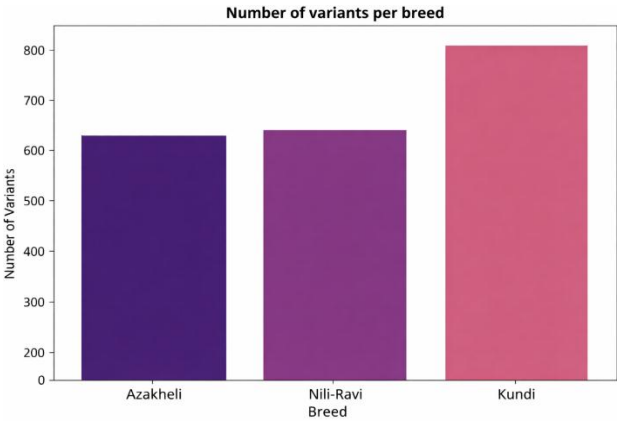

Fig. 2: Distribution of identified genetic variants across different buffalo breeds

Protein model effect

The amino acid replacement involves the replacement of the natural residue by one chemically different side-chain, which results in an apparent change in local side-chain geometry and physicochemical properties. The introduction of the residue alters the size, polarity, and the capacity to form H-bands, causing structural changes in the local interactions of neighboring residues, despite the overall polypeptide backbone being structurally conserved. It can modify the

Table 3: Identified missense mutation in *CSN3* gene in Nili-Ravi buffalo

Ch	Transcript	Chromosomal Location	Exon	Genetic Vartation	Protein
7	ENSBTAT00000150859.1	31,808,036	5	c. 467 C>T	p. T156I

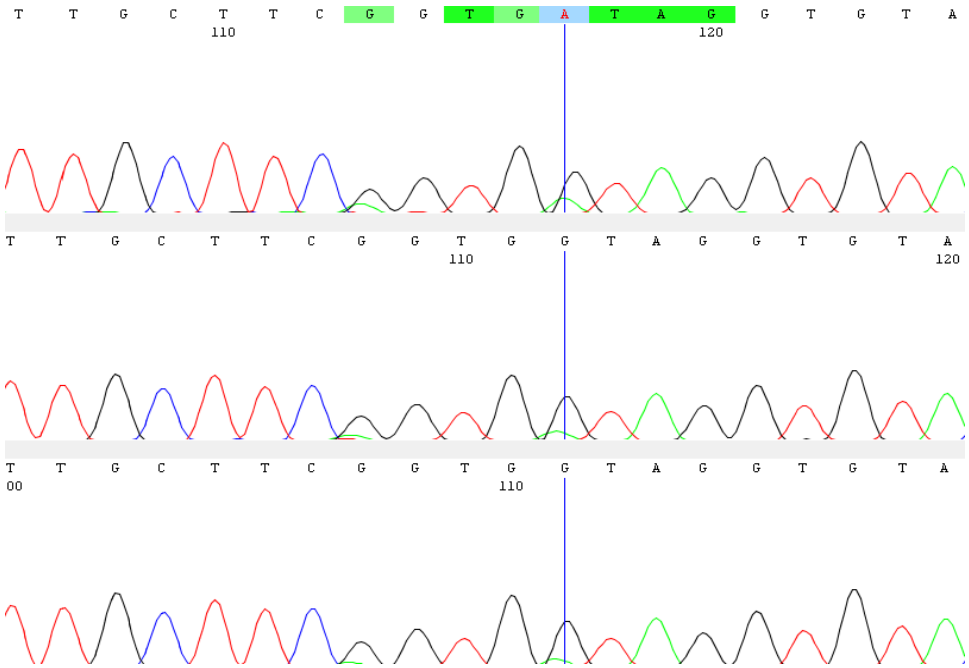

Fig. 3: Change in nucleotide in reverse strand of *CSN3* gene

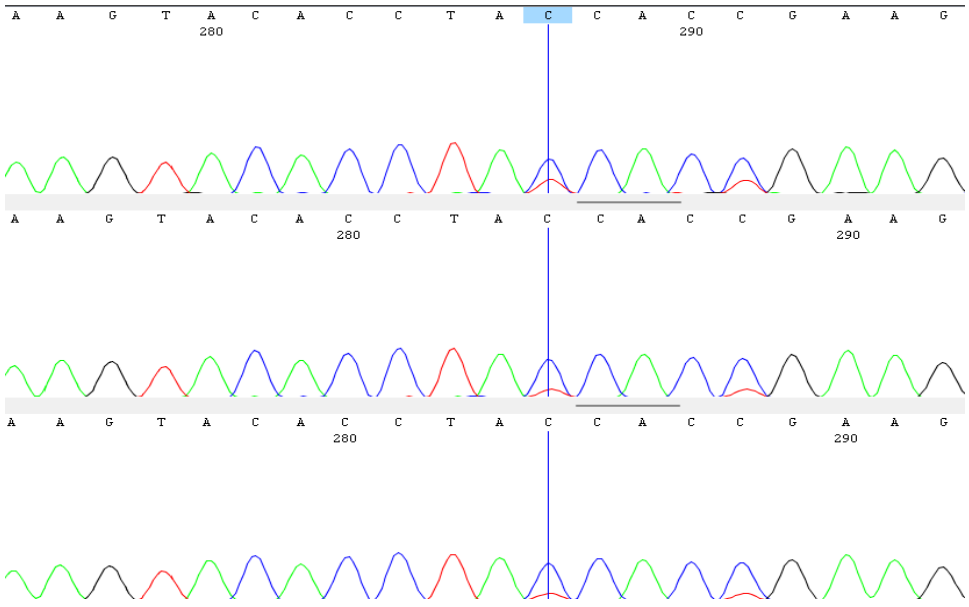

Fig. 4: Change in nucleotide in forward strand of *CSN3* gene

steric packing, the electrostatic interactions, or the solvent accessibility within the surrounding region and this can affect the local structural stability. Therefore, the substitution might also have functional consequences in disturbing residue-specific interactions having roles in protein conformation, molecular recognition, or catalytic efficiency (Fig. 5 and 6).

Phylogenetic analysis

A phylogenetic tree was drawn on each of the casein genes to depict genetic relationship between the buffalo samples and the respective reference sequences. The trees give an insight into the patterns of evolutionary separation and

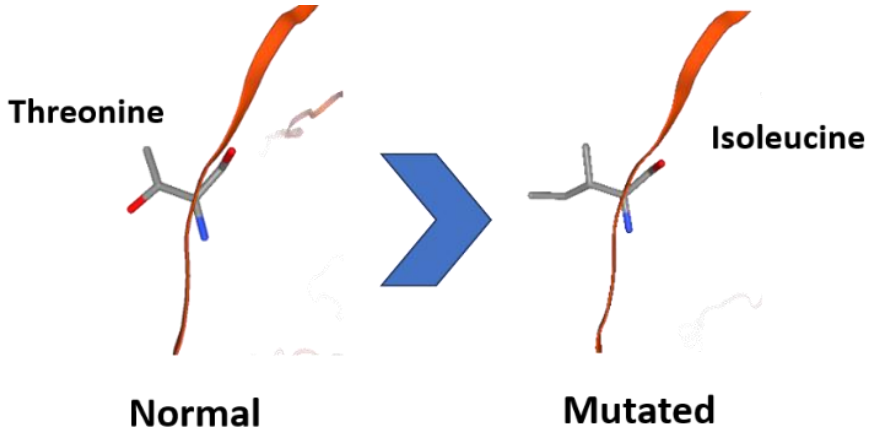

Fig. 5: Protein model of showing change in amino acid at position 156

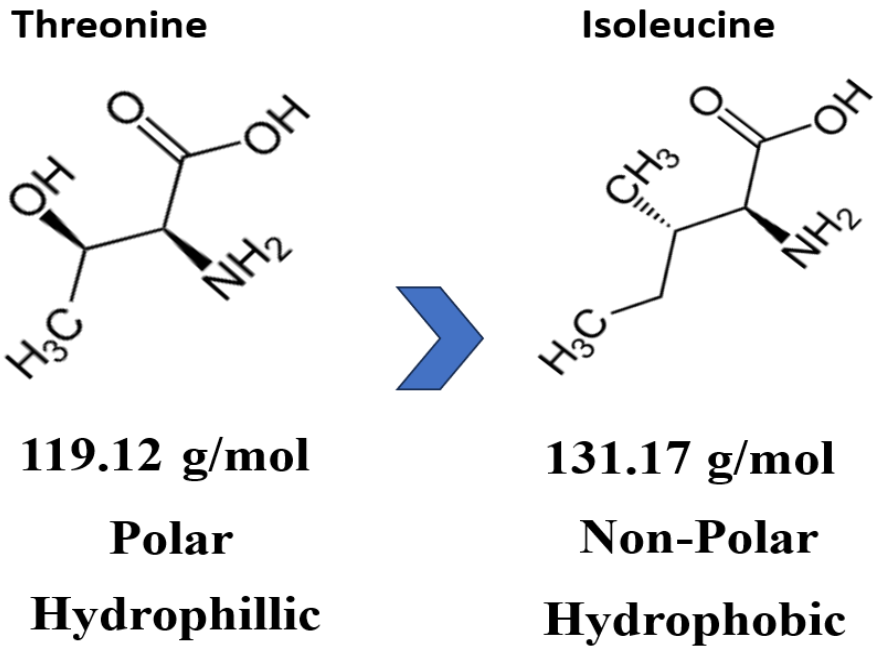

Fig. 6: Change in structure and function of amino acid caused by mutation p.T156I

clustering of the sequences. The phylogenetic tree of *CSN1*, *CSN2* and *CSN1S1* genes are indicated in Fig. 7, 8 and 9.

Discussion

This research provided a genetic and structural analysis of the large casein gene family, *CSN1S1*, *CSN2* and *CSN3*, in three Pakistani buffalo breeds of economic and genetic significance, *i.e.*, Nili-Ravi, Kundi and Azakheli. Casein proteins form the major protein component in milk and are at the heart of determining milk yield, nutritional value, micelle formation and processing properties including coagulation and efficiency in cheese making among others. Genetic diversification in casein genes has therefore been known a long time as a major predictor of milk quality phenotypes and a valuable marker-assisted breeding goal in dairy animals (Rehman *et al*. 2021).

The analysis of the three casein genes has established a great degree of polymorphism among the Pakistani buffalo populations as 2254 genetic variants were detected. *CSN1S1* had the most variants which was followed by *CSN2* and *CSN3*. This disproportionality among the variants of caseins loci may be a result of the dissimilarity between the functional limitations, the length of the caseins genes, the complexity of their regulation, and the selection pressure imposed on each gene singularly, and may be part of the reason that casein micelles form variably among cattle and buffalo (*CSN1S1*), a major protein constituent of milk, has been reported to exhibit high polymorphism at the locus in both cattle and buffalo (Demirel and Çak 2025). The relatively fewer variants in *CSN3* than in *CSN1S1* can be attributed to the vital nature of κ-casein in stabilizing casein

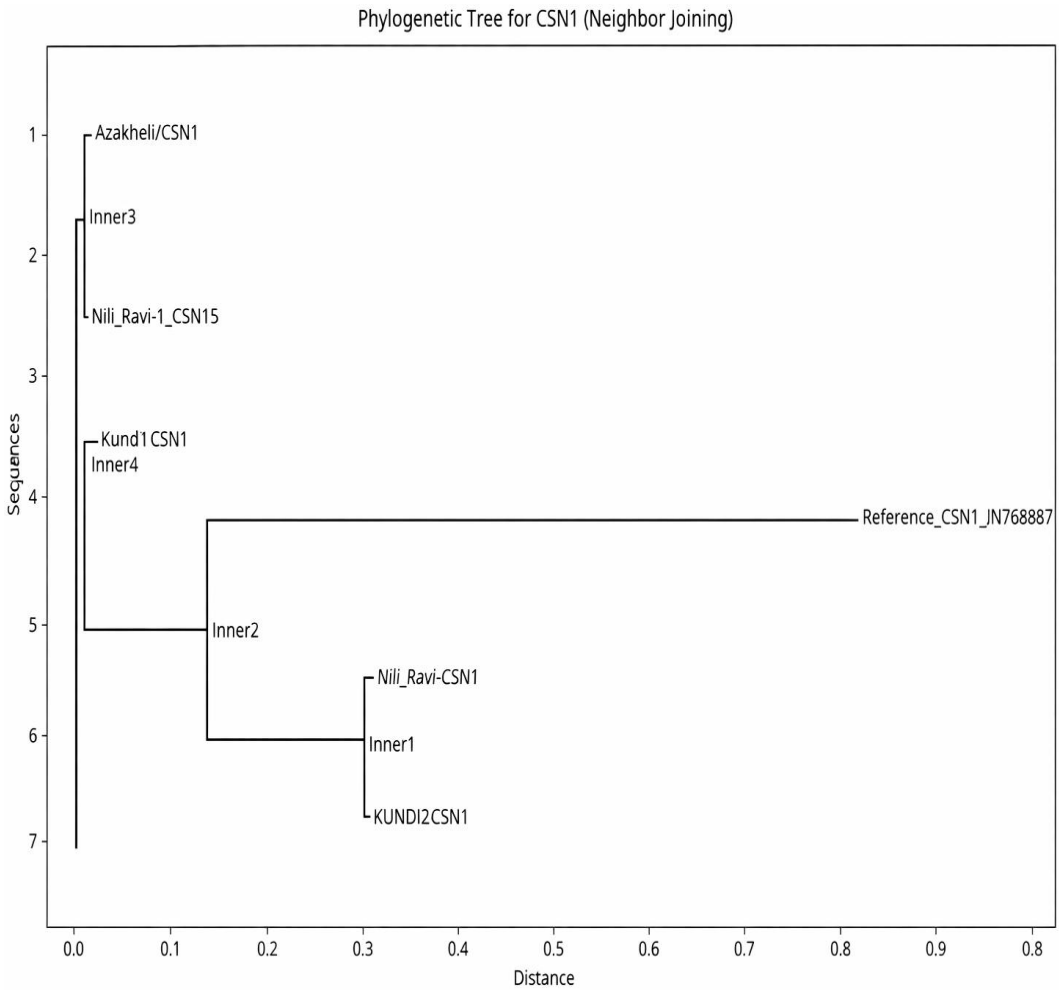

Fig. 7: Phylogenetic tree for *CSN1S1* gene sequences, constructed using the Neighbor Joining method

micelles. κ-casein plays a crucial role in ensuring that micellar aggregation is repelled by steric and electrostatic repulsion and thus the mutations in this gene are more vulnerable to purifying selection. However, even a small change in *CSN3* can cause significant phenotypic changes especially on the coagulation character and processing characteristics of milk (Sharaz and Rabindrakumar 2024).

The breed wise analysis showed that Kundi buffalo had the most variants followed by Nili-Ravi and Azakheli breeds. This observation indicates that there was a higher degree of genetic heterogeneity among the Kundi population, which could be the variation in breeding patterns, population size, geographic isolation, or historical introgression, consistent with genome-wide analyses that reported the fastest linkage-disequilibrium decay in Kundi among Pakistani buffalo breeds, indicating it is the most genetically diverse (Anas *et al.* 2023). Less intensive artificial selection has historically been applied to native breeds like Kundi and Azakheli in comparison to Nili-Ravi, a stable herd breeding type of dairy. Severe selection may reduce the evolutionary pressure, permitting a larger range of genetic variation to be maintained, such as infrequent or breed-specific alleles. The discovery of 221 breed specific variants in addition to this also works in favor of the genetic uniqueness of these buffalo groups. These variants can be used as useful molecular markers to identify breeds, trace and conservation genetics, consistent with recently validated casein-gene SNP panels reported across 11 buffalo populations for dairy improvement and conservation purposes (Pauciullo *et al.* 2025). Breed-specific polymorphisms in casein genes have also been observed in Mediterranean buffalo, Murrah buffalo and in other cattle breeds where they have been associated with local environmental adaptation and the use of milk (Antonopoulos *et al.* 2021). On the other hand, the fact that there are few variants that are common in all the breeds indicates that there are conserved polymorphic sites that could be maintained following neutral evolution or through selection.

Among other things, one of the most remarkable conclusions of the present research is the presence of a missense mutation (c.467 C>T) in exon 5 of the *CSN3* gene in Nili-Ravi buffalo with a threonine-isoleucine replacement

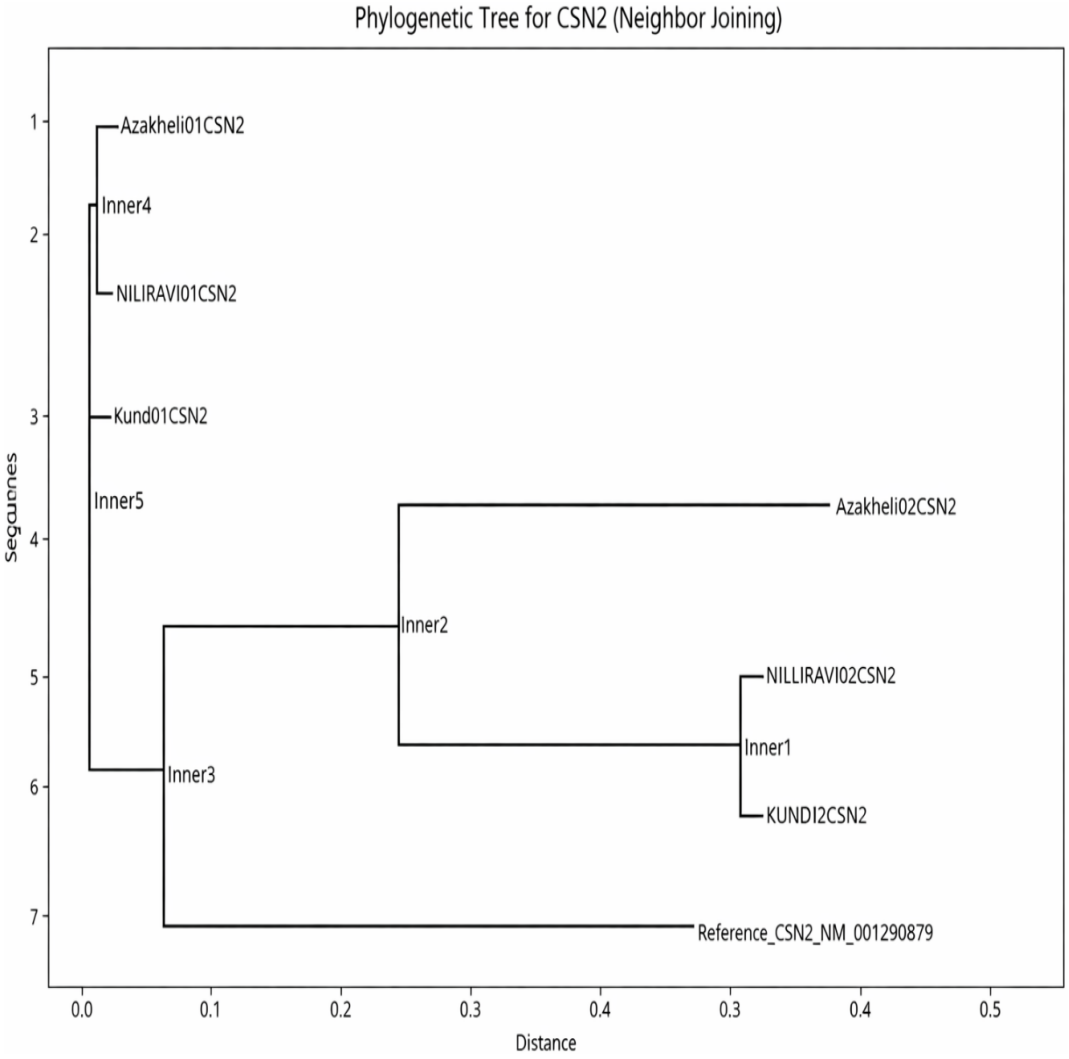

Fig. 8: Phylogenetic tree for *CSN2* gene sequences, constructed using the Neighbor Joining method

at position 156 (p.T156I). κ-casein is a well-characterized milk protein with a vital function in casein micelle stabilization and coagulation and, as such, is highly relevant to dairy processing properties, including cheese yield and curd firmness. Replacement of threonine, which is a polar amino acid and can form hydrogen bonds, by isoleucine, which is a nonpolar hydrophobic residue, is a significant physicochemical alteration. This type of substitution will tend to affect local interactions of residues, change solvent access, and alter the microenvironment in the protein. Past research has also established how even the changes in one amino acid in κ-casein can significantly influence the milk coagulation parameters, such as the rennet coagulation time and the curd strength in both cattle and buffalo (Lavon *et al*. 2023; Pauciullo *et al*. 2024).

Notably, the mutation was noted in Nili-Ravi buffalo but not in all other breeds implying that it could be breed specific. This particular variant could be a functionally relevant allele since Nili-Ravi is a high-yield dairy breed in Pakistan, and the approach to identifying a breed-specific trait with milk quality. Its functional significance will however be necessitated by the need to conduct additional population-level genotyping and phenotypic association studies.

The use of protein structural modeling also gave more information about the possible effect of the p.T156I substitution. The discussion has shown that although the backbone of polypeptides over time is structurally conserved, modification of the amino acid triggers localized changes in the geometry of side chains and physicochemical characteristics. The observation is in line with the general rule that missense mutations tend to have their effects on a local perturbative, but not structural rearrangement, as similarly reported for structurally modelled missense variants in other livestock candidate genes (Zeng *et al*. 2019). A substitution of polar residue by a hydrophobic residue could destabilize local hydrogen bonding patterns and alter steric packing between the residue and its neighbors. These alterations may affect the association of

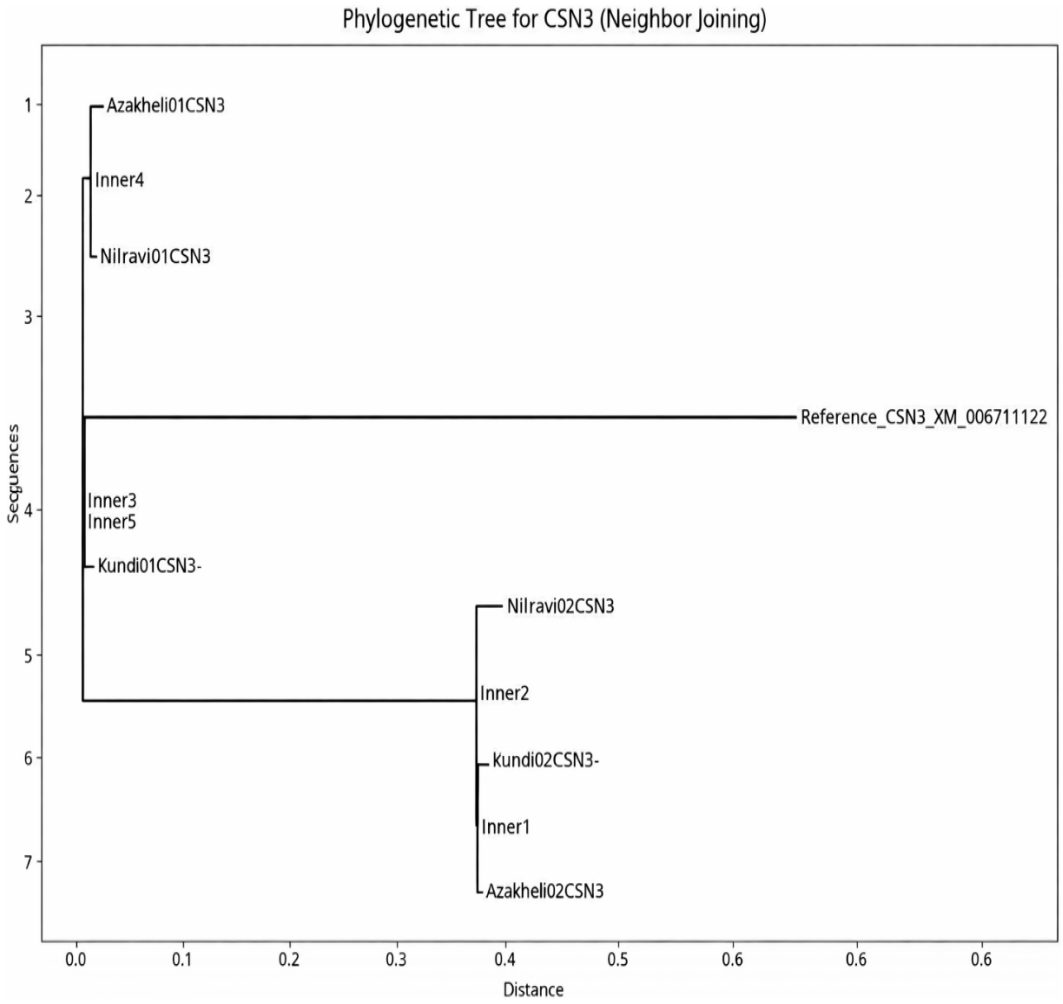

Fig. 9: Phylogenetic tree for *CSN3* gene sequences, constructed using the Neighbor Joining method

κ-casein with other casein proteins, as well as calcium phosphate nanoclusters, which are important in stability of the micelles. Czerniawska-Piątkowska *et al*. (2023) have highlighted that the small structural alterations in κ-casein can give rise to detectable changes in the micellar behavior and milk processing performance, which highlights the probable functional importance of the identified mutation.

CSN1S1, *CSN2* and *CSN3* gene sequences have shown that phylogenetic analysis can show distinct clustering patterns at the level of buffalo samples and reference sequences which indicates the evolutionary conservation and breed-level divergence. The high level of consistency in the overall topology of the various loci of casein speaks in favor of the strength of these genes as molecular markers to evaluate genetic relationships among buffalo populations. The phylogeny divide between breeds that was observed is in agreement with previous research findings that show that milk protein gene captures domestication history and breeding selection in dairy species (Lavon *et al*. 2023). Casein genes are likely to be maintained in a balance between purifying and diversifying selection due to the essential milk activities and to human preferences towards the milk yield and processing characteristics, respectively.

In future the studies should be carried out on large buffalo populations to establish the frequency of this mutation, its relationship with milk composition and processing parameters, and its interaction with other casein gene variants. Such molecular information will be significant in the integration of genomic data and detailed phenotypic records to convert these results into feasible breeding practices that not only improve productivity but also help preserve genetic diversity.

Conclusion

This study has shown a marked genetic variability within the casein gene family (*CSN1S1*, *CSN2* and *CSN3*) of Pakistani buffalo, where *CSN1S1* exhibits the greatest variability and evident variants burden differences across the breeds. Kundi buffalo had the most variants, whereas Azakheli and Nili-Ravi exhibited relatively low polymorphisms, which is in favor of breed-level genetic distinction among national groups.

Notably, a functionally significant nonsynonymous mutation was identified in *CSN3* (c.467C>T; p.T156I) that results in a threonine-isoleucine change that alters the local residue chemistry to change the polarity to hydrophobicity. The structural modeling suggests that the given change will have a high probability of affecting local interactions without the global fold being disrupted, which suggests that the behavior of κ-casein will be affected and, thus, milk micelle stability and qualities related to the processing. The findings have given genetic markers that are breed informative and given a candidate *CSN3* mutation of potential technological and nutritional interest that forms a useful basis of future genotype/phenotype association studies and the utilization of molecular information in buffalo breeding, conservation and milk quality enhancement programs.

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Authors' Contribution

Hammad Qaiser and Umar Aziz contributed equally in Writing, Methodology and Original draft. Muhammad Wasique Shahid, Zainab Mubeen and Ali Mujtaba Shah contributed in Methodology. Xueming Zhao and Tanveer Hussain contributed as Investigator and writing, reviewing and editing.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Consent for sample collection and publication

Consent was taken from all farm owners for blood sample collection and publication of data.

Data Availability

Data will be available upon reasonable request to the corresponding author.

Declaration of Generative AI Use

No generative AI tools were used in the preparation of this manuscript, including for content generation, language improvement, editing, or translation. The authors take full responsibility for the content, accuracy, originality, and integrity of the manuscript.

Ethics Approval

Ethical approval was taken from IRB, Virtual University of Pakistan.

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