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Research Article

Exploring Genetic Variability of the Partial Kappa-Casein Gene in Nili-Ravi Buffalo and Sahiwal Cattle of Pakistan

Muhammad Naeem Riaz[1], Sarfraz Mehmood[1], Abubakar Siddique[1], Hafiz Muhammad Bilal Akhtar[1], Khansa Jamil[1], Aatka Jamil[1], Jai Parkash kolhi[2], Ghulam Muhammad Ali[1]

[1] *Animal Biotechnology Program, National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Center (NARC), Islamabad, Pakistan.*

[2] *Department of Animal Product Technology, Sindh Agricultural University Tandojam, Pakistan.*

ABSTRACT

Recent years have seen an immense spike in scientific interest in the kappa casein gene (κ-CN) polymorphism because of its strong association with the milk composition, processing properties of milk and breed characterization etc. In the current study, two breeds of Pakistan, i.e., Nili-Ravi buffalo (n= 45) and Sahiwal cattle (n= 60), were genotyped for the κ-CN gene polymorphism using Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Animals of Nili-Ravi buffalo were found homozygous, showing BB genotype. However, genotypes AA & AB were observed in Sahiwal cattle with the genotypic frequencies 0.6 and 0.4, respectively. Sequence analysis of Nili-Ravi buffalo samples with already published sequences has shown two single nucleotide polymorphisms (SNPs) with no effect on protein functionality. However, on sequence comparison with Indian Murrah buffalo replacing Threonine with Isoleucine at codon 136, which affects protein functionality, also confirmed through PROVEAN software. Moreover, in Sahiwal cattle, both synonymous (mutations without codon substitution) as well as non-synonymous substitutions (mutations with codon substitution) were found. This work paves the door for future studies to characterize breeds and identify unique SNPs within the breed.

Keywords: Kappa-casein, SNP, Nili-Ravi Buffalo, Sahiwal cattle.

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Correspondence
Muhammad Naeem Riaz
naeem_nibge@yahoo.com

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INTRODUCTION

The ultimate goal of any dairy farm is to optimize both animal production and financial gain. The dairy industry's goal is to maximize milk output from existing herds without increasing the number of cows in each herd. "Milk," a lacteal secretion, contains all the necessary nutrients for development. It has been established that both genetic and environmental factors affect milk quality (Haug *et al.*, 2007; Heck *et al.*, 2009). Casein (αS1, αS2, β, and κ) and whey protein (α-LA & β-LG) are two major proteins found in bovine milk (Pesic *et al.*, 2012).

Polymorphisms in milk protein genes interest scientists because of the correlation between milk production and quality. The proteins in bovine milk are predominantly caseins (78-82%); kappa-casein (KCN) accounts for around 12% of the total (Bonfatti *et al.*, 2019). The genes for bovine casein are clustered together on chromosome 6. The κ-CN gene is over 13 kb long, although most of the protein's coding sequence is in exon IV.

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κ-CN stands apart from other caseins in terms of structure and function. In the process of milk curdling, it serves as a stabilizing factor during the formation of micelle structures. (Zhang *et al.*, 2023; Awad *et al.*, 2016). Most κ-CN in milk is concentrated on the outer surface of casein micelles. In its hydrolytic activity, chymosin destabilizes casein by cleaving it into the hydrophilic glycopeptide and the insoluble para κ-CN (macro-peptide) (Freitas *et al.*, 2019; Hobor *et al.*, 2008). Cheese-making rely on this stage of milk's coagulation process (Chen *et al.*, 2021). For eutherians, κ-CN is the only glycosylated casein that contains the sulfur amino acids cysteine and methionine (Matjek et al.2008). The threonine and serine residues of κ-CN can bind to carbohydrates via O-glycosidic linkages. The diversity in milk protein content is widely studied because of its practical importance in breed selection and characterization. Casein protein polymorphism impacts the structure, processing, and quality of milk. (Zhang *et al.*, 2023; Tsiaras *et al.*, 2005; Alipanah *et al.*, 2008). Breastfeeding duration, milk production, and composition have all been linked to κ-CN differences. Variations in the κ-CN gene locus have been found in many cattle breeds. Multiple alleles of the κ-CN gene, including A, B, C, E, F, G, H, I, and A1 (Caroli *et al.*, 2009; Djedović *et al.*, 2015; O'Riordan *et al.*, 2014). Only the A and B alleles are particularly common. Previous research found two amino acid changes, Thr136 IIe and Asp148 Ala, between alleles A and B (Alexander *et al.*, 1988). The genotype with allele B produces better curdles and cheese-making qualities due to its higher thermal resistance and shorter coagulation time (Heck *et al.*, 2009). Also, cheese made from milk of the κ-CN BB genotype produces roughly 10% more cheese from the AA genotype (Marziali and Ng-Kwai-Hang, 1986). Moreover, the KCN BB genotype animals produce significantly more milk protein than the AA animals (Bijl *et al.*, 2014). Several nations have begun incorporating selection for the κ-CN B allele into their animal selection programs because of its beneficial effect on milk production and quality. Dairy production in Pakistan heavily relies on the Nili-Ravi buffalo and Sahiwal cattle, which may also be found in other regions worldwide. These milch breeds are the best in the tropics due to their high feed conversion ratios, disease resistance, and ability to thrive in harsh environments. The kappa casein genotype of Nili-Ravi buffalo and Sahiwal cattle is analyzed using PCR-RFLP and sequence analysis because milk production is important to Pakistan's economy. Extensive research into κ-CN gene polymorphism in these breeds is currently underway.

MATERIALS AND METHODS

Samples Collection

The animals were selected randomly from the government as well as private livestock farms of Punjab, Pakistan. In the current study, 105 animals (Nili-Ravi = 45 and Sahiwal = 60) each had 3-5 ml of peripheral blood collected from them in sterile, EDTA-coated vacutainers.

DNA extraction

Gene JET Whole Blood Genomic DNA Purification Mini Kit (Thermo, MBI Fermentas) was used to extract genomic DNA from the blood. DNA samples were analyzed through NanoDrop (BioSpec-nano Shimadzu Biotech, Life Sciences) and on an agarose gel. The Purified DNA samples were vacuum-dried and dissolved in 100-200 µl elution buffer.

Polymerase chain reaction (PCR)

Bovine κ-CN gene exon IV was partially amplified by polymerase chain reaction (PCR) using genomic DNA as a template with primers (Barroso *et al.*, 1998), Forward: 5- TGTGCTGAGTAGGTATCCTAGTTATGG-3 and Reverse: 5-GCGTTGTCTTCTTTGATGTCTCCT-3. 100 ng template DNA, 1X Taq buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 U Taq DNA polymerase (Thermo, MBI Fermentas, USA), and 50 pmol of each forward and reverse primer were included in the PCR mixture (25 ul). There was a 5-minute denaturation at 94 °C, then 35 cycles of 1-minute denaturation at 94 °C, 1-minute annealing at 65 °C, and 2-minute extension at 72 °C, and finally a 10-minute extension at 72 °C.

Restriction fragment length polymorphism (RFLP)

For κ-CN genotype differentiation, each amplicon was digested with the Fast-digest *Hinf* 1 restriction enzyme, as reported by (Barroso *et al.*, 1998). In the reaction, 8 µl of PCR product, 5 U of Fast digest restriction enzyme (Thermo, MBI Fermentas), and 1 µl restriction enzyme buffer in a total of 20 µl was made. The incubation period for the reaction was 56^{o}C for 30 min. 15 µl of restriction digest result was electrophoresed on a 2% agarose gel for each sample, and band patterns were examined under UV light stained with ethidium bromide.

Statistical Analysis

The genotypic and allelic frequencies were calculated in the current study. The Hardy-Weinberg equilibrium of the animal populations used in this study was assessed using a chi-square test with one degree of freedom (P≤0.05).

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Sequence Analysis

Amplified PCR products of Nili-Ravi buffalo and Sahiwal cattle were sent for sequencing.Several bioinformatics tools have been utilized to analyze the sequencing data obtained. Sample sequences of buffalo and cattle have been checked and trimmed using FinchTV 1.4.0 (Geospiza Inc., Seattle, Washington, USA) (http:// www.geospiza.com). For sequence analysis of Nili-Ravi buffalo samples, two reference sequences (Accession No. MF679163 and FJ770200) were obtained through NCBI BLAST search, and one reference (Accession No. U96662) was chosen from the previous study (Mitra *et al.*, 1998). Regarding cattle samples, two reference sequences were chosen (Accession no. MG581713 and EU295526). Reference sequences were selected based on (1) similarity with sample sequences, (2) based on breed and (3) Geographical location. Sequence alignment of various sequences was carried out in 'CLC Sequence Viewer '8. (Knudsen *et al.*, 2007). Aligned sequences with SNPs were subjected to EBOSS Transeq 6.6.0(https://www.ebi.ac.uk/Tools/st/emboss_transeq/,lastaccess:8june2021), and nucleotide sequences were translated to their respective amino acids to identify if any SNP resulted in the change of amino acid causing non-Synonymous substitution. Similarly, PROVEAN was used to identify the effect of non-synonymous substitution on Protein functionality (http://provean.jcvi.org/index.php, last access: 8 June 2021). Moreover, phosphorylation and O glycosylation sites were analyzed through the NetOGlyc 4.0 server (http://www.cbs.dtu.dk/services/NetOGlyc/, last access: 9 June 2021) and NetPhos server 3.1(http://www.cbs.dtu.dk/services/NetPhos/, last access: 9 June 2021) (Fan *et al.*, 2019b). The SNP-based phylogenetic tree was constructed using UPGMA through CLC sequence viewer 8. Cattle reference sequences were used as outgroups for buffalo samples and vice versa.

RESULTS

PCR-RFLP analysis:

Electrophoretic examination of the amplified PCR result on a 1.8 percent agarose gel revealed a 453 bp fragment of the κ-CN gene on exon-IV, which covers the mutation area in all buffalo and bovine samples depicted in (Figure 1). The PCR results showed that the DNA was of good quality for PCR-RFLP analysis. When the PCR products of buffalo animals were digested with Hinf I, two DNA fragments (426 and 27 bp) were generated with just one restriction site for Hinf I, indicating the presence of the BB genotype only (Figure 2). Similarly, DNA fragments of Sahiwal cattle generated two types of banding patterns when digested with *Hinf* I: 326, 100 and 27 bp and 426, 326, 100 and 27 bp DNA fragments, respectively shown in (Figure 3), indicating the presence of two genotypes i.e. AA and AB with genotypic frequencies 0.6 and 0.4 respectively shown in Table 1. And allele A and B allelic frequencies were 0.8 and 0.2, respectively.

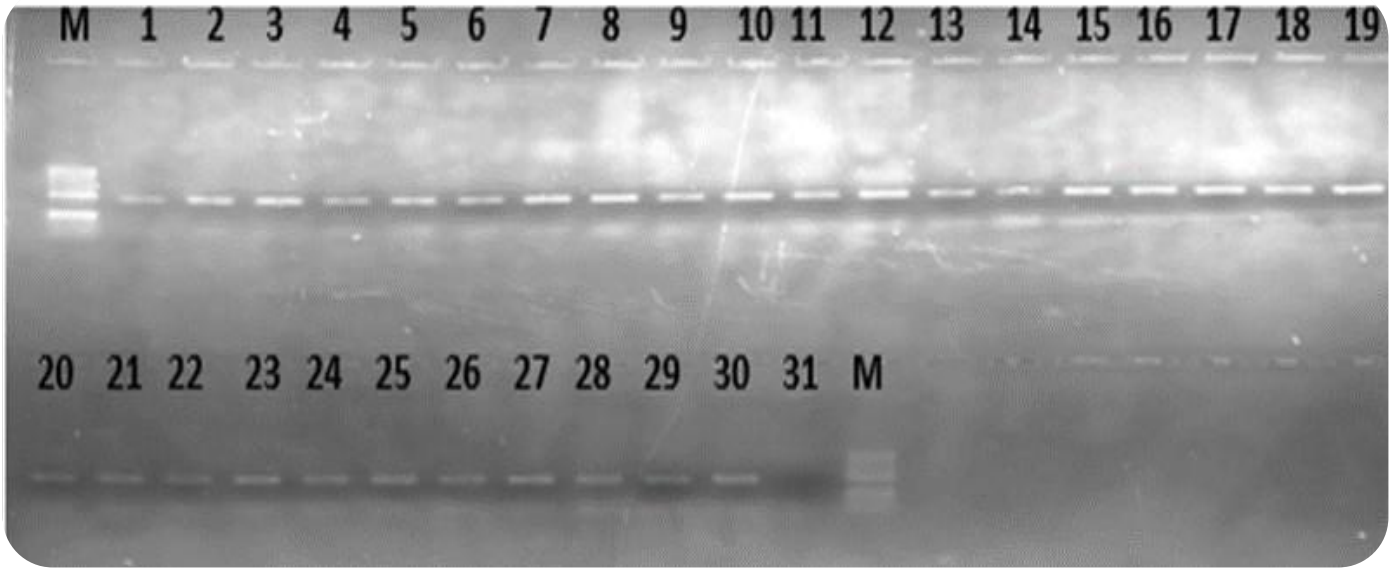

Figure 1. Amplified PCR products of κ-CN gene (453bp) analyzed on 1.8 % agarose gel. 50 bp DNA ladder (Thermo, MBI, Fermentas) indicated as Lane M. Lane 1-20: *Nili-Ravi* buffalo, Lane 21-30 samples from *Sahiwal* cattle has shown 453 bp PCR product Lane 31: Negative control.

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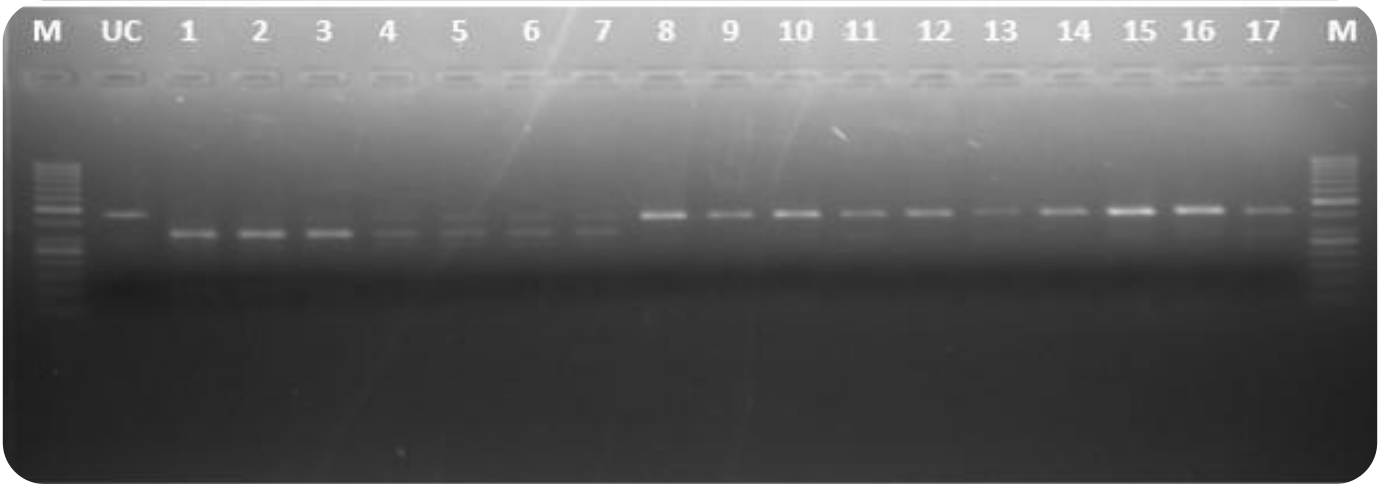

Figure 2. Nili-Ravi buffaloes with BB genotypes as identified through restriction analysis of a κ-CN gene fragments (426 and 27 bp) on a 2% agarose gel using the Hinf I restriction endonuclease. Lane M: 50 bp DNA ladder (Thermo, MBI, Fermentas), Lane 2: UC Uncut PCR product & Lane 8-17: restricted product of κ-CN gene.

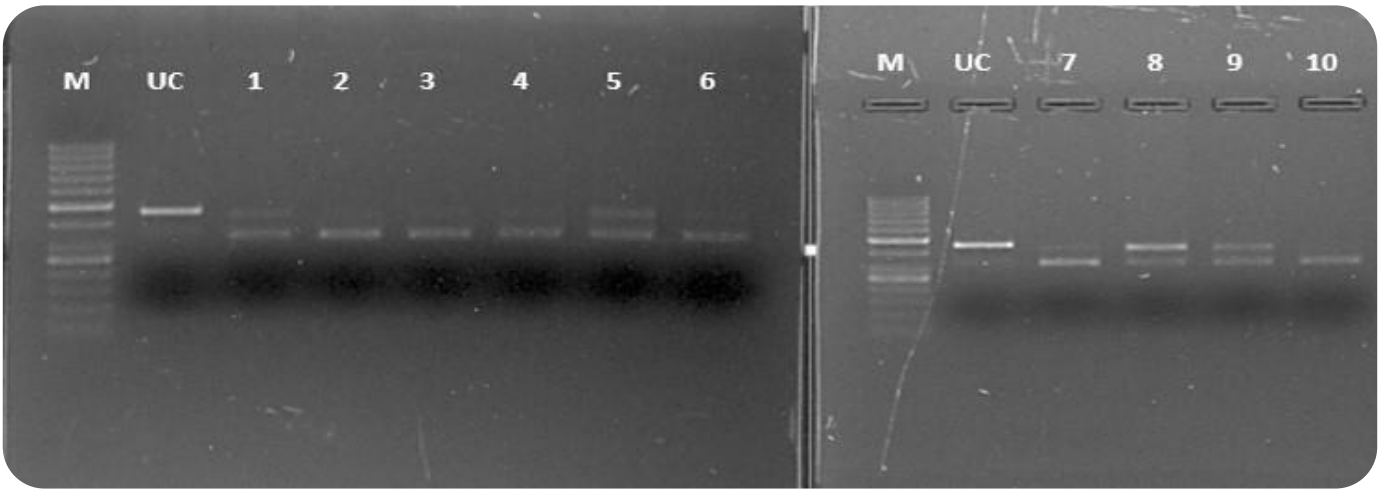

Figure 3. Restriction analysis of κ-CN gene fragments with (326, 100 & 27 bp and 426, 326, 100 & 27 bp) from Sahiwal cattle with AA and AB genotypes using Hinf I restriction endonuclease on 2% agarose gel. Lane M: 50 bp DNA Ladder (Thermo, MBI, Fermentas); Lane UC: uncut PCR product; Lanes 1-10: restricted Sahiwal bovine samples.

Table 1. Genotypic and allelic frequencies of buffalo and cattle**.**

Animals	Genotypic frequencies				Allelic frequencies	
	N	AA	AB	BB	A	B
Nili-Ravi buffalo	45	0.00	0.00	1.00 (45)	0.00	1.00
Sahiwal Cattle	60	0.60 (36)*	0.42 (24)	0.00	0.80	0.20

*No. of animals

Sequence Analysis

Multiple sequence alignment of buffalo samples with already published sequences has shown different SNPs with buffalo breeds (Figure 4). Sequence alignment of the κ-CN gene has shown 100 % similarity with Chinese buffalo (Accession No. MF679163). However, alignment with the already published sequence of Nili-Ravi buffalo (Accession No. FJ770200) has shown two SNPs at 102 and 111 positions as G>C and A>C, respectively. Nucleotide translation into amino acids has shown the replacement of Asparagine (N) with lysine (K) due to the replacement of 'G' with 'C' at position 102 in Nili-Ravi buffalo, thus showing non-synonymous substitution with no effect on Protein functionality. However, SNP at position no. 111 has shown synonymous mutation as GCC replaces GCA; both translate Alanine

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without affecting amino acid (Table 2).

Multiple sequence alignments of the samples with Murrah buffalo (Accession No. U96662) have shown two SNPs at nucleotide positions 317 and 321. SNP at nucleotide position 317 was identified as a non-synonymous substitution as it causes the replacement of Isoleucine with Threonine, as shown in Table 2. However, SNP at nucleotide position 321 was synonymous (ACT>ACC), both translated for threonine. Multiple sequence alignments of Sahiwal cattle with reference sequences (Accession No. EU365834 and MG581713) resulted in two mutations with no potential effect as shown in (Figure 5). However, the mutation in one of the cattle samples' OL439895/S-75', as shown in (Figure 6), was found to be a potential mutation as T>C with the amino acid transition from isoleucine (ATC) to threonine (ACC) at nucleotide position 410.

Figure 4. Multiple sequence alignment of buffalo (Nili-Ravi breed) samples with reference sequences (MF679163, FJ770200 & U96662) showing different SNPs at different Nucleotide positions. *Dots (.) shows the similarity of sequences. *Gaps (-) shows missing sequences.

Table 2. Effect of substitutions in κ-CN gene in buffalo samples (Nili-Ravi) compared to reference sequences.

SNPs and their positions with respect to buffalo samples	Nilli-Ravi breed (FJ770200)				Murrah breed (U96662)			
	SNPs in Nucleotide position	Substitution	PROVEAN Score	Prediction (Cuttoff= -2.5)	SNPs in Nucleotide position	Substitution	PROVEAN Score	Prediction (Cuttoff= -2.5)
	G102C	Lys34 Asn	-2.426	Neutral	C317T	Thr136Ile	-3.118	Deleterious
	A111C	GCC>GCA= Ala (Synonymous mutation)	-	-	C317T	ACT.ACC= Thr (Synonymous mutation)	-	-

Red color indicates potential SNP

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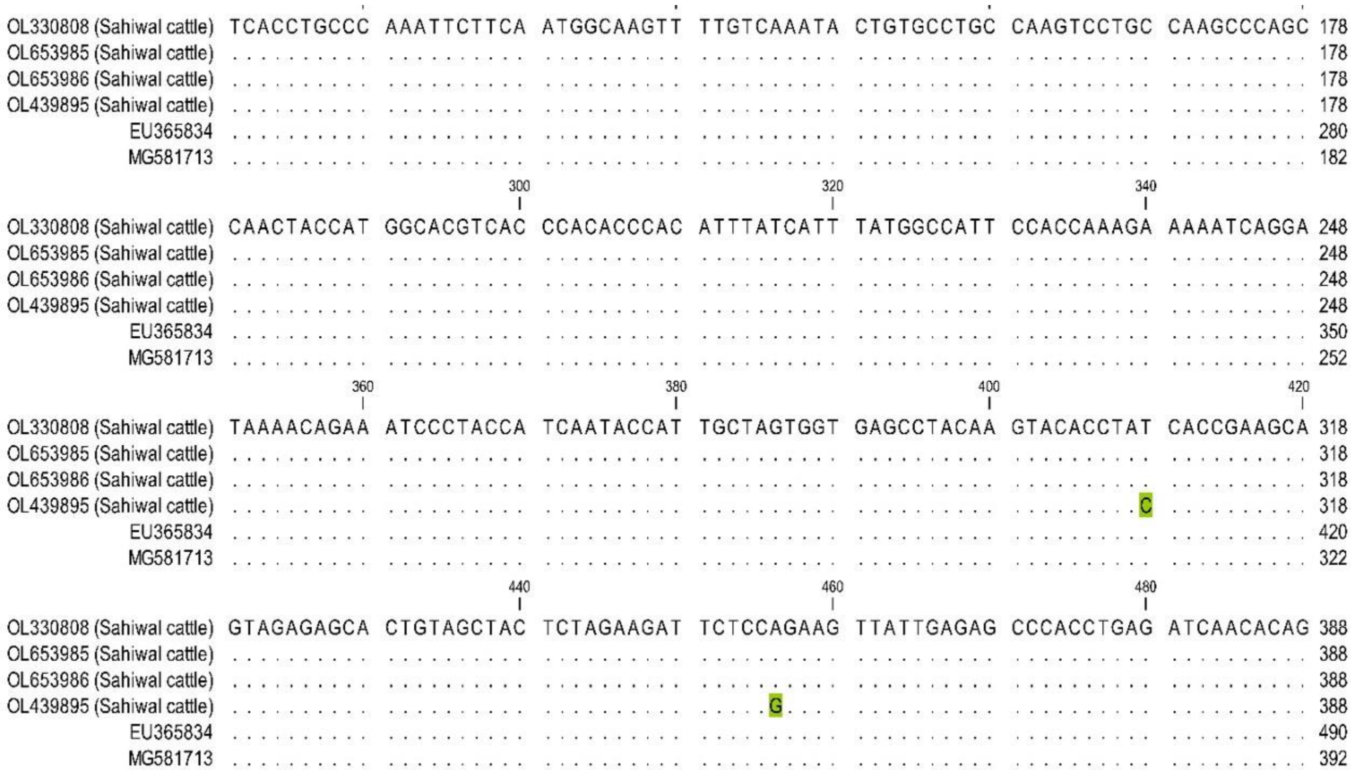

Figure 5. Multiple sequence alignment of cattle (Sahiwal) samples with reference sequences (MG581713, & EU365834) showing different SNPs at different Nucleotide positions. *Dots (.) shows the similarity of sequences. *Gaps (-) shows missing sequences.

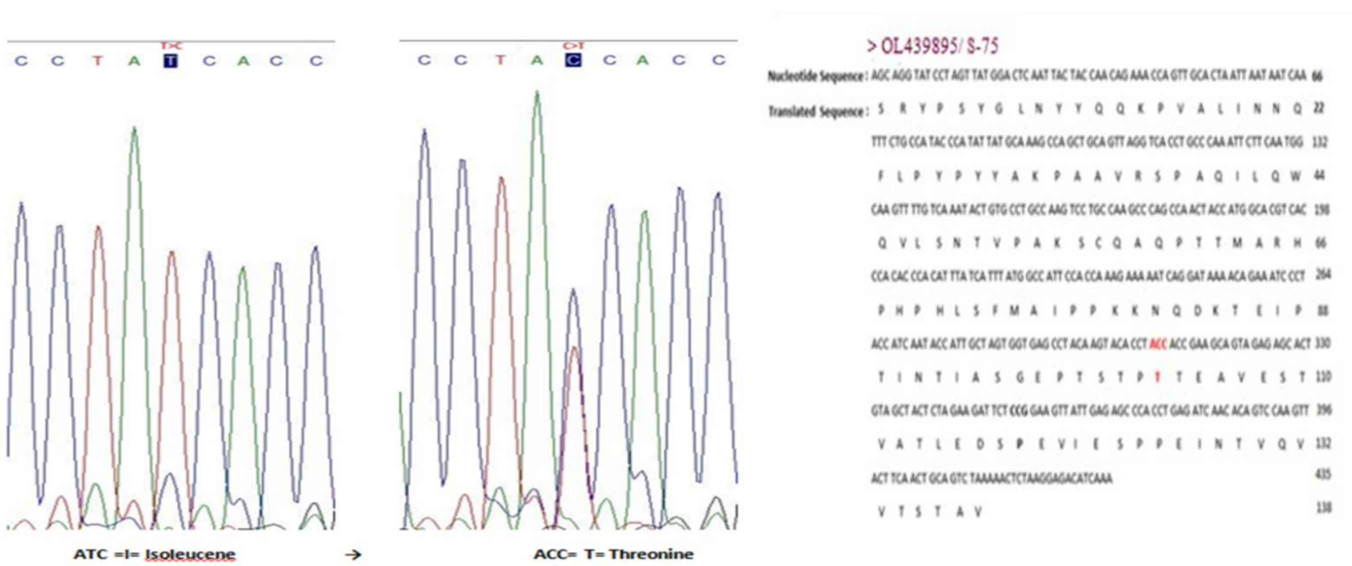

Figure 6. Chromatogram and protein translation of nucleotide sequence into respective amino acid of Sahiwal cattle representing nucleotide substitution (T>C) of coding region and amino acid substitution of isoleucine into threonine.

Phylogenetic analysis of buffalo samples has shown differences within the breed and with other breeds, as shown in (Figure 7-A). The cattle sequences were used as outgroups sharing different clades with buffalos, indicating the species difference in the κ-CN gene. The Nili-Ravi buffalo samples shared different branches with the already published Sequence of Pakistan. Moreover, samples have also shown divergence with the Murrah buffalo of India. Similarly, phylogenetic analysis of cattle samples has also shown differences as three samples shared the same clade (Accession No. MG581713 & EU295526). However, sample 'OL439895/S-75' shared a different branch from

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other samples, indicating SNPs and showing more close relatedness with reference (Accession No. EU365834) as shown in figure (Figure 7-B).

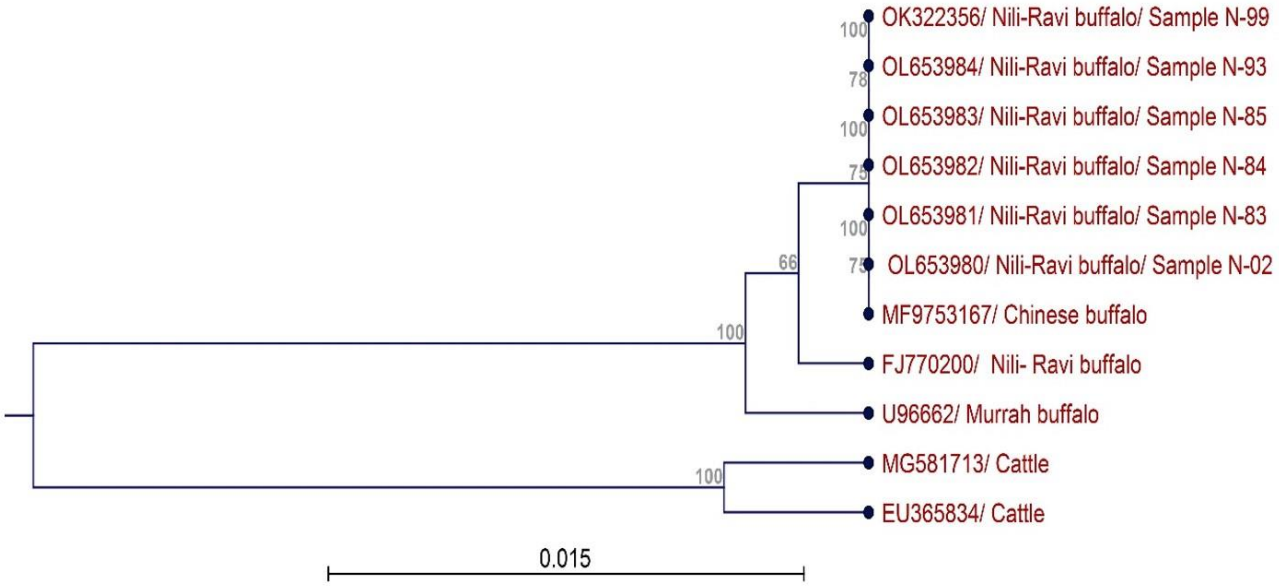

Figure 7-A. Phylogenetic analysis of Nili-Ravi buffalo isolates using partial kappa casein (453bp) sequences. The phylogenetic tree was constructed using UPGMA method in CLC sequence viewer 8 software. The GenBank accession numbers used as reference were (Accession no. MF679163, FJ770200 & U96662) and outgroup references (Accession no. MG581713 & EU365834).

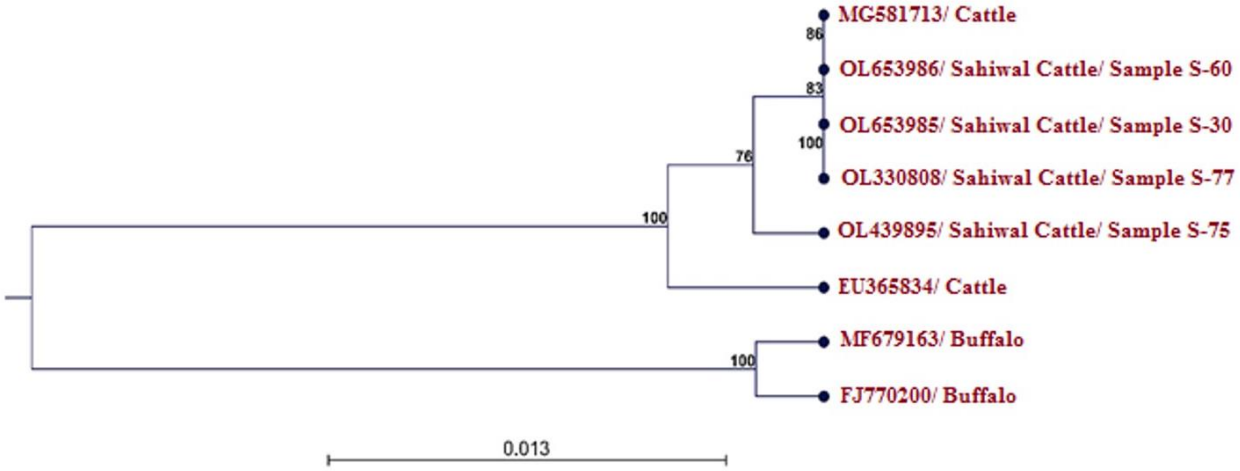

Figure 7-B. Phylogenetic analysis of Sahiwal cattle isolates using partial kappa casein (453bp) sequences. The phylogenetic tree was constructed using UPGMA method in CLC sequence viewer 8 software. The GenBank accession numbers used as reference were (Accession no. MG581713 & EU365834) and outgroup references (Accession no. MF679163 & FJ770200).

DISCUSSION

The Kappa casein gene (κ-CN) is crucial to milk protein output and the stability of casein micelles, and it encodes around 12% of the total casein in bovine milk. Milk protein polymorphism has recently garnered much scientific interest because of the correlation between milk output and quality (Zhang *et al.*, 2023). Micelle production is stabilized by polymorphism in the κ-CN gene, which also helps in breeds characterization (Awad *et al.*, 2016; Bonfatti

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et al., 2019). Our goal was to examine the influence of SNPs on protein translation of the κ-CN gene in Nili-Ravi buffalo and Sahiwal cattle by combining the PCR-RFLP approach with sequence analysis.

The amplified products from Nili-Ravi buffalos only had one restriction site when digested with the HinfI restriction enzyme, showing only BB genotype. In previous studies, only KCN BB genotype was reported in the Nili-Ravi buffalo and Egyptian buffalo (Ghafoor *et al.*, 2015) and Egyptian buffalo (Abdel Dayem *et al.*, 2009; El Rafey and Darwish, 2008) was also observed in a study conducted in Pakistan. Cheese production, milk protein production, etc., can all be attributed to the BB monomorphic form of the κ-CN gene (Othman *et al.*, 2011). Table 1 displays that the allelic frequencies for alleles A and B were 0.8 and 0.2, respectively, showing that the A allele was more common than the B allele. In previous study, in Sahiwal cattle only AA and AB genotypes were detected (Riaz *et al.*, 2012). Similarly, the AA genotype was dominant for the κ-CN gene in cattle, with allele A found in 82% of total samples (Sumaiya *et al.*, 2020). Furthermore, Allele B was less prevalent in all *Bos indicus* types (Sindhi, Gyr, Guzerat, and Nelore), with values that vary from 0.01 to 0.30 (Azevedo *et al.*, 2008).

To find the polymorphism, sequence analysis of Nili-Ravi buffalo and Sahiwal cattle as compared to sequences of other previously reported breeds. The results showed that Nili-Ravi has SNPs in several places. Multiple sequence alignments with the published Nili-Ravi sequence (Accession No. FJ770200) have uncovered two SNPs in buffalo samples (Nili-Ravi). In contrast, PROVEAN findings have revealed no risk to protein function. No SNPs were discovered when the sequences were compared to that of the Chinese buffalo (Accession No. MF679163). A nucleotide change at position 317 led to a non-synonymous mutation in which threonine replaced isoleucine. Sequence alignment of buffalo samples with the partial κ-CN gene of Murrah buffalo revealed two SNPs. The PROVEAN analysis verified the substitution as a potential SNP, indicating that the κ-CN gene in the two breeds likely has distinct physiochemical properties. Previous research has revealed that nucleotide SNPs can functionally impact the buffalo casein gene by altering the amino acid sequence (Fan *et al.*, 2019a). More amino acid variations in κ-CN protein have been found in riverine buffalo (Nili-Ravi, Murrah, etc.) compared to other breeds, suggesting a causal relationship between genetic variants in κ-CN genes and milk composition and milk product quality (Masina *et al.*, 2007; Azevedo *et al.*, 2008; Massella *et al.*, 2017; Rangel *et al.*, 2017). Post-translational changes, including O glycosylation and phosphorylation, have a major impact on κ-CN biological function (Bijl *et al.*, 2014). O glycosylation occurs mainly on threonine and serine residues in macro-peptides. The amino acids threonine, serine, and tyrosine phosphorylation sites (Fan *et al.*, 2019a).

The creation of these sites during the hydrolysis of milk chymosin κ-CN has been shown to directly alter macro-peptide's solubility and biological function, affecting milk processing (Fan *et al.*, 2019b; O'Riordan *et al.*, 2014). More O glycosylation sites were found in the Nili-Ravi buffalo than in the Murrah buffalo, according to the results of the current study. Nili-Ravi buffalo have a threonine instead of isoleucine at codon 136 when compared with Murrah buffalo, which has isoleucine at that position. Consistent with a previous investigation on the buffalo κ-CN gene, this one found that O glycosylation occurred primarily at threonine and that replacing threonine with isoleucine altered the protein's biological function after translation (Fan *et al.*, 2019a). More glycosylation sites in milk improve its cheese-making potential by decreasing micelle size on average and increasing its capacity for coagulation (O'Riordan *et al.*, 2014). In addition, SNP at O' glycosylation sites corroborates previous research findings (Bijl *et al.*, 2014; Fan *et al.*, 2019a; Othman *et al.*, 2011) and demonstrates that the B variant possesses a greater number of glycosylation sites than do other variants.

One of the SNPs, a non-synonymous SNP T>C resulting in an amino acid transition from isoleucine (ATC) to threonine (ACC) at nucleotide position 410 and codon 136 in one of the cattle samples, as shown in (Figure 6), indicates the presence of o glycosylation and phosphorylation site. The *Bubalus bubalis* and the *Bos indicus* have been the subject of several phylogenetic investigations. The phylogram of buffaloes shows that they are most closely related to Chinese buffalo in their DNA. (Accession No. MF679163) than between Nili-ravi buffalo sequences and Murrah. As seen in (Figure 7-A), there are clear differences in the genetic makeup of the κ-CN gene between buffalo and outgroups (*Bos indicus*), with buffalo samples belonging to a distinct clade than *Bos indicus*. Because of the divergance from the Sahiwal cattle samples in the phylogram caused by a non-synonymous mutation, the 'OL439895/S-75' sample also appeared as a distinct clade.

CONCLUSION

The Nili-Ravi buffalo and Sahiwal cattle undergo PCR-RFLP and nucleotide sequencing for κ-CN gene polymorphism. Genotype analysis revealed that all Nili-Ravi buffalo are BB homozygotes, while Sahiwal cattle are

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AA/AB heterozygotes. Multiple sequence alignment has revealed several SNPs in both Nili-Ravi buffalo and Sahiwal cattle. One potential SNP found in Nili-Ravi buffalo i-e Threonine>Isoleucine at nucleotide position 317 has a significant effect on protein functionality. Moreover, better cheese processing properties of milk can be attributed to the SNPs found in BB-genotyped animals of the Nili-Ravi buffalo. The Nili-Ravi buffalo has more O glycosylation sites when compared with the Murrah buffalo i-e replacing threonine with isoleucine which affects better cheese-making processing. This study paves the way for further breed characterization which can be useful for breed improvement programs.

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AUTHOR CONTRIBUTIONS

All authors contributed equally to this research.

COMPETING OF INTEREST

The authors have not declared any conflict of interest.

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