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# Molecular Analysis of Kappa Casein Gene in Kundhi Buffalo

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**JPG conducted research and wrote the manuscript. AHS directed and provided technical suggestions for manuscript. GBK contributed in drafting the manuscript. MNR guided to conduct molecular study. MAJ helped in sample collection and handling. GSB facilitated in farmer's meeting, animal identification and sample collection. AS helped in primer designing, and material arrangement. S Mehmood helped in RFLP, sequencing and applying Bio-informatics tool. S Memon did sequence alignment and SNP identification.**

**Key words**
**Kappa casein gene, Kundhi buffalo, Genotyping; RFLP, Sequence analysis, Haplotype analysis**

## ABSTRACT

In dairy sector, animal selection is mainly done on the basis of milk quality and quantity produced by the particular animal whereby protein is considered as a major marker for measuring the quality of milk. Among the milk protein, kappa casein attains major attention. The present study aimed to analyze kappa casein gene (CSN3) in Kundhi buffalo. To achieve the objectives, a total of 100 samples belonging to Kundhi buffalo were genotyped through PCR-RFLP analysis using *Hinf1* restriction enzyme. The restriction digestion pattern indicates the presence of only BB genotype in all analyzed samples. Similarly, characterization of amplified CSN3 gene fragment has also been carried out through nucleotide sequencing which showed variation when compared to Nili-Ravi and Murrah breeds of Pakistan and India, respectively. A potential SNP was observed in Kundhi buffalo sequences when compared with Murrah buffalo of India *i-e* Thr 136 Ile, the variation in post-translational modification affects the average micelles size, which may influence biological functioning of protein. In order to analyze heterogeneity within breed, haplotype analysis detected seven haplotypes in ten Kundhi buffalo samples with main haplotype distinct from others with three mutations comprising of 40% (AGK-1, AGK-2, AGK-5 and AGK-8) of total samples. A total of eleven polymorphic sites were detected with analysis showed nucleotide diversity of 0.01015 ± 0.00398 while Haplotype (gene) diversity of 0.867 ± 0.107 for partial kappa casein gene. Collectively, this is the first study to document Kappa casein gene polymorphism in Pakistan's Kundhi buffalo, which lays the groundwork for future research on the influence of genotype on milk production and its quality.



## INTRODUCTION

Improvement of milk quality and quantity has remained the major objective of animal selection in dairy (Caroli *et al*., 2009). Milk composition is expressed in terms of protein, fat, ash, total solids and water contents. Milk protein is of major importance due to its contribution in milk product development. The of the milk varies due to various factors such as breed, genetics, environmental conditions, and some of the managerial affects (Stoop *et al*., 2009).

Protein is considered as a major indicator for measuring milk quality (Ju *et al*., 2011). Among the milk proteins, kappa casein which is being determined by the gene positioned at chromosome number BTA6 (Bangar *et al*., 2021) has got major attention. It consists of five exons spread over about 13.06 kilobases, with most of the protein-coding region located in exon 4 (Bangar *et al*., 2021). Kappa casein protein is encoded by the CSN3 gene located on chromosome 06 in cattle (Chessa *et al*., 2007) and on chromosome 07 in water buffalo (Iannuzzi *et al*., 2003) in the region of 250kb, near to position 6q 31-33. Various other studies have also been conducted on genetic analysis of kappa casein of various breeds (Ceriotti *et al*., 2004; Dadhich *et al*., 2006; Jõudu *et al*., 2007; Alipanah *et al*., 2008; Akyuz *et al*., 2011; Volkandari *et al*., 2017; Barbosa *et al*., 2019). These studies have shown polymorphic types of kappa casein. Casein genetic polymorphisms are important for milk production traits and thus have received considerable attention (Hamza *et al*., 2010). Kappa casein type A and B are common as well as important from technological aspect of milk (Patel *et al*.,

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2007; Dinc *et al*., 2013; Djedović *et al*., 2015). The alleles A and B, may be used as a potential marker for uplifting the milk yield and its physico-chemical characteristics (Deb *et al*., 2014).

Research on kappa casein genetic polymorphism in buffaloes has been conducted in many countries by using molecular techniques of restricted fragment length polymorphism and nucleotide sequencing. Monomorphic (BB) buffaloes were reported by Mahmoud et al. (2010), Abdel-Dayem *et al*. (2009), Othman (2005) using the PCR–RFLP method. Also, there have been evidences of silent mutation due to nucleotide variation at codons 135 threonine (ACC)/Ileunine (ATC) and 136 threonine (ACC/ACT) (Bonfatti *et al*., 2012; Beneduci *et al*., 2010; Masina *et al*., 2007) when analyzed through nucleotide sequencing. In Pakistan, Nili-Ravi buffaloes have been reported as monomorphic (BB) (Riaz *et al*., 2008). Similarly, Indian Pandharpuri breed (Shende *et al*., 2009), both South Kanara and Surti buffalo breeds (Gangaraj *et al*., 2008) as well as Murrah buffalo breed and related crossings in Brazil (Otaviano *et al*., 2005) been reported monomorphic for *CSN3* gene. On contrary, some other Indian buffaloes have been reported as polymorphic for kappa casein gene (Patel *et al*., 2007).

Kundhi buffalo, being indigenous breed, is habitated in Sindh province of Pakistan and producing a significant quantity of milk in rural as well as in urban dairying. Up to yet, to the best of researchers' knowledge, none of molecular study has been done for analysis of kappa casein gene in Kundhi buffalo. Considering the growing interest in molecular analysis of *CSN3* gene in livestock globally, the production potential of milk in Kundhi buffalo and influence of kappa casein gene on the properties of milk required for value added products making. Current study was conducted to characterize the kappa casein gene in Kundhi buffalo on molecular basis.

## MATERIALS AND METHODS

*Collection of blood samples*

A survey was conducted throughout the Sindh province to identify Kundhi buffalo (phenotypically) following the description of breeds by Shah (1994). A total of one hundred lactating Kundhi buffaloes (1st to 3rd parity) were identified, tagged and blood samples were collected from jugular vein, stored in sterile EDTA-coated vaccutainers and transferred to genomic laboratory for DNA extraction.

*DNA extraction and purification*

DNA was extracted from blood samples by using GeneJET Genomic DNA Purification Kit (Thermo Scientific) according to manufacturer's instructions. The quantitative and qualitative measurement of isolated DNA was done on agarose gel (1.8%) by using ultraviolet transilluminator.

*PCR amplification*

Following primers were used as suggested by Barroso *et al*. (1998).

F 5'- TGTGCTGAGTAGGTATCC TAGTTATGG - 3'
R 5' - GCGTTGTCTTCTTTGATGTCTCCTTAG - 3'

The polymerase chain reaction was performed as described by Barroso *et al*. (1998). In brief, amplication was performed in a total volume of 50µL per reaction containing 25µL of DreamTaq (TM) Green PCR Master Mix, 2.0µL (each) of forward and reverse primer, 1µg of template DNA (10pg) and nuclease-free water upto (50µL). Amplification was done in a thermalcycler (Bio-Sys; Australia) with cycling conditions: initial denaturation temperature 95°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, primer annealing at 65°C for 30 s, extension at 72°C for 1 min and one cycle of final extension at 72°C for 15 min. PCR products of the kappa casein gene were finally run-on agarose gel (1.8%) for obtaining required size of DNA bands of kappa casein gene with support of DNA ladder.

*PCR-RFLP*

For genotypic and allelic analysis of kappa casein in Kundhi buffalo, the PCR products were digested by endonuclease restriction enzyme *HinfI* (Thermo Scientific). Digestion solution consisting of PCR product (10µL), nuclease-free water (18µL), 10X Buffer R (02µL), digestion endonuclease (1-2µL) was mixed gently for a few seconds. The digestion solution was incubated at 37°C for 1-16 h.

After the successful digestion of PCR products, the RFLP assay was carried out for the detection of kappa casein gene variants through Gel Electrophoresis. The digested DNA fragments were loaded on 2.0% agarose gel in 1× Tris-acetate buffer (TAE) containing ethidium bromide and left for fragmentation for 50 min at 100 volts. The size of different bands produced was measured with support of DNA marker (MBI Fermentas) of 100kb.

The amplicons of different size resulting from restriction pattern were confirmed by their size. The size of unrestricted PCR product was kept as control.

*DNA sequencing*

Amplified DNA samples were delivered to Macrogen (Korea) for sequencing. After obtaining the sequences of the amplified products, various bioinformatics tools were used to analyze the data. Nucleotide sequences

were viewed and trimmed by using software FinchTV 1.4.0 (Geospiza Inc., Seattle Washington, USA) (http://www.geospiza.com). Then these sequences of kappa casein gene were subjected to BLAST tool on NCBI to compare with already published sequences with Accession No. OL653980, OL653981, OL653982, FJ770200 and U96662. These reference sequences were related to Nili-Ravi breed of Pakistan and Murrah breed of India, respectively. To perform multiple sequence alignment and SNP detection, the sequences were subjected to CLC sequence viewer 8 (Knudsen *et al*., 2007). The FASTA file of aligned sequences was then subjected to EMBOSS Transeq 6.6.0 (https://www.ebi.ac.uk/Tools/st/emboss_transeq/,lastaccess:8june2021) for nucleotide translation into amino acids. Potential SNPs causing change in protein translation were identified by using PROVEAN (http://provean.jcvi.org/index.php). Moreover, in order to analyze the O-glycosylation and phosphorylation sites in translated amino acid sequences, NetOGlyc 4.0 server (http://www.cbs.dtu.dk/services/NetOGlyc) and NetPhos server 3.1 (http://www.cbs.dtu.dk/services/NetPhos) (Xinyang, 2019) were used. Single nucleotide polymorphism (SNP) based phylogenetic tree for Kundhi buffalo breed was constructed in CLC sequence viewer 8 by using UPGMA method.

*Haplotype analysis*

Haplotype analysis was done for assessment of genetic heterogeneity within Kundhi buffalo breed. The detection of haplotype diversities within buffalo breed was carried out by using, Popart 1.7 (population analysis with reticulate trees) (Leigh *et al*., 2015). The data was then subjected to DnaSP 6 software, to determine nucleotide diversity ($\pi$), haplotype diversity (Hd), haplotype (h) and neutrality indices including Tajima's D (Tajima, 1989; Rozas *et al*., 2017).

*Statistical analysis*

Allele and genotype frequencies of kappa casein gene in Kundhi buffalo were calculated according to (Rosner, 2005). A Chi-square test was performed for knowing the statistical difference between (*Ho*) and (*He*) to prove Hardy-Weinberg genetic equilibrium.

$x^2 = \sum (O - E)^2 / E$

Where O is observed frequency; E is expected frequency; while degree of freedom (DF) was 1 at ($P < 0.05$) significance level.

## RESULTS

Amplified segment of kappa casein gene yielded band of 453bp when visualized on agarose gel (Fig. 1). Amplified products were exposed to restriction digestion by utilizing the *HinfI* restriction enzyme. The digestion resulted into two different sized fragments with an approximate size of 426 and 27bps, which indicates the homozygosity among all Kundhi buffaloes and related to the BB genotype of the kappa casein gene (Fig. 2) with genotype frequency (1.0) and allele B with frequency of (1.0) (Table I).

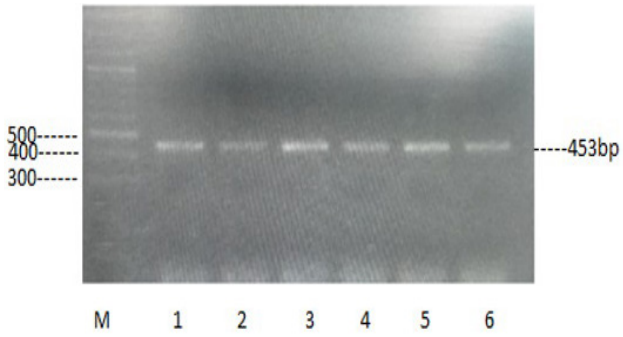


Fig. 1. PCR products of amplified partial Kappa casein gene (453 bp) on 1.8 % agarose gel. Lane M, 100bp DNA ladder (Thermo, MBI, Fermentas). Lane 1-6, Kundhi buffalo.

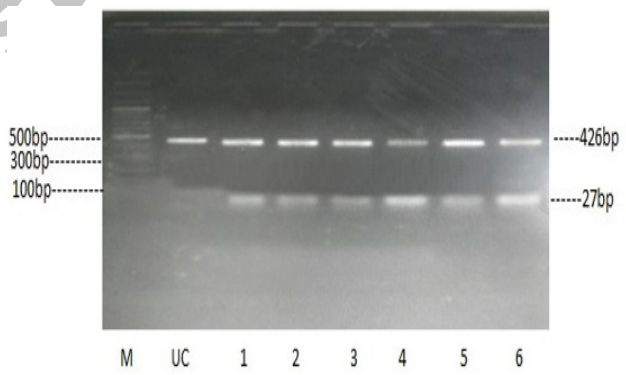


Fig. 2. PCR-RFLP results of kappa casein gene fragment, Kundhi buffalo showing BB genotypes on 02% agarose gel with *Hin* restriction endonuclease. Lane M, 100bp DNA ladder (Thermo, MBI, Fermentas); Lane 2, UC Control PCR product and Lane 3-7, restricted product of kappa casein gene.

**Table I. Genotypic and allelic frequencies of kappa casein locus in Kundhi buffalo.**

| Breed | No. of samples | Genotype/ Allele frequencies | | | | |
|---|---|---|---|---|---|---|
| | | Genotype | | | Allele | |
| | | AA | AB | BB | A | B |
| Kundhi Buffalo | 100 | 0.00(0) | 0.00(0) | 1.00(100) | 0.0 | 1.0 |

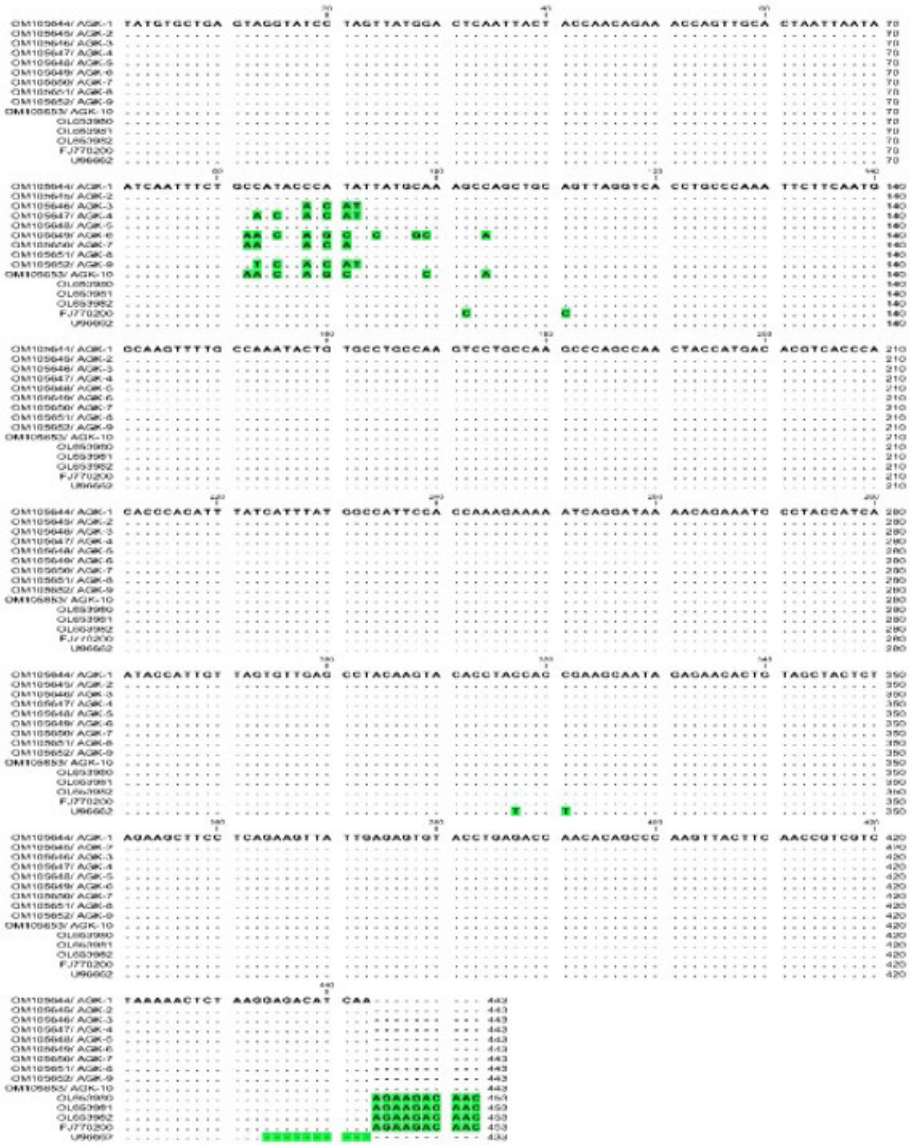

Fig. 3. Comparison of Kundhi buffalo samples with reference sequences (Accession no. OL653980, OL653981, OL653982, FJ770200and U96662) through multiple sequence alignment, showing different SNPs at different Nucleotide positions. *Dots (.) shows the similarity of sequences. *Gaps (-) shows missing sequences.

*Sequence analysis*

The resulting sequences of kappa casein gene were deposited in the NCBI database with accession numbers (OM105644, OM105645, OM105646, OM105647, OM105648, OM105649, OM105650, OM105651, OM105652 and OM105653). The Multiple sequence alignment of Kundhi buffalo samples have shown different SNPs within breed, moreover sequence alignment of samples with reference sequences have also shown SNPs at different positions (Fig. 3). When comparing with Nili-Ravi buffalo (Accession No. FJ770200), sequence alignment of Kundhi samples has shown two SNPs at nucleotide 102 and 111 positions (G > C and A > C, respectively). Moreover, nucleotide translation has shown replacement of asparagine (N) with lysine (K) due to the replacement of base ‘G’ with ‘C’ at position 102 in Kundhi samples indicating non-synonymous substitution with no effect in biological functioning of protein proven through PROVEAN. In addition, SNP at position 111 has shown synonymous mutation as GCC replaces GCA both translate Alanine with no effect on amino acid (Table II).

**Table II. Mutations in amino acid translation of protein and the PROVEAN score of each mutation in Kundhi buffalo.**

| Samples | Mutations at amino acid positions (Proven score) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 28 | 29 | 30 | 31 | 32 | 33 | 35 |
| K3 | | | T30P (3.341) | I31Y (4.759) | | | |
| K4 | Q28P (4.143) | H29Y (2.187) | T30P (4.027) | I31Y (4.936) | | | |
| K6 | K28P (4.339) | H29Y (2.304) | T30P (3.968) | H31Y) (3.274) | H32Y (3.386) | G33A (2.727) | Q35P (4.330) |
| K7 | K28P (4.358) | | T30P (3.791) | N31Y (5.338) | | | |
| K9 | L28P (4.863) | H29Y (2.187) | T30P (3.968) | I31Y (4.897) | | | |
| K10 | K28P (3.968) | H29Y (1.732) | T30P (3.618) | H31Y (3.407) | | | Q35P (4.145) |

While comparing Kundhi sequences with Murrah buffalo (Accession No. U96662), two SNPs were detected at nucleotide positions 317 and 321. As shown in (Table II), the SNP at nucleotide position 317 results in the substitution of Threonine for Isoleucine at nucleotide 106 which after post-translational modifications results in 136 amino acids containing mature polypeptide protein, identifying it as a non-synonymous substitution. While the SNP at nucleotide position 321 was synonymous (ACT>ACC) both ACT and ACC translate to Threonine.

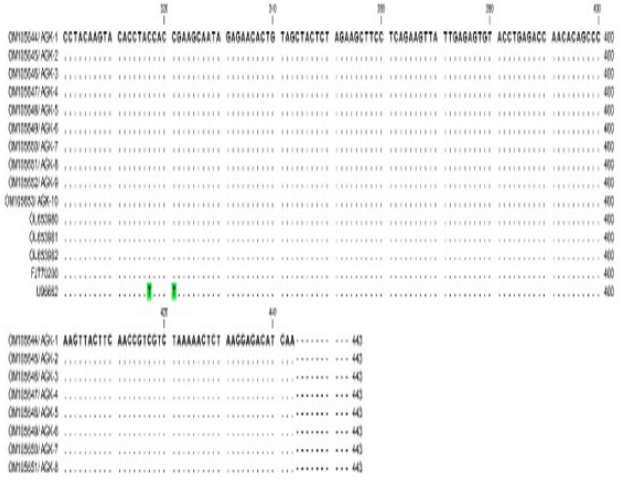

Fig. 4. Multiple sequences alignment of Pakistani buffalo breeds (Kundhi and Nili-Ravi) with Murrah buffalo (Accession no. U96662) indicating two SNPS in the sequence. *Dots (.) shows the similarity of sequences.

*Phylogenetic tree analysis*

Phylogenetic analysis of Kundhi buffalo samples has shown differences within and among breeds (Fig. 5). Out groups used in phylogenetic tree were of cattle breeds retrieved from NCBI gene bank forming different clade with buffalo samples of kappa casein gene. Four of the Kundhi buffalo samples (AGK-1, 2, 5 and 8) shared close relatedness as sharing same clades, as these samples also share clades with Nili-Ravi sequences (OL653980, OL653981 and OL653982). The remaining samples of Kundhi have shown more close relatedness with Nili-Ravi sequence (Accession No. FJ770200) and Murrah buffalo (Accession No. U96662). Moreover AGK-3 and AGK-7 were not sharing branch with any of another sample. Samples have also shown divergence with the Murrah buffalo (Accession No. U96662) of India shown in (Fig. 4). Murrah buffalo shared separate clad in the phylogenetic tree.

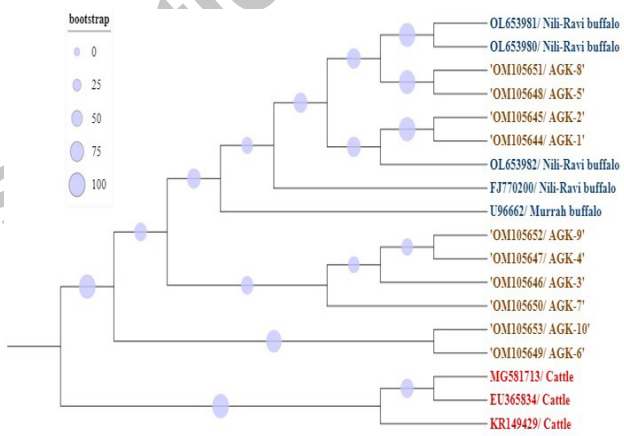


Fig. 5. SNP based phylogenetic analysis of partial kappa casein gene in Kundhi buffalo. The phylogenetic tree was constructed using UPGMA method. The GenBank accession numbers used as reference were (Accession No. OL653980, OL653981, OL653982, FJ770200 and U96662) and outgroup references (Accession No. KR149429, EU365834 and MG581713). * Brown color indicates the samples, blue color indicates the reference sequences, and red color indicates the outgroups. Bootstrap values indicated by blue circles with smallest circle indicating the lowest value and large circle indicating high bootstrap value.

*Haplotype analysis*

In order to investigate the heterogeneity within breed, haplotype analysis was performed. Haplotype analysis detected seven haplotypes in ten Kundhi samples with main haplotype distinct from others with three mutations comprising of 40% (AGK-1, AGK-2, AGK-5 and AGK-8) of total samples as shown in (Fig. 6, Table III). In assessment,

ten polymorphic sites were detected as the analysis has shown nucleotide diversity of 0.01015±0.00398. While, the overall haplotype (gene) diversity of 0.867±0.107 for partial kappa casein gene was detected. The overall neutrality indices were positive (D=1.16841, P>10).

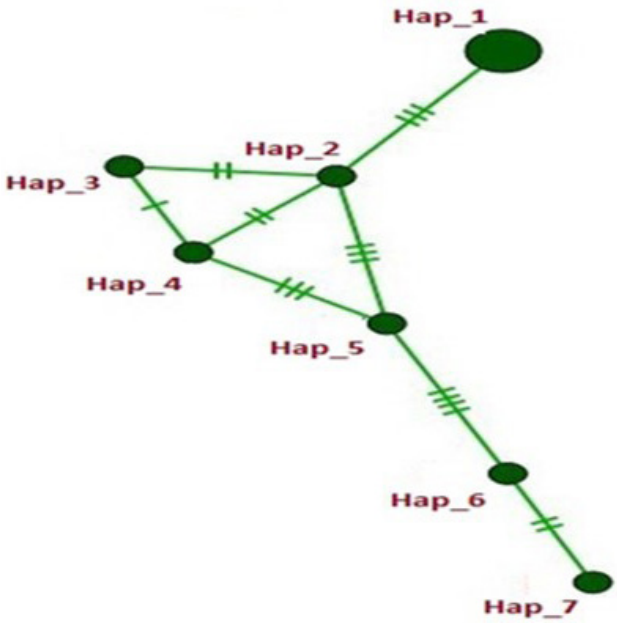


Fig. 6. Kappa casein gene haplotype isolates of Kundhi buffalo. The size of the circles indicates the haplotype frequency. Small circles indicate additional mutational areas. The number of 'hatch marks indicate the number of mutations.

**Table III. Haplotypes in partial kappa casein gene sequences (443bp) of the Kundhi buffalo.**

| Haplotypes | No of isolates | Sample names |
|---|---|---|
| Hap_1 | 4 | AGK-1, AGK-2, AGK-5 and AGK-8 |
| Hap_2 | 1 | AGK-3 |
| Hap _3 | 1 | AGK-9 |
| Hap _4 | 1 | AGK-4 |
| Hap _5 | 1 | AGK-7 |
| Hap _6 | 1 | AGK-10 |
| Hap _7 | 1 | AGK-6 |

## DISCUSSION

Kappa casein accounts for 12% of total casein in bovine milk, performs a crucial part in the formation of milk proteins, generation and stabilization of casein micelle. This enhances the ability to digest milk protein in neonates and also the industrial milk processing (Martin *et al*., 2002; Boland *et al*., 2001). During recent years, increasing interest has been observed in research to investigate milk protein polymorphism since it has a significant relationship with milk yield and composition. Additionally, the breed characterization and milk processing properties *i-e* cheese making *etc* are strongly influenced by kappa casein gene polymorphism, which even serves as a component of stabilization during formation of the casein micelle (Awad *et al*., 2016). The goal of study was to use the PCR-RFLP technique for genotyping in conjunction with sequence analysis to identify possible SNPs and investigate their influence on the protein translation in Kundhi buffalo.

Genotyping of milk protein genes in livestock is well documented. Pinders *et al*. (1991) suggested polymerase chain reaction is the best and reliable technique used for genotyping of economic traits in bovine. While (Soria *et al*., 2003) suggested RFLP technique for targeted DNA segment separation and genotyping of *CSN3* gene in bovines. During the current study conducted in Kundhi buffalo, it was observed that PCR amplification of target fragment yielded 453bp size as predicted which after digestion with *Hinf*I restriction enzyme resulted in the appearance of two bands of predicted size of 426 and 27bps. Restriction pattern analysis of Kappa casein amplified gene fragment indicates the existence of allele B and BB genotype for kappa casein in population studied (Table I). Results of study on Kappa casein gene characterization in Kundhi buffalo are in agreement with (Ozsensoy, 2020; Ghafoor *et al*., 2014; Riaz *et al*., 2008; Otaviano *et al*., 2005; Pipalia *et al*., 2001; Mitra *et al*., 1998), as they all reported BB genotype of *CSN3* gene in buffaloes and declared monomorphism.

However, two alleles A and B for kappa casein locus were reported by (Singh *et al*., 2005) in Bhadawari and Murrah buffalo breeds but also reported monomorphism with only B allele in Mehsana and Surti buffalo breeds. Likewise, Patel *et al*. (2007) also reported both alleles A and B with polymorphic genotype in Murrah, Surti and Pandharpuri buffalo breeds.

Higher cheese yield and milk protein yield are caused by monomorphism of the kappa casein BB genotype (McLean, 1987). There are evidences of increased cheese yield (by 10%) when the milk of cows having BB genotype of CSN3 was used (Marziali and Ng-Kwai-Hang, 1986). The results of the current study indicated homozygous population of Kundhi buffaloes for kappa casein having B allele and BB genotype.

Sequence analysis of Kundhi buffalo revealed SNPs at different positions within and among breeds (Nili-Ravi buffalo and Murrah buffalo). Previous studies revealed

more amino acid variations in kappa casein gene for riverine buffalo breeds (Kundhi, Nili-Ravi, Murrah, etc.), implying an effective relationship between genetic variants in CSN3 gene and milk composition as well as quality of milk products (Fan *et al*., 2020; Miluchová *et al*., 2018; Massella *et al*., 2017; Rangel *et al*., 2017; Azevedo *et al*., 2008; Masina *et al*., 2007). In present study, Kundhi buffalo sequences have shown one potential SNP at nucleotide position 317:A>T when compared with Murrah buffalo. This SNP results in transition of "Isoleucine with Threonine". In addition, multiple sequence alignment revealed the same SNP in Nili-Ravi buffalo, demonstrating that both Pakistani breeds of buffalo contain Threonine while Murrah has Isoleucine at that position, resulting in the absence of glycosylation and phosphorylation sites in Murrah breed. In kappa casein Threonine and Serine are the O-glycosylation sites. Serine, threonine and tyrosine are phosphorylation sites and these sites are formed by the hydrolysis of milk chymosin of kappa casein affecting the solubility and biological function of protein (Bijl *et al*., 2014). This results in shorter micelle size formation and better coagulation which results in better cheese processing property of milk (Fan *et al*., 2020; O'Riordan *et al*., 2014).

In current study SNP based phylogenetic analysis of Kundhi samples has shown divergence and similarity with reference sequences as shown in (Fig. 5). Samples AGK-8, AGK-5, AGK-2 and AGK-1 formed two clades with Nili-Ravi reference sequences (Accession No. OL653981, OL653980 and OL653982) indicating similarity with these samples and divergence from other Kundhi buffalo samples. However other Kundhi buffalo samples have shown more close relatedness with Murrah (Accession No. U96662) and Nili-Ravi buffalo (Accession No. FJ770200). Cattle sequences used in Phylogenetic tree were used as out groups and have formed different clade however both species *Bubalus bubalis* and *Bos indicus* have shown same origin indicating that both species were originated from same ancestors.

In study, heterogeneity within Kundhi breed was checked through haplotype analysis which has shown seven haplotypes in ten Kundhi samples with ten polymorphic sites. Analysis has shown nucleotide diversity of 0.01015 ± 0.00398 while haplotype (gene) diversity of 0.867 ± 0.107 for partial kappa casein gene. Heterogeneity within Kundhi buffalo samples point-out that the samples might not be pure except four samples (AGK-1, AGK-2, AGK-5 and AGK-8) forming main haplotype shown in (Table III). The current study is the first to use popart haplotype 1 to identify heterogeneity through haplotype analysis in Kundhi buffalo. Previously, heterogeneity identification through same haplotype software was performed on *Echinococcus granulosussensostricto* in Turkey (Mehmood *et al*., 2020).

## CONCLUSION

It was concluded that the Kundhi buffalo was found homozygous showing BB genotype only. Multiple sequence alignment has shown variation not only with reference sequences but also within breed. Potential SNP was observed in Kundhi buffalo sequences with Threonine (glycosylation site) at 106 nucleotide positions compared to Murrah buffalo, resulting in more glycosylation sites in Kundhi buffalo compared to Murrah buffalo. By integrating the PCR-RFLP approach with sequence analysis, the current study has opened the doors for breed improvement as well as for the identification of various SNPs affecting the production traits in different indigenous breeds of livestock.

## DECLARATIONS

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*Ethical statement*

The study protocol was reviewed and approved by the Board of Studies and Ethical Committee of Sindh Agriculture University, Tandojam, Pakistan.

*Generative AI and AI-assisted technology statement*

The authors have declared that no generative AI or AI-assisted technologies were used to create this manuscript.

*Statement of conflict of interest*

The authors have declared no conflict of interests.

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