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Molecular characterization and phylogenetic analysis of *κ-casein* (*K-cn*) gene of indigenous cattle of Assam

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Abstract
The study was conducted to investigate the polymorphism in *κ-Casein* (*κ-Cn*) gene in indigenous cattle of Assam. Genomic DNA from 53 indigenous cattle was extracted and used to study the polymorphism in *κ-Cn* gene using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique. A PCR product of 351 bp obtained upon amplification was subsequently digested with restriction endonuclease *Hinf*I and yieldedtwo types of restriction pattern; three fragments of 131, 131 and 89 bp representing AA genotype and, four fragments of 262, 131, 131 and 89 bp representing AB genotype. The frequencies of alleles A and B were found to be 0.6510 and 0.3491 and those of AA and AB genotypes 0.4237 and 0.4545 respectively. The population under study did not show the presence of genotype BB for *κ-Cn* locus. From the present study it could be concluded that the frequency of allele A was higher than that of allele B in the indigenous cattle with a higher frequency of AB genotype (AB>AA) and Chi-square (χ^2) test revealed that the population under study was not in Hardy-Weinberg Equilibrium.

Keywords: *κ-casein*, gene frequency, genotyping, indigenous cattle

Introduction
The primary goal of dairy industry has been to identify an efficient and economical way of increasing milk production and its constituents without increasing the size of the dairy herd. Selection of animals with desirable genotypes and mating them to produce the next generation has been the basis of livestock improvement. The use of polymorphic genes as detectable molecular markers is a promising alternative to the current methods of trait selection once these genes are proven to be associated with traits of interest in animals. Milk protein genetic polymorphism has received considerable interest in recent years because of possible associations between milk protein genotypes and economically important traits in dairy cattle, which could be detected before actual target traits are being expressed. Therefore, milk protein genes could be useful as genetic markers for additional selection criteria in dairy cattle breeding. The discovery of PCR-RFLP generated renewed interest in the use of genetic marker loci as an aid to selection programme for improvement of livestock (Soller and Beckmann, 1983) [18].

β-Lg and κ-Cn are the most important proteins expressed in milk. The proteins κ-Cn and β-Lg are known to possess great effects on milk production and milk constituents (Marziali andNg-Kwai-Hang, 1986; Grosclaude, 1988; Aleandri *et al*., 1990; Erhardt, 1996) [11, 8, 1, 6]. So, if the DNA polymorphisms of *β-Lg* and *κ-Cn*genes are associated with milk production traits, they could serve as genetic markers for genetic improvement in future marker-assisted selection programme in indigenous cattle of Assam.

κ-Cn constitutes approximately 12% of the casein and is a constituent of bovine milk. *κ-Cn* is found in a number of genetic variants of which A and B variants are predominant (Prinzenberg *et al*., 1999). The casein genes are tightly linked and inherited as a cluster so they have a potential value and can play an important role in MAS for milk traits (Lien and Rogne, 1993) [10]. They are located on chromosome 6 within a 200-kb fragment in the order *αS1*, *β*, *αS2*and *κ*. *κ-Cn* variant B is reported to be favourable for milk quality and is considered to be included in breeding strategies of dairy animals.

Selection for the B allele of *κ-Cn* gene is integrated into cattle breeding programs in many countries. Nevertheless, most of the studies have been done with Taurine breeds and little is known about this gene in *indicus* breeds. Knowledge of the *κ-Cn* allele frequency distribution in *indicus* breeds is of major importance for cattle breeding in tropical countries.

The indigenous cattle (*Bos indicus*) of Assam is smaller in size and has comparatively lower milk production ability. However, these animals are well adapted to their habitat and environment and are capable to withstand harsh climatic condition, tropical diseases and scarcity of feeds and fodder. So far, very little attention has been paid towards the development of these indigenous cattle in Assam. This is in spite of the fact that the indigenous cattle of Assam constitute an integral component of revised cattle breeding policy of the state.

In view of the above, the present study has been planned in indigenous cattle of Assam to study occurrence of polymorphism in sequences of *κ-Cn* genes and to analyze the gene sequences for determining gene and genotype frequencies.

Materials and Methods

Animals and samples

The present study was conducted on indigenous cattle of Assam from3 different districts, viz., Darrang, Silchar and Kamrup. 5 ml of blood was aseptically collected from 53 apparently healthy indigenous cattle using EDTA (2.7%) as anticoagulant. The samples were properly labelled and transferred to the laboratory and genomic DNA was extracted immediately (Sambrook and Russell, 2001) [16].

DNA extraction and purity

The purity of the genomic DNA was assessed by UV spectrophotometer (Nanodrop Spectrophotometer, Model-UV/VIS 916) by checking the optical density (OD) value at 260 and 280 nm. The samples having OD ratio (260 nm/ 280 nm) 1.7 to 1.9 were used for the experiment. Horizontal submarine agarose gel electrophoresis (0.8%) was performed to check the integrity of DNA and the DNA was visualized under gel documentation system. The genomic DNA samples having good quality DNA (Intact bands without smearing in gel) were used for further analysis.

PCR amplification and gel electrophoresis

The primer sequences used for the amplification of *κ-Cn* gene were F: 5'-ATTTATGGCCATTCCACCAA-3′ and R: 5′-ATTAGCCCATTTCGCCTTCT-3′ (Medrano and Aguilar-Cordova, 1990). The primers were diluted for working concentration of 20 pmol/ µl.The PCR reaction was carried out in 0.2 ml PCR tubes in a thermal cycler (BIO-RAD Model S100, USA). PCR was carried out in a final reaction volume of 25 µl. Each reaction volume contained DNA template 1.0µl, forward and reverse primer 0.5 µl each, master mix 12.5 µl and nuclease free water 10.5 µl. Cycle condition for amplification was, initial denaturation at 95°C for 5 mins, followed by denaturation at 94°C for 1 min, annealing at 51°C for 1 min, extension at 72°C for 30 sec and final extension was performed at 72°C for 5 mins. Thirty five cycles were run to have optimum amplification. PCR amplification was confirmed by 1.5% agarose gel electrophoresis and amplified product was visualized under gel documentation system.

PCR-RFLP

The PCR product was purified using PCR purification kit and digested with *Hinf*I (Thermo Scientific) as per the manufacturer's protocol. Digested PCR product was run in 12% polyacrylamide gel electrophoresis was visualized the band pattern in Gel documentation system (Bio-rad).

Sequencing and data analysis

The purified PCR products were sent for sequencing to SciGenom Pvt. Ltd., Cochin, Kerala. (ABI Genetic Analyser) following Sanger's dideoxy chain termination method (Sanger *et al*., 1977) [17]. The sequences were analyzed by using Clustal W method of DNASTAR Software (Lasergene, USA) and MEGA 6 Software to generate sequence alignment reports, sequence distance, residue substitution and phylogenetic analysis.

Results

The amplification of *κ-Cn* gene resulting in a 351 bp (Figure. 1). Screening of the indigenous cattle was carried out to detect polymorphism in the amplified segment of *κ-Cn* gene. The studied population with *Hinf*I indicated the presence of two types of restriction pattern (Figure. 2), AA genotype with three fragments of 131 bp, 131 bp and 89 bp and AB genotype with four fragments of 262 bp, 131 bp, 131 bp and 89 bp. The gene and genotypic frequencies of AA and AB are presented in the Table. 1. The sequences obtained were analyzed by BLAST and submitted to NCBI for Accession Numbers (KF571745 and KF571746). In sequence of *κ-Cn* gene, two *Hinf*I restriction sites were detected for AA genotype and three for AB genotype. All the sequences showed 99-100% similarity with the sequences of other *Bos indicus* and *Bos taurus* breeds. The frequencies of A and B alleles of *κ-Cn* gene were 0.6510 and 0.3491 and genotypic frequencies of AA homozygote and AB heterozygote were found to be 0.4237 and 0.4545 respectively in indigenous cattle of Assam. Phylogenetic analysis of partial sequence of *κ-Cn* gene of Assam indigenous cow with all ready published sequences *κ-Cn* gene of different cow was done (Figure. 3). It revealed that the sequences of Assam indigenous cow of the present study lies in a common cluster with *κ-Cn* gene sequences of *Bos taurus* which differs from that of the other cattle breeds.

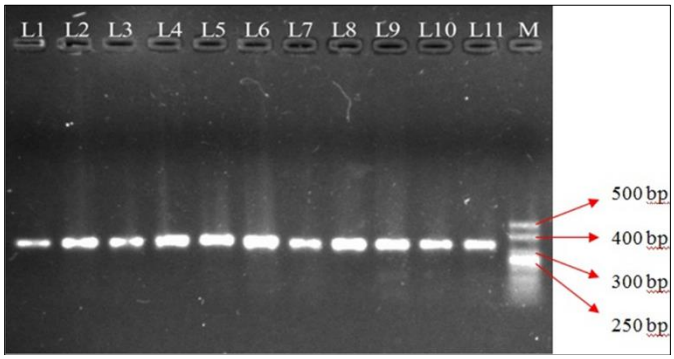

Fig 1: PCR amplicon (351 bp) of *κ-Cn* gene in 1.5% Agarose gel. L1, L2, L3, L4, L5, L6, L7, L8, L9, L10, L11: PCR product M: 50 bp ladder

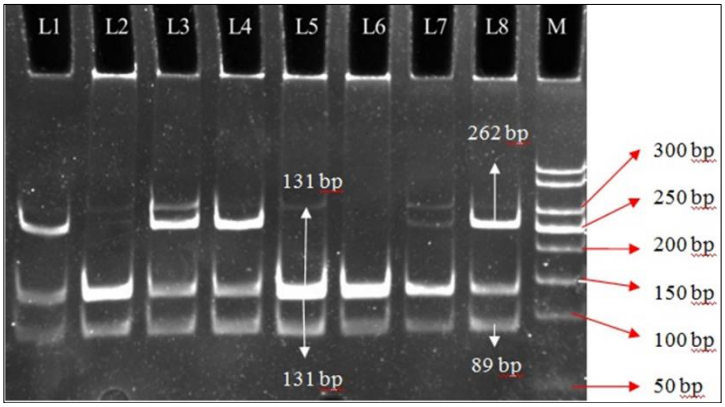

Fig 2: PCR- RFLP of *κ-Cn* gene in 12% non-denaturing PAGE. L1, L2, L3, L4, L5, L7 and L8: AB genotype (262, 131, 131 and 89 bp) L6: AA genotype (131, 131 and 89 bp) M: 50 bp ladder

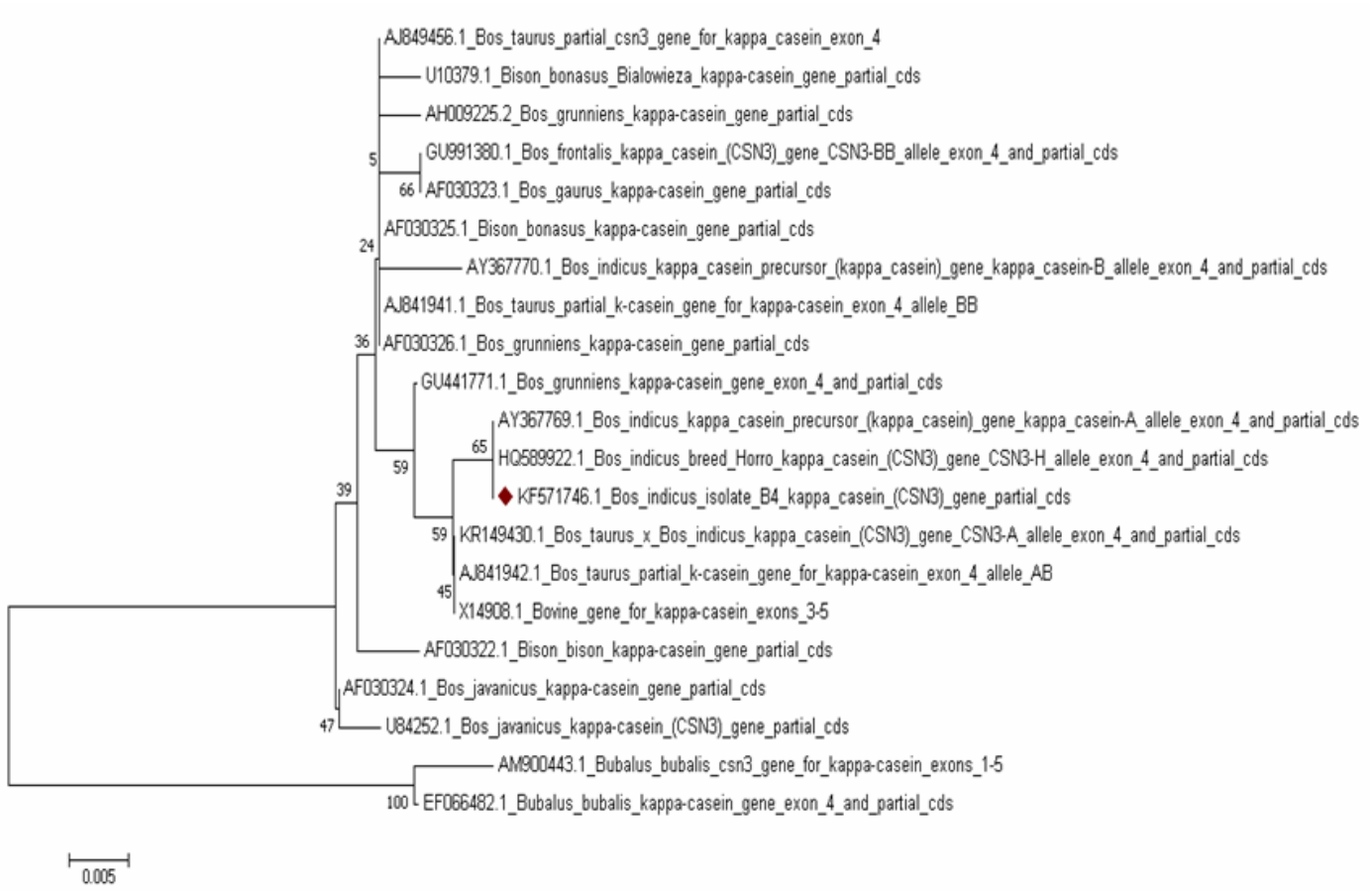

Fig 3: Phylogenetic analysis of *κ-Cn* gene of indigenous cattle of Assam with all ready known sequences in the database

Table 1: The distribution of *κ-Cn* genotypes, their allele frequencies in indigenous cattle of Assam and Hardy-Weinberg Equilibrium.

LOCI		Genotype			Allele frequency	
		AA	AB	BB	A	B
κ-Cn	Observed frequency	16	37	0	0.6510	0.3491
	Expected frequency	22.4561	24.0885	6.4607		
	Genotype frequency	0.4237	0.4545	0.1219		
	χ^2	$15.24^{N.S}$				

N.S. = Non significant

Discussion

The amplification of *κ-Cn* gene resulting in a 351 bp PCR product in indigenous cattle of Assam was in accordance with earlier reports (Dogru and Ozdemir, 2009) [5]. The studied population with *Hinf*I digestion indicated the presence of two types of restriction pattern, AA genotype with three fragments of 131 bp, 131 bp and 89 bp and AB genotype with four fragments of 262 bp, 131 bp, 131 bp and 89 bp. Similar finding was also reported in previous studies (Dogru and Ozdemir, 2009) [5]. The frequencies of A and B alleles of *κ-Cn* gene were 0.6510 and 0.3491 and, genotypic frequencies of AA homozygote and AB heterozygote were found to be 0.4237 and 0.4545, respectively in indigenous cattle of Assam. The present findings revealed a higher frequency of AB genotype for *κ-Cn* gene in indigenous cattle of Assam. The frequency found for allele A was higher than that of

allele B, which was in close agreement to the results of earlier studies performed in *Bos taurus* (Rachagani and Gupta, 2008) [14] and in Chinese Holstein and Jersey (Ren *et al*., 2011) [15]. AA genotype was the most frequent for Brazilian cattle (Azevedo *et al*., 2008) [2]. However, a higher frequency of *κ-Cn* variant B over variant A was observedin Brown Swiss cattle breed (Dogru and Ozdemir, 2009) [5]. Cows of AB and BB genotypes showed a higher milk fat content when compared to the AA genotype (Botaro *et al*., 2009) [3]. Because of the effects of genetic variants of *κ-Cn* on cheese yield, selection of animals with the favourable *κ-Cn* B allele is considerable. However, among the animals examined, BB homozygote could not be detected. This finding was in agreement with an earlier report which also showed a rare occurrence of homozygote BB in Holstein (Galila *et al*., 1996). The absence of BB genotype in the population studied, however, might be due to limited sample size. In studies of genetic characterization of cattle breeds, it was found that the B allele of *κ-Cn* occurred at higher frequencies in breeds originating from *Bos taurus* than in those of *Bos indicus* origin (Del Lama and Zago, 1996) [4]. The χ^2 test revealed that the calculated value for *κ-Cn* gene (15.24) was greater than the tabulated value at 5% and 1% level of significance with 2 degrees of freedom. Hence the population under study was not found to be in Hardy-Weinberg Equilibrium with respect to *κ-Cn* gene. This finding was consistent with those determined in Brazilian *Bos indicus* (Del Lama and Zago, 1996) [4] Pantaneiro cattle (Lara *et al*., 2002) [9] Sarabi and Holstein-Friesian (Toorchi *et al*., 2006) [20] Iranian Sistani cattle (Tahmoorespur *et al*., 2007) [19] Brazilian cattle (Azevedo *et al*., 2008) [2] and Brown Swiss cattle (Dogru and Ozdemir, 2009) [5].

Conclusion

From the present study it could be concluded that the frequency of allele A was higher than that of allele B in the indigenous cattle with a higher frequency of AB genotype (AB>AA). However future study need to conduct with more numbers of sample and and covering more areas.

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