SHORT REPORT

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# Molecular characterization of kappa-casein gene in buffaloes

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**ABSTRACT**: The genetic variations in $\kappa$-casein gene locus exon IV of three buffalo breeds, i.e. South Kanara, Surti, and Murrah, were investigated. PCR amplification of the $\kappa$-casein gene using primers specific for *Bos taurus* yielded DNA products of a similar size. Upon restriction digestion with *Hinf* I, two fragments having 266 bp and 84 bp were obtained for all the samples studied. Single strand conformation polymorphism (SSCP) and nucleotide sequence analysis also did not reveal genetic variation. BLAST alignment of 350 bp nucleotides of buffalo $\kappa$-casein gene showed homogeneity with the $\kappa$-casein gene of *Syncerus caffer nanus*. Analysis of the $\kappa$-casein gene using PCR-restriction fragment length polymorphism and SSCP techniques including DNA sequencing data revealed a monomorphic pattern which is buffalo specific.

**KEYWORDS**: polymorphism, oligonucleotide primers, PCR amplicons, BLAST, monomorphic haplotype

## INTRODUCTION

More than 95% of the proteins contained in ruminants' milk are encoded by 6 structural genes. These proteins are now well-characterized: $\alpha$-lactalbumin and $\beta$-lactoglobulin (the two main whey proteins in ruminants) and the four caseins, $\alpha_{S2}$, $\beta$, $\alpha_{S1}$, and $\kappa$ which are encoded by four tightly linked and clustered genes[1,2] in that order in a 250-kb genomic DNA fragment. The four casein genes have been mapped on chromosome 6 in cattle and goats[2,3] and on chromosome 7 in buffaloes[2], whereas the genes encoding $\alpha$-lactalbumin and $\beta$-lactoglobulin have been mapped on chromosomes 5 and 11, respectively[3].

Polymorphisms of caseins are well documented[4]. Out of six known $\kappa$-casein genetic variants, the A and B are most common in the majority of cattle breeds[5]. The majority of researchers believe that the $\kappa$-casein B variant is associated with higher fat, protein, and casein in the milk and has a significant influence on cheese making properties of milk and superior rennet coagulation properties in comparison to AA or AB variants. The genotypes BB and AB are used in artificial insemination programs to obtain a greater increase of the frequency of these alleles in cattle populations of commercial interest[6]. Effective genotyping of the $\kappa$-casein gene of cattle and buffalo requires fast, efficient, and low cost methods, which are applicable irrespective of sex and age. Molecular genetic approaches are more useful for identifying milk protein genotypes than direct milk testing, which is restricted to mature lactating females, and indirect genotyping of sires is time consuming. Differences associated among variants have enabled the development of several PCR-restriction fragment length polymorphism (PCR-RFLP) tests for A and B[7]. Orita et al[8] developed a simple method for detecting mutations in DNA: the PCR-single stand conformation polymorphism (PCR-SSCP) method, which is less expensive and easily distinguishes different variants. Barroso et al[9] used PCR-RFLP and PCR-SSCP analysis to screen the most frequent variants (A, B, C, and E) of the bovine $\kappa$-casein gene. They used 8 reference samples initially and 40 anonymous samples of different cattle breeds and observed no discrepancies between the PCR-SSCP results. They concluded that SSCP is a cost-effective, sensitive, and fast test and strongly recommended this technique for routine screening of the $\kappa$-casein variants. Pipalia et al[10] and Otaviano et al[6] used the same two techniques and genotyped the $\kappa$-casein genetic variants in buffaloes. Currently, direct sequencing is one of the high-throughput methods for mutation detection, and is the most accurate method for determining the exact nature

of a polymorphism. Sanger dideoxy sequencing can detect any type of unknown polymorphism and its position when the majority of DNA contains that polymorphism[11]. Hence, sequencing was employed to detect the mutation in the present study.

## MATERIALS AND METHODS

A total of 150 lactating buffaloes (50 South Kanara, 50 Surti, and 50 Murrah buffaloes) belonging to different buffalo breeding farms from Karnataka state, India were used for the study.

DNA was isolated from blood by adopting the high salt method[12]. The purity and concentration of DNA samples were estimated by spectrophotometry (Shimadzu). The absorbance ratio of 260/280 nm was calculated. A ratio of 1.7–1.9 was considered as high purity of DNA.

### PCR amplification

The primers JK5 (5′-ATCATTTATGGCCATTCC-ACCAAAG-3′) and JK3 (5′-GCCCATTTCGCC-TTCTCTGTAACAGA-3′) were from Sigma-Aldrich, Bangalore. The sequences of the primers correspond to the flanking regions of the 350 bp fragment of the $\kappa$-casein gene locus in the exon IV and a part of intron IV between the locations +5211 and +5561[13].

All the reactions were carried out in 200 µl reaction tubes. Each single PCR amplification reaction (20µl) contained 1× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3; MBI Fermentas), 2.0 mM $MgCl_2$ (MBI Fermentas), 100 mM dNTP's (MBI Fermentas), 1.0 unit of *Taq* DNA polymerase (MBI Fermentas), 2.5 nM of each primer, 100 ng of DNA, and filtered Milli Quartz (FMQ) water.

The PCR conditions used were as follows: 94 °C for 2 min (initial denaturation) and then 35 cycles of the sequence 94 °C for 60 s, 58.4 °C for 60 s, and 72 °C for 60 s, and then, a final primer extension was carried out at 72 °C for 10 min. Finally the samples were cooled to 15 °C until retrieved.

### PCR-RFLP analysis

A 20 µl volume of digestion mixture, consisting of 15 µl of the PCR product, 1× of recommended buffer, 2 µl of FMQ water, and 6 units of restriction enzyme *Hinf* I was placed in a 0.5 ml microcentifuge tube. The digestion mixture was mixed thoroughly in a vortex mixer and incubated at 37 °C for 3.5 h in a dry bath. The digested product was run on a 2% agarose gel (Bangalore Genei) along with the standard DNA marker (MBI Fermentas) at 50 V for 3 h and observed under a UV transilluminator. Agarose gel photographs were taken using a gel documentation instrument (Bio-Rad).

### SSCP analysis

First, 5 µl of sample was aliquoted into separate tubes and an equal volume of 2× SSCP gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamide, 20 mM EDTA) was added and mixed. Then, the mixture was denatured at 94 °C for 4 min in a heating block (Bangalore Genei). The samples were snap cooled on ice to prevent heteroduplex formation, and then subjected to electrophoresis in an acrylamide/bis-acrylamide (37.5:1) gel, using a Bio-Rad mutation detection unit. Acrylamide/bis-acrylamide (37.5:1 acrylamide to bisacrylamide), 10× TBE-buffer, N,N,N′,N′-tetramethylethylenediamine (TEMED) and water were mixed in a clean, fat-free beaker, and stirred well, carefully avoiding the air-bubbles. A final concentration of 0.09% (v/v) each of ammonium persulphate and TEMED solutions were used. The running time and polyacrylamide concentrations were standardized to observe clear fragment separations. DNA visualization in the polyacrylamide gel after electrophoresis was done by ethidium bromide staining.

### Nucleotide sequencing

The PCR products were concentrated to 50 ng/µl by pooling several tubes and then precipitating by the isopropanol procedure. In order to obtain a clean DNA fragment for sequencing, the PCR products were separated by electrophoresis in a TAE agarose gel containing ethidium bromide using standard protocols. The desired PCR product band was excised using a clean, sterile razor blade or scalpel (the band was visualized in medium or long wavelength ($\geqslant$300 nm) UV light, and excised quickly to minimize exposure of the DNA to UV light). The minimum agarose slice was transferred to a 1.5 ml micro centrifuge or screw cap tube and then purified by using a commercially available gel extraction kit (Qiagen). Quantification was done by loading 1 µl of eluted sample in 1% Agarose gel and compared with standard molecular marker ($\phi$X174 DNA ladder or 100 bp DNA ladder). Only samples with a concentration $>$50 ng/µl were selected. Samples were labelled and sent for sequencing. Sequencing was done by Avesthagen, Bangalore and Macrogen, Seoul.

### Sequence data analysis

Sequences were edited and initially aligned using SEQUENCHER (demo version) and then optimally aligned visually. Multiple sequences were aligned by

the Clustal format for the T-COFFEE version 1.41 online application. Polymorphic sites were analysed and were confirmed by electropherogram results. Coding sequences were translated to amino acids using the European Bioinformatics Institute online translation tool (www.ebi.ac.uk).

### Database search

The database search of sequences for a possible match to the DNA sequence of casein gene was conducted using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI, Bethesda, MD). Translated protein sequences of different casein genes were also subjected to the BLAST algorithm.

## RESULTS AND DISCUSSION

### PCR-RFLP using *Hinf* I digestion

The extracted DNA samples, with a 260/280 nm absorbance ratio of 1.7–1.9, were used as the templates for PCR amplification using the procedure as described by Medrano and Cordova[13]. The PCR conditions were optimized with respect to annealing temperatures, primer extension times, $MgCl_2$, and template concentrations. The amplified product (350 bp) showed no variations in size either within or between the buffalo breeds studied, suggesting conservation of the $\kappa$-casein gene between *Bos taurus* and *Bubalus bubalis* species. The product was digested with the restriction enzyme *Hinf* I to detect the polymorphism. Upon digestion of the 350 bp fragment from exon IV and a part of intron IV using the *Hinf* I restriction enzyme, a homozygotic type defined by a major fragment of 266 bp and a minor fragment of 84 bp was observed (Fig. 1). This allelic pattern, classified as BB genotype, is in agreement with the earlier reports[6, 10, 14], and was observed in all the 150 animals belonging to South Kanara, Surti, and Murrah breeds without any variation.

Unlike the BB genotype which has only one restriction site for *Hinf* I and thus results in two fragments of 266 bp and 84 bp upon digestion with the enzyme, the AA genotype has two *Hinf* I sites at the 350 bp amplicon and yields two major fragments of 134 bp and 132 bp[13], while the AB genotype yields fragments of 266, 134, 132, and 84 bp. Burzynska et al[15]genotyped exon IV of the $\kappa$-casein gene of *Bison bonasus* (European bison), and found only BB variants with the allele 'A' being totally absent. They concluded that the homozygosity could be due to the loss of genetic variability in the small and isolated population studied. Similar results were also obtained

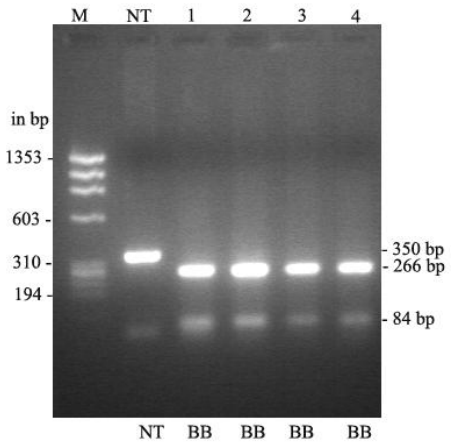


**Fig. 1** PCR-RFLP pattern of *Bubalus bubalis* $\kappa$-casein gene by restriction enzyme *Hinf* I in South Kanara (lanes 1 and 2), Surti (lane 3), and Murrah (lane 4) breeds. M is $\phi$X174 ladder in bp.

by Lama and Zago[14] in buffaloes. Mitra et al[16], screened 57 Sahiwal cattle, 53 Murrah, 19 Nili-Ravi, and 11 Egyptian buffaloes and obtained a frequency of 0.16 for the CSN3 B allele in the Sahiwal cattle with no homozygous BB animals. No polymorphism was observed in exon IV of the buffalo CSN 3 gene when the same set of primers was used with the restriction enzymes *Hind* III / *Hinf* I and *Taq* I. However, DNA sequencing of the amplified fragment revealed one polymorphism at codon 135 [Threonine (ACC) $\rightarrow$ Isoleucine (ATC)] in buffalo, which is in contrast to the observation of the present study. The frequencies of 135 Thr/Ile alleles were estimated as 0.88 and 0.12, respectively. Malik et al[17] studied the frequencies of the CASK and CASB alleles in cross-breds and Sahiwal breed of cattle using PCR and sequence specific oligonucleotide probes. The results showed a predominance of CASK-A allele in Sahiwal while the CASK-B allele was predominant in cross-bred cattle. At the CASB level, the CASB-A2 allele was predominant in both Sahiwal and cross-bred cattle. However, in the present study it was found to be monomorphic for both $\kappa$- and $\beta$-caseins in buffaloes. Pipalia et al[10] scanned the 379 bp fragment of exon IV of the $\kappa$-casein gene in Jaffarbadi, Mehsana, Surti, and Pandharpuri buffalo breeds using *Hind* III and *Hinf* I enzymes. Restriction analysis revealed 288 and 91 bp for *Hind* III and 225 and 154 bp size fragments for *Hinf* I. They classified the above fragment pattern as genotype BB and concluded that all the buffalo breeds were monomorphic for BB genotype, which is confirmed by the results of the present study.

Otaviano et al[6] studied 115 lactating Murrah buffaloes for the exon IV of the $\kappa$-casein gene. They amplified *CSN3* K2 and *CSN3* KY of the *CSN3* gene with 400 and 280 bp, respectively. RFLP analysis by enzymes *Alu* I and *Hind* III showed two fragments of 280 and 120 bp for *CSN3* K2 and two fragments of sizes 180 and 100 bp, corresponding to the *CSN3* KY gene. SSCP analysis allowed the visualization of only one allele for the gene *CSN3* and hence no association between the $\kappa$-casein gene and milk trait could be detected. They concluded that all the individuals were homozygous (monomorphic) with genotype BB. The present study utilizing the restriction enzyme *Hinf* I also yielded similar results.

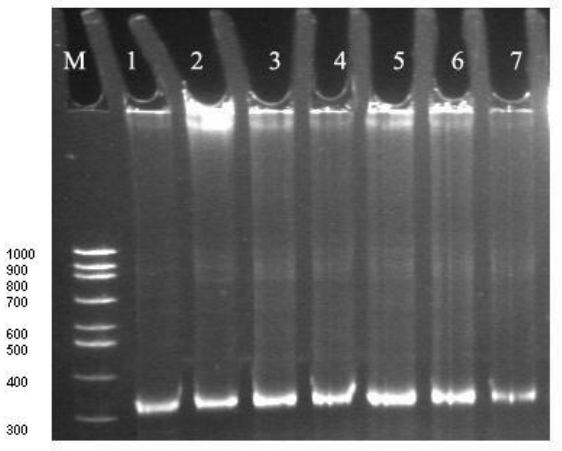


**Fig. 2** PCR-SSCP pattern of *Bubalus bubalis* $\kappa$-casein gene in South Kanara (lanes 1–3), Surti (lanes 4 and 5), and Murrah (lanes 6 and 7) breeds.

## PCR-SSCP

The 150 amplified samples of $\kappa$-casein gene subjected to SSCP did not show significant variation in mobility of the fragments (Fig. 2), suggesting a homozygous banding pattern. No discrepancies between PCR–RFLP and PCR-SSCP patterns were detected for any of the three buffalo breeds studied. The variant $\kappa$-casein genotype BB identified in the present study was similar to the result obtained by Otaviano et al[6] who were the first group to use the SSCP technique along with the RFLP technique to analyse the *Bubalus bubalis CSN3* ($\kappa$-casein) gene. They observed only one allele for both *Alu* I and *Hind* III restriction endonucleases. Their SSCP results also yielded only one allele for the *CSN3* gene. Thus it can be concluded that the part of the $\kappa$-casein gene studied in buffaloes is monomorphic.

## Sequencing and genotyping of $\kappa$-casein gene

All the 12 sequence reports of $\kappa$-casein gene were subjected to restriction mapping using the enzyme *Hinf* I (G↓ANTC). The 350 bp fragment showed a restriction site at the 265th nucleotide position in all the sequence reports. Restriction mapping yielded two fragments of sizes 266 bp and 84 bp for all the samples studied. The restriction enzyme did not cleave at the 134th nucleotide position because of transversion from 'A' (adenine) to 'C' (cytosine), resulting in the loss of restriction site for the enzyme *Hinf* I (NCBI gene bank accession no. EF066482). The restriction analysis indicated a single restriction site at the 266th bp position, which resulted in two fragments of 266 and 84 bp. The $\kappa$-casein gene showed cytosine at the +5345 nucleotide position, which was seen in genetic variant 'B' only. This feature confirmed that all the animals studied were of the BB genotype. The translation of all the twelve sequences yielded 66 amino acids. The 45th amino acid was alanine which was present only in homozygous genotype BB.

Medrano and Cordova[13] reported that bovine AA variant $\kappa$-casein genotype has two restriction sites for *Hinf* I at the 350 bp amplicon, yielding two major fragments of 134 bp and 132 bp and a minor fragment of 84 bp. However BB genotype had only one restriction site resulting in two fragments of sizes 266 bp and 84 bp. The AB genotype had fragments of 266, 134, 132, and 84 bp. They reported that bovine AA genotype had aspartic acid (GAT) at the 45th amino acid position, which had a restriction site for *Hinf* I and bovine BB genotype had alanine (GCT) at the 45th amino acid position, which lacks a restriction site for *Hinf* I.

## BLAST search for $\kappa$-casein gene

The BLAST search of sequences for a possible match to the DNA sequence of $\kappa$-casein gene yielded 106 hits on the query sequence in the nucleotide databases at the NCBI. The highest match was with the $\kappa$-casein gene of *Syncerus caffer nanus*. The alignment of 350 total nucleotides showed 98% identity. The second highest match was with the *Bubalus bubalis* $\kappa$-casein mRNA sequence and it also matched the $\kappa$-casein gene of *Boselaphus tragocamelus* (Nilgai) and *Bos javanicus* (Banteng).

There was dissimilarity in amino acids at different positions between *Bubalus bubalis*, *Syncerus caffer nanus*, and *Bos taurus*. The exact effect of this mutation has to be detected in the case of *Bubalus*

*bubalis*. Translation data of 350 bp exon IV and part of intron IV of $\kappa$-casein gene yielded 66 amino acids and the 45th amino acid was alanine (NCBI gene bank accession no. EF066482). There was dissimilarity in amino acids of $\kappa$-casein exon IV at different positions between the *Bubalus bubalis* and *Syncerus caffer nanus*[18]. There were 3 amino acids that were different between the two species and 11 amino acids were different between *Bubalus bubalis* and *Bos taurus*[14].

## CONCLUSIONS

Buffalo casein genes were successfully amplified using primers designed for cattle, indicating that the genes coding for casein of both animals are conserved. All the 150 animals belonging to South Kanara, Murrah, and Surti were homozygous (monomorphic) for the $\kappa$-casein gene. The monomorphic pattern of the $\kappa$-casein gene revealed by PCR-RFLP, SSCP, and sequencing data analysis may be a buffalo specific character. The milk component and milk yield parameters could not be associated with buffalo casein genotypes due to its monomorphic haplotype. There was a significant difference in nucleotide sequences between the $\kappa$-casein milk protein gene of *Bubalus bubalis* and *Bos taurus*. Further studies are required to understand the differences and variations in milk constituents in these two species.

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