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# Genotyping of *κ-casein* and *β-lactoglobulin* genes in native cattle from Barishal region of Bangladesh

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**ABSTRACT**

The study was conducted to determine the genetic variants of *κ-casein* and *β-lactoglobulin* genes in native cattle. DNA was extracted from blood samples ($n = 80$) collected from Babuganj, Barishal followed by PCR with gene-specific primers. Genotyping was done by RFLP with *HindIII,* and *HaeIII* restriction enzymes. Allelic and genotypic frequencies, genetic diversity, heterozygosity and Hardy–Weinberg equilibrium were estimated using the Popgen32 software. A total 80 samples were genotyped and three genotypes, namely AA, AB and BB, were detected for both the genes. In case of *κ-casein* gene, higher frequency was observed for AA genotype (0.73) followed by AB (0.23) and BB (0.04) genotype. A allele (0.84) was found to dominate over B allele (0.16). For *β-lactoglobulin* gene, BB genotype (0.66) was found more frequently than AB (0.18) and AA (0.16) genotypes. Highest frequency was found for B (0.75) followed by A (0.25) allele. The average genetic diversity (He) was 0.38. The result indicated differences between observed (Ho) and expected (He) heterozygosity and it was out of equilibrium genetics, assumed that selection pressure was in population. To the best of our knowledge, this is the first reported study on *κ-casein* and *β-lactoglobulin* gene variants analysis in cattle in Bangladesh.

**KEYWORDS**

Native cattle; *κ-casein*; *β-lactoglobulin*; PCR-RFLP; genotype

## Introduction

Bangladesh is rich in native/indigenous (*Bos indicus*) animal genetic resources and is distributed all over the country. Among the indigenous farm animals non-descript Deshi, Red Chittagong, Pabna, North Bengal Gray, Madaripur and Munshiganj cattle are notable among other livestock and poultry species. Besides, imported exotic breeds and/or commercial strains and crossbreds derived thereof are available especially cattle, chicken and duck. In general, indigenous genetic resources are in declining trend due to their lower production efficiency, increased demand, urbanization, population growth, etc. However, there is distinctive demands of animal products like milk, meat, egg of indigenous animal origin due to their flavor & taste and thus brings higher price than the products derived from high yielding varieties.[1] However, there is little detailed study on the indigenous cattle of Bangladesh though they form significant part of cattle population (70%) in the country.[2] Cattle included in this study are indigenous. These types of cattle have been originated in this county over the centuries for natural selection and farmers' interest on draft power to perform agricultural practices. They are using for multiple purposes, like, milk, meat and draft. This type of cattle commonly called deshi and has no definite characteristics to define as breed. They are highly adaptive to varying conditions i.e., temperature, humidity, rainfall and natural calamities. Their production performances in terms of meat and milk are lower than local improved varieties of cattle found in Chattogram and Pabna district of the country as well as far below than the high yielding breeds.[2]

Milk is one of the most important diets to human. It is a complex mixture of proteins, carbohydrates, vitamins, minerals, and other constituents dispersed in water. Major portion of milk is water (about 85–87%) in which other nutrients, i.e., fat, protein; lactose, minerals and vitamins are present in solution, colloidal suspension or emulsion in water. Milk proteins are divided into two main groups; (i) caseins (80% of the milk proteins) and (ii) whey proteins. Casein is the insoluble fraction and is composed of

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four different caseins; *alphas$_1$ casein (CSN1S1)*, *alphas$_2$-casein (CSN1S2)*, *beta-casein (CSN2)* and *kappa-casein (CSN3/κ-CN)*. Whey proteins make up the soluble fraction and they are composed of several different proteins, the most important of which are *α-lactalbumin* and *beta-lactoglobulin (β-LG)*[3] Other minor part is made by peptones/low molecular weight peptides (3%) and milk fat globule membrane (MFGM) proteins (1%).[4] For all six major milk protein genes, there are autosomal and codominant alleles, which are called genetic variants. Several variants of milk protein genes have been reported.[5,6]

Identification of milk protein genetic polymorphism is important because of its possibility in association of milk protein genotypes and economically important traits in dairy cattle.[7] Therefore, milk protein genes could be useful as genetic markers for additional selection criteria in dairy cattle breeding. The *κ-CN* constitutes approximately 12% of total casein.[8] It is of great interest because it correlates to milk quality and composition.[9] The *κ-CN* gene is located in chromosome 6p31, approximately 13 kb long and separated into 5 exons.[10] The *κ-CN* fragment, single chain polypeptide of 169 amino acids has molecular weight of 19.2 kDa.[11] Mutations in exon IV have determined two allele variants, A and B, which are differed in the amino acids of 136 and 148. Variant A has threonine (ACC) and aspartic acid (GAT) amino acid at positions 136 and 148, respectively. In variant B, isoleucine (ATC) substitutes threonine and aspartic acid is substituted by alanine (GCT).[10,12] *β-LG* is the major whey protein in milk of cows and other ruminants e.g., deer, bison and buffalo, and in some non-ruminants such as pigs, horses, dogs, dolphins and whales. However, it is not an endogenous part in human milk.[13] It exists at the normal pH of bovine milk as a dimer with a molecular weight of 36 kDa. At least 12 variants are known for *β-LG*, out of which A and B variants are more frequent.[14] The variants are differing by 2 amino acid substitutions in the polypeptide chains and 2 single nucleotide substitutions in the *β-LG*. Variant A has aspartic acid (GAT) and valine (GTG) at positions 64 and 118, whereas variant B has glycine (GGT) and alanine (GCG). Milk produced by *β-LG* AA-genotype was found to contain more lactoglobulin, less casein, and less fat than that obtained from BB-genotype cows.[15] There is no report about *κ-CN* and *β-LG* gene variants of cattle in Bangladesh. Therefore the present study was taken to determine the κ-CN and *β-LG* gene variants along with genotype and allelic frequencies of these two milk protein genes in cattle.

## Materials and methods

### Study area

The study was conducted at Babugonj Upazila, Barishal (22°42′17.89″N and Longitude: 90°22′12.47″E) district that lies on the bank of Kirtankhola river in south-central Bangladesh. Animals were selected randomly and samples were collected. The study was approved by the ethical review committee (ERC) of National Institute of Biotechnology (NIB).

### Sample collection

A total of 80 blood samples, 21 from male and 59 from female, were collected from jugular vein of cattle brings to the Veterinary Hospital of Patuakhali Science and Technology University for treatment purposes. About 3 ml blood was collected aseptically from each of the animal using 10 ml syringe. Soon after collection the sample was transferred to vacuum tube (Vacuette) containing ethylene di-amine tetra acetate (0.5 M, pH:8) and labeled properly. After proper mixing by inverting the tube several times, the sample was preserved at −20°C until transferred to NIB.

### DNA extraction and quantification

DNA was extracted from blood samples using modified phenol-chloroform organic extraction method.[16] Briefly, 400 µl of blood was mixed with 700 µl water, vortexed and centrifuged at 10,000 rpm for 10 min. After discarding supernatants 200 µl of lysis buffer and 2 µl of proteinase K were added and mixed by inverting the tube several times and then incubated at 58 °C for 4 h. Upon incubation, 100 µl of 4.5 M NaCl was added and mixed by inverting the tube. Then about 225 µl chloroform was added and mixed by shaking for 10 min. The mixture was then centrifuged at 14,000 rpm for 10 min and about 200 µl upper phase was transferred into a new tube. Then 200 µl of isopropanol was added and mixed by inversion of the tube and centrifuged at 14,000 rpm for 15 min. The supernatant was discarded and 500 µl of 70% ethanol was added and incubate at room temperature for 15 min. Upon incubation, the mixture was centrifuged at 14,000 rpm for 15 min and then decants the alcohol. About 100 µl of TE buffer was added and incubated at 56°C for 5 h and then mixed by pipetting. Extracted DNA was stored at −20°C until use. The concentration and purity of DNA was assessed with a Nanodrop spectrophotometer (Nanodrop 2000c). The samples having OD ratio ($A_{260}/A_{280}$ nm) between

1.7–1.9 were considered good and used for polymerase chain reaction (PCR).

### Amplification of κ-CN and β-LG genes by polymerase chain reaction

The PCR reaction was performed in 25 µL reaction scale. *κ-CN* gene was amplified with primers *κ-CN*-F:5′-AGCGCTGTGAGAAAGATG-3′and *κ-CN*-R: 5′-GTGCAACAACACTGGTAT-3′ while *β-LG* gene was amplified with *β-LG*-F 5′-TGTGCTGGACACCG ACT ACAAAAAG-3′ and *β-LG*-R 5′-GCTCCCGG TATAT GACCACCCTCT-3′ primers reported earlier.[17,18] The reaction mixture consisted of 12.5 µL of 2x master mix (Tris-HCl 20 mM, dNTPs 400 mM), $MgCl_2$ 3 mM, Taq DNA polymerase 0.1 U/µL), 1 µL forward primer, 1 µL reverse primer, approximately 50 ng of extracted DNA and molecular grade water to make final volume of 25 µL. Thermal conditions for κ-CN gene were: initial denaturation at 94 °C for 5 min; followed by 32 cycles of 94 °C for 1 min, 61 °C for 1 min and 72 °C for 1 min and final extension at 72 °C for 10 min.[17] Thermal condition for β-LG gene was same except annealing temperature which was 65 °C. Amplicons were analyzed by gel electrophoresis in a 1.5% agarose gel using TAE buffer and stained with ethidium bromide. These primers supposed to amplify 935 and 247 bp fragments from κ-CN and β-LG genes, respectively.

### Genotyping of κ-CN and β-LG gene by restriction fragment length analysis

Genotyping of *κ-CN* gene was performed using *HindIII* and *HaeIII* endonucleases as described by Soria et al.,[17] and *β-LG* gene using *HaeIII* endonuclease as described by Medrano and Aguilar-Cordova.[18] All the restriction enzymes were derived from New England Biolabs, USA. Each digestion reaction (10 µl) consisted of 1.5 µl nuclease-free water, 1 µl of compatible 10X buffer, specific restriction enzyme 0.5 µl and PCR product 7 µl. The reaction mixture was incubated at 37 °C in water bath for 1 h. Fragment sizes were resolved in 4% agarose gel and genotyped considering the fragment sizes depicted in Table 1.

### Data analysis

Polymorphic amplicons were considered to estimate the allelic diversity and effective number of alleles. Allelic and genotypic frequencies were estimated using the software Popgen32.[19]

**Table 1.** Expected fragment pattern of PCR products of *κ-CN* and *β-LG* genes digested with different restriction enzymes.

| | Fragment length | | |
|---|---|---|---|
| | *κ-CN* (935 bp) | | *β-LG* (247 bp) |
| Genotype | *HindIII* | *HaeIII* | *HaeIII* |
| AA | 935 | 641 + 294 | 148 + 99 |
| AB | 935 + 520 + 415 | 641 + 294 | 148 + 99 + 74 |
| BB | 520 + 415 | 641 + 294 | 99 + 74 |

**Table 2.** Genotype and allele frequencies *of κ-CN* and *β-LG* genes in native cattle ($n = 80$).

| | Genotype frequency | | | Allele frequency | |
|---|---|---|---|---|---|
| Gene | AA | AB | BB | A | B |
| κ-CN | 0.73 $n = 58$ | 0.23 $n = 1\ 9$ | 0.04 $n = 3$ | 0.84 | 0.16 |
| β-LG | 0.16 $n = 13$ | 0.18 $n = 14$ | 0.66 $n = 53$ | 0.25 | 0.75 |

## Results

### Determination of κ-CN gene variants

PCR on extracted genomic DNA yielded 935 bp products from total DNA. Out of 80 DNA samples tested, specific band was found in 100% ($n = 80$) samples. For genotyping, PCR product of *κ-CN* gene was digested with two different restriction enzymes namely *HindIII* and *HaeIII*. Based on digestion pattern the samples were genotyped (Table 2 and Fig. 1a). Out of 80 samples, 72.50 (58/80), 23.75 (19/80) and 3.75% (3/80) samples were genotyped as AA, AB and BB, respectively. AA genotype (0.73) and A allele (0.84) of *κ-CN* was found dominant.

### Determination of β-LG gene variants

PCR of extracted genomic DNA was performed and 247 bp PCR product was obtained from total DNA. A total of 50 DNA samples were tested and specific band was found in 100% (50/50) samples. For genotyping, PCR product of *β-LG* gene was digested with restriction enzyme *HaeIII*. Based on digestion pattern the samples were genotyped (Table 2 and Fig. 1b). Out of 80 samples, 16.25 (13/80), 17.50 (14/80) and 66.25% (53/80) samples were genotyped as AA, AB and BB, respectively. Overall BB genotype (0.66) and B allele (0.75) of *β-LG* gene was found dominant.

### Genetic diversity

Differences were found between observed (Ho) and expected (He) heterozygosity (Table 3) in the population analyzed. Expected (He) heterozygosity was almost double than observed (Ho) in *β-LG* locus.

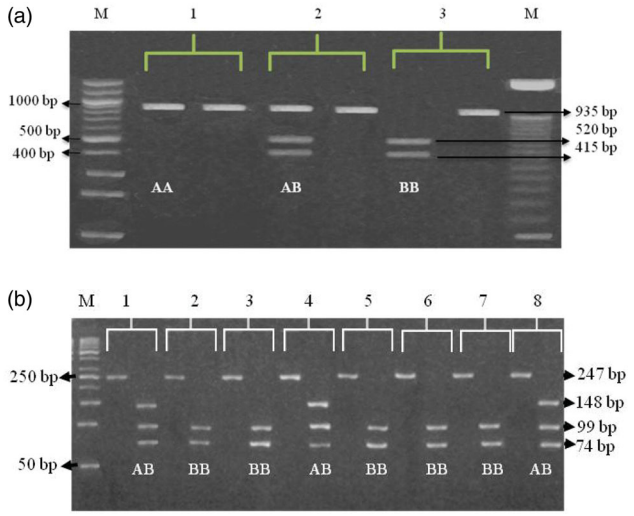


**Figure 1.** (a) Genotyping of *κ-CN* gene. Portion of *κ-CN* gene (935 bp) from genomic DNA of cattle is amplified by PCR, digested with *HindIII* and resolved in 1.5% agarose gel. Lane M: DNA marker. Lane 1–3: Test sample. In each lane-digested PCR product (left) and undigested PCR product was run simultaneously. Genotype of each sample is indicated by AA, AB and BB. Fragment size (after digestion with mentioned restriction enzymes) and genotype determination guide is shown in Table 1. (b) Genotyping of *β-LG* gene. Portion of *β-LG* gene (247 bp) from genomic DNA of cattle is amplified by PCR, digested with *HaeIII* restriction enzyme and resolved in 4% agarose gel. Lane M: DNA ladder; Lane 1–8: In each lane-undigested PCR product (left) and PCR product digested with *HaeIII* (right). Genotype of each sample is indicated as AB and BB. Fragment size (after digestion with mentioned restriction enzymes) and genotype determination guide is shown in Table 1.

**Table 3.** Observed heterozygosity (Ho) and expected heterozygosity (He) of *κ-CN* and *β-LG* genes in native cattle.

| Locus | Ho (observed) | He (expected) | Chi-squire ($\chi^2$) |
|---|---|---|---|
| *κ-CN* | 0.22 | 0.25 | 0.27 |
| *β-LG* | 0.18 | 0.38 | 0.0002 |

Based on a Chi-square test ($\chi^2$), heterozygosity were out of Hardy–Weinberg disequilibrium ($p > 0.05$) in *β-LG* locus and were within in *κ-CN* locus.

## Discussion

Milk yield is a polygenic trait which is also affected by environmental factors. The protein composition of bovine milk is predominantly determined by genetic factors.[20] Hence, milk protein polymorphisms have been studied intensively to understand the genetic relationship between alleles of milk protein loci and milk protein composition and concentration.[21,22] Lack of appropriate breeds is identified as one of the major constraints in dairy farming in Bangladesh (National Livestock Development Policy, 2007, http://old.dls.gov.bd/files/Livestock_Policy_Final.pdf, Accessed on 10/07/2018). Hence, molecular study based information can be employed during cattle breeding activities with the intention of increasing the proportion of cows producing milk with improved values such as higher levels of casein that will ultimately enhance the yield and processing properties of milk and its products.[21,22] Many researchers found that *κ-CN* gene is associated with milk yield; milk fat and cheese yield.[8,23] In buffalo, *κ-CN* gene is observed for milk production traits.[24] We found higher frequency of A allele (0.84) along with AA genotype (0.73) in native cattle in case of *κ-CN* gene (Table 2). Though association analysis was not performed in this study, our genotyping findings seem to have a relation with the findings of Rahman et al.,[25] who reported that local cow contains higher fat (5.05%), protein (3.78%), lactose (5.37%) and solid-not-fat (9.94%) than Pabna and crossbred animals. On the other hand, BB genotype (0.66) and B allele (0.75) of *β-LG* gene was found to dominate in the same group of cattle (Table 2). Indigenous cattle of different breeds were reported to have higher A-allele (0.78) for *κ-CN* and B-allele (0.66) for *β-LG* gene.[11,26–28] *β-LG* B allele, presumably associated with high fat and casein content and more desirable for cheese making, was observed with the highest frequency in our study. Heck et al.,[22] reported that native cattle populations with higher frequencies of *β-LG* B allele are more desirable for milk production traits. Higher frequencies of *β-LG* B allele are also reported from zebu cattle in Brazil with a frequency range of 0.559–0.955[29] and 0.43–0.83[30] and to those of zebu cattle in India with frequency values of 0.83 and 0.61 for Indian Sahiwal and Indian Tharparkar cattle populations, respectively.[31]

On the contrary high allelic frequency of B-allele (0.65) *κ-CN* was reported in a Jersey cattle population.[32] Besides, polymorphism studies conducted in dual purpose Gyr and Nelore breeds showed higher frequencies of B allele of *κ-CN* than beef breeds.[30] *κ-CN* B allele was reported to have a favorable and significant effect on both milk and milk protein yield.[33] The milk produced by *β-LG* AA-genotype cows was reported to contain more lactoglobulin, less casein and less fat than that obtained from BB-genotyped cows.[34] Whereas, monomorphic form BB of *κ-CN* is responsible for higher yield in cheese making as well as milk and milk protein yield.[35] The cheese production can be increased by 10% if milk from cows of genotype BB of *κ-CN* is used.[36] Broadly, B variants of *κ-CN* and *β-LG* proteins were recognized

as superior for milk quality in European cattle breeds. Thus, it may be assumed that AA genotype of *κ-CN* and BB genotype of *β-LG* can be used as genetic markers. The genotypes (AA, BB, and AB) and 2 alleles (A and B) were observed in the study for both the genes that could be a potential genetic marker to improve the production performance of Bangladeshi cattle population. Differences in observed (Ho) and expected (He) heterozygosity (Table 3) in the population analyzed implied that, somehow artificial selection was applied for reproductive management and genetic improvement programs in the studied population for *β-LG* locus or it was the result of other genetic improvement selection pressure. Further studies using long term production data and in vitro biological analysis should be conducted in order to check the effects of such polymorphisms and validate its function on production traits.

## Disclosure statement

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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