Original Research

Genotyping of Indigenous Cattle Breeds and their Exotic Crosses for β - Casein Milk Type using PCR-RFLP and Sequencing

Ashish C Patel*, Chitra Patel, Hinal Zala, Yasin Sheikh, Tripti Dadheech, Pooja Mankad, S.V. Shah[1] and D. N. Rank

Department of Animal Genetics and Breeding, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand- 388 001, Gujarat, INDIA

[1]Livestock Research Station

***Corresponding author:** aashishvet@gmail.com

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Abstract

The role of A1 and A2 ß casein milk variants and human health is a subject of concern for scientific investigations. The status of A1/A2 ß-casein variants in Bos taurus cattle breeds from several countries have shown presence of A1 variant in European cattle, which has been linked to range of illness. However, information on A1/A2 ß-casein variants in Bos indicus and their crosses is limited. Bulls can influence the population structure more profoundly. Hence, the intend of the study was to know the prevalence of variants of ß-casein (β-CSN2) in Gir and Kankrej, prominent Bos indicus breeds of the country and crossbred bulls (HF crossbreds) used for artificial insemination. Semen / blood samples of 226 Gir, 88 Kankrej and 263 crossbred bulls and cows were collected from three semen stations and three non-government breeding farm, in Gujarat. PCR–RFLP and allele specific PCR analysis of the ß casein region revealed all three genotypes suggesting prevalence of A1 variant in Gir and Kankrej also. Frequency of A1 allele in the indigenous breeds was much higher than believed earlier. We also report the first case of A1A1 Gir bull. This is probably the first report of prevalence of A1A1 genotype in any of the zebu Bos indius breeds. Crossbred cattle showed prevalence of both variants, A1 and A2 with higher frequency of A2. Further, allelic frequencies confirmed deviation from H-W equilibrium in HF crossbreds but not in Gir and Kankrej. The sequence analysis confirmed SNP 'C' to 'A' causing replacement of proline by histidine between A1 and A2 ß casein. The present study confirmed the earlier theory that A2 allele of ß casein is predominant in Bos indicus.

Key words: β-Casein, Crossbred Cattle, Indigenous Cattle, PCR-RFLP, Sequencing

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Introduction

Milk from a variety of animal species has been included in the human diet as a source of animal protein, fat and minerals. Cow serves as major source of milk to the human diet. Among the two types, casein forms the major (80%) part of cattle milk proteins. Casein group contains four proteins, αs1-casein, β-casein, αs2-casein, and κ-casein, located on chromosome number 6, in which β-casein, containing 209 amino acids constitutes 25-30% (Rijnkels, 2002). Total 13 genetic variants of ß-casein are known; A1, A2, A3, A4, B, C, D, E, F, H1, H2, I, and G, out of which A1 and A2 are the most common; B is the less common while others are rare (Farrell *et al*., 2004). A2 ß-casein is recognized as the original ß-casein protein because it is believed to be existed before a mutation caused the appearance of A1 ß-casein in European cattle (*Bos taurus*) a few thousand years ago (Malamathi *et al*., 2014). Polymorphism in one of the ß-casein gene codons – CCT → CAT – causes substitution of proline (A2) by histidine (A1, B) at position 67 in the amino acid sequence. β-casein has received considerable research interest in the dairy industry; the polymorphic nature of β-casein and its association with milk production traits, composition, and quality also attracted several efforts in evaluating the allelic distribution of β-casein locus as a potential dairy trait marker. The A2 variant has a positive relationship with milk performance traits especially protein and milk yield while the A1 variant gives opposite results (Ikonen *et al*., 1999, 2001; Olenski *et al*., 2012). It was also a focus of research in animal breeding in recent years as a source not only of high quality protein, but also of a bioactive peptide that may be linked to health effects. It is thought that ß-casein variant A1 yields the bioactive peptide ß-casomorphin-7 (BCM-7) on digestion in the gut, which may play an unclear role in the development of some human diseases. At the end of the 1990s, some reports suggested consumption of milk having casein variant A1 as a risk factor of type 1 (insulin independent) diabetes mellitus (Elliot *et al.*,1999; Thorsdottir *et al.*,2000) and ischemic heart disease in humans (McLachlan, 2001). It was also associated to incidence of sudden infant death syndrome (SIDS) (Sun *et al*., 2003), coronary heart disease (McLachlan, 2001), arteriosclerosis (Tailford *et al*., 2003), autism, schizophrenia (Woodford, 2011). Considering role of ß-casein variants of A1 and A2 on human health, and its association with quality and quantity of milk, present study was conducted to know the prevalence of A1/ A2 ß casein types in Gir, Kankrej and their HF crosses in Gujarat especially in breeding bulls.

Gir and Kankrej, both transboundary breeds are native to Gujarat and are among the best dairy and dual-purpose breeds of the country. As per 2007 census Gir with 1.4 million heads in 2007 (https://gldb.gujarat.gov.in/) accounting for 37 percent of total cattle population in Gujarat, its home state, has the highest population among four dairy breeds of India. Kankrej is one of the heaviest and important dual-purpose breed known for powerful bullocks. With 2.68 million population 2007 census (https://gldb.gujarat.gov.in/) it is the most populous among all 41 Indian cattle breeds. Further, Kankrej has been used extensively in crossbreeding with HF.

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Because of recent public interest on health issues associated with A1/A2 ß casein variants, large many European breeds have been screened for ß casein composition. The most widely distributed breed HF possesses high frequency of A1 (Kaminski *et al.*, 2007). The Southern European breeds and the Jersey carry the A1 allele at about 35 % (Boro *et al.*, 2016). Exceptionally, Guernsey breed appears to carry the A1 allele at less than 10% while, A1 β-casein is nearly absent in the milk of pure Asian and African cattle (Ng-Kwai-Hang and Grosclaude, 2002). It is also indicated that *Bos indicus* breeds harboured predominantly A2 type ß casein (Mclachlan, 2003). Recently, there is a sudden spurt of activities on screening Indian cattle breeds for ß casein variants. Since the first report on the prevalence of ß casein variants in Indian breeds by Mishra *et al*. (2009), it is firmly established that most of the Indian cattle and buffalo breeds carry exclusively A2 variant (Joshi, 2011).

However, only scanty information is available on Gir, Kankej and the HF crossbreds. As extensive use of bulls with a specific genotype can rapidly change the population structure for a particular gene, it is important to know the frequency of ß casein genotype in breeding bulls used for AI. In the present investigation, we present the information on Gir and Kankrej bulls from three important semen stations of the state for ß casein genotypes and proportion of A1/A2 alleles in Gir, Kankrej and HF x Kankrej crossbred females on three non-government breeding farm.

Materials and Methods

Experimental material for the present study comprised semen/blood samples of 29 Gir, 60 Kankrej and 52 crossbred (HF X Gir & HF X Sahiwal) bulls from three semen stations. Blood samples were also collected from the Jugular vein of 197 Gir, 28 Kankrej and 211 HF x Kankrej crossbred females from three well manage non-government breeding farm in 9 ml capacity vacutainer (EDTA, K3). Genomic DNA was extracted by John's method (John *et al*., 1991). Genomic DNA from semen samples was extracted by modified John's method. The quality and quantity of DNA were checked by Nanodrop ND-1000 Spectrophotometer V3.5 (Nano drop Technologies, Inc. USA) and 0.8 % agarose gel electrophoresis.

PCR amplification was carried out using primers (Forward primer) 5´-CCTTCTTTCCAGGATGAACTCCAGG-3´, (Reverse primer) 5´ -GAGTAAGAGGAGGGATGTTTTGTGGGAGGCTCT- 3´described to cover β-casein (CSN2) exon seven by McLachlan (2006). The reaction mixture in the total volume 25 µl included 100 ng DNA, 10 pmole of each primer with PCR master mix containing *Taq*I polymerase (Emerald), $MgCl_2$ and dNTPs. Amplification was achieved by thermal cycling at 95 °C for 5 minutes followed by 30 cycles: 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds. The reaction was completed by the final extension at 72°C for 10 minutes. The 121 bp PCR amplicon product of CSN2 gene was digested with 5 units of *Dde I* restriction enzyme (Promega) for 1.5 hours at 37 ^{0}C. Restriction digestion fragments were loaded on 3 per

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cent agarose gel containing ethidium bromide (10mg/10ml) and the gel was analyzed under the UV transilluminator. The samples were also subjected to allele specific PCR using forward primer of 5'- GCC CAG ATG AGA GAA GTG AGG -3', and the reverse primer 1 of 5′- GAT GTT TTGTGG GAG GCT GTT AT -3′ for A1 and the reverse primer 2 of 5′-GAT GTT TTG TGG GAG GCT GTT AG -3′ for A2 allele (Keating *et al.*, 2008). The observed genotype frequencies were tested for Hardy–Weinberg equilibrium by Chi-square test. The observed and expected frequencies were analyzed for goodness of fit with level of significance at probability $P< 0.05$.

Representative samples genotyped as A1A1, A1A2 and A2A2 based on RFLP patterns were selected for cloning and sequencing. For cloning of CSN2 fragment, two representative samples each from three different RFLP patterns were cloned in pTZ5R/T (Thermo Fisher) vector. Ligation was confirmed by performing M13 primer PCR. Ligated products were transformed into competent *E. coli* Top 10 cells and transformed cells were cultured on LA agar plates containing X-Gal and IPTG for blue white screening. White colonies obtained were further selected and grown on LB broth followed by plasmid extraction from overnight grown culture. The recombinant plasmid DNA was isolated and sequenced on ABI-PRISM - 3500 DNA sequencer (Thermo Fisher). All three RFLP variants were sequenced at least two times. The CSN2 sequences were analyzed for homology search against nr/nt database of NCBI using BLAST. The BLAST analysis was used to find variation in the sequences obtained with the reference sequences of bovines and of other species in the NCBI database.

Results and Discussion

A large number of reports have specifically expressed the association of A1 β-casein to a range of illnesses as it preferentially releases an opioid peptide called β-casomorphin-7 (BCM-7) upon digestion (Jinsmaa and Yoshikawa, 1999). In the present investigation, PCR-RFLP and allele specific PCR were employed to explore polymorphism in allelic variants (A1, A2) of ß-casein gene in Gir, Kankrej and HF crossbred (HF × Gir& HF × Sahiwal) cattle. The causative nucleotide change responsible for A1/A2 ß casein type was confirmed by cloning and sequencing. A single base mutation at codon CCT → CAT causes substitution of proline by histidine at position 67th in the amino acid sequence leading to abolition of restriction site of *Dde I* in A1 ß casein gene. Hence, a 121 bp fragment of ß casein exon 7 when digested with *Dde I* produces two fragments of 86 bp and 35 bp in A2 but leaves 121 bp fragment uncleaved in A1.

The allelic and genotypic frequencies of β-casein gene obtained through PCR-RFLP and allele specific PCR are presented in Table 1. Aim of the present investigation was to estimate the gene frequency of A1 and A2 alleles specifically in the breeding bulls of prominent indigenous breeds, Gir and Kankrej and HF crossbreds. There was predominance of A2 allele in Gir (82%) and Kankrej (92%) breeds, respectively (Table 1). A1 allele was mainly present as heterozygous (carrier) in both the indigenous breeds. The

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frequency of A1 and A2 alleles in the organised herds (females only) was found to be 0.172 and 0.107 in Gir and Kankrej breeds, respectively. In HF crossbreds also A2 allele was predominant with 0.610 frequency in breeding bulls and the frequency of A2 allele in cows is also nearly similar, 0.632. Because of sizable proportion of A1 allele, it was also represented in homozygous state. Thus, unlike Kankrej, all three genotypes A1A1, A1A2 and A2A2 genotypes were present in HF crossbreds (Table 1). Of course, these crossbreds did not have inheritance of Kankrej but of Gir and Sahiwal. For Hardy-Weinberg Equilibrium testing, the Chi-square value of the frequency revealed that allele frequencies in only Gir and Kankrej population confirmed to HW equilibrium while, crossbred populations were not in Hardy-Weinberg equilibrium (Table 1).

Table 1: Gene and genotype frequencies of CSN2 gene in Gir, Kankrej and Crossbred cattle

Breed (Number of Animals)	Genotype Frequency			Allele Frequency	
	A1A1	A1A2	A2A2	A1	A2
Gir Bulls (54)	2 (0.037)	20 (0.370)	32 (0.593)	0.222	0.778
Gir Cows (172)	1 (0.006)	57 (0.331)	114 (0.663)	0.172	0.828
Total Gir (226)	**3 (0.013)**	**77 (0.341)**	**146 (0.646)**	**0.184**	**0.816**
	$\chi 2 = 4.188^{ns}$				
Kankrej Bulls (60)	0	8 (0.154)	52 (0.867)	0.067	0.933
Kankrej Cows (28)	0	6 (0.214)	22 (0.786)	0.107	0.893
Total Kankrej (88)	**0**	**14 (0.159)**	**74 (0.841)**	**0.08**	**0.92**
	$\chi 2 = 0.650^{ns}$				
Crossbred Bulls (73)	9 (0.123)	39 (534)	25 (0.342)	0.39	0.61
Crossbred Cows (190)	15 (0.079)	110 (0.579)	65 (0.342)	0.368	0.632
Total HF crossbred (263)	**24 (0.091)**	**149 (0.567)**	**90 (0.342)**	**0.375**	**0.625**
	$\chi 2 = 11.51^{**}$				
Grand Total: 567	**27 (0.048)**	**240 (0.423)**	**300 (0.529)**	**0.259**	**0.741**
	$\chi 2 = 5.9^{ns}$				

It was earlier reported that in both these breeds A2 allele is fixed and there is absence of A1 allele (Mishra *et al*., 2009). However, the present study revealed much higher frequency of A1 allele than believed earlier in both the breeds. Interestingly one Gir bull was found to be A1A1, homozygous for A1 allele. This is probably the first report of A1A1 individual in any of the Indian cattle breed. Breeding bull can have profound influence on genetic structure of the population and can rapidly change the gene frequency of a given gene under selection. So, we also studied the frequency of A1 and A2 alleles in the cows from three organised herds. Although frequency of A1/A2 in the organised herds may not be true representative of the population but the frequency of A1 and A2 alleles in the organised herds (females only) was found to be not far different from that of bulls in the semen stations. This finding confirms the earlier reports of predominance of A2 allele in *Bos indicus*. However, frequency of A1 allele in Gir and Kankrej is much higher than all previous reports. While there are fewer reports available suggesting low frequency of A1 allele in the indigenous breeds, i.e, Malanad Gidda (0.096) and Kherigarh (0.109) (Mishra *et al.*, 2009),

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Vechur (0.20) (Muhammed and Stephen, 2012), Ongole (0.06) (Ganguly *et al*., 2013), Malnad Gidda (0.014) and Kasargod (0.042) Ramesha *et al.*, 2016), there are several reports implying absence of A1 allele in various indigenous cattle breeds like Dangi, Sahiwal (Shahlla *et al*., 2014), Kangayam (Malamathi *et al*., 2014), Nimari, Red Kandhari, Malvi, Amritmahal, Kankrej, Gir, Hariana, Tharparker, Rathi, Mewati, Red Sindhi (Mishra *et al*., 2009). Not getting the A1 allele in the Gir by Mishra *et al*., 2009 could be due to limited sampling, many of them might be coming from a common source. Unlike others, Muhammed and Stephen (2012) reported much higher frequency of A1 allele in Vechur (0.2) and Kasargode dwarf (0.39). Not many reports are available on *Bos taurus x Bos indicus* crossbred cattle for comparison. Muhammed and Stephen (2012) reported frequency of A1 allele in Vechur crossbred cattle as 0.46; Sodhi *et al*. (2012) found mean frequency of 0.645 in several exotic and crossbred bulls. Ramesha *et al.* (2016) observed the frequency of A1 allele in HF crossbred males as 0.169 while, Malarmathi *et al.* (2014) observed 40% A1 in HF crossbred cattle.

Large many reports are available suggesting predominance of A1 allele in the several European cattle breeds including HF *viz*. Pinzgau (Beja-Pereira *et al*., 2003), Black and-White breed (Bech *et al*., 1990), Red-and-White breed (Ehrmann *et al*., 1997), Ayrshire (Ikonen *et al*., 1999), Holstein (Hanusova *et al*., 2010). However, predominance of CSN2 A2 allele (0.764) was detected in Carora (Caroli *et al*., 2008), Slovak Spotted breed (Miluchova*et al*., 2013) and Czech Spotted and Czech Holstein breed (Manga *et al*., 2010). Although not confirmed, due to possible health risk associated with A1 allele, frequency of A1 can further be reduced by genotyping and avoiding use of A1A2 bulls in the breeding can be established. A1A1 are extremely rare and normally will not be encountered due to extremely rare chances of homozygous A1A1 genotype (<1% and <3% in Gir and Kankrej in our case). However, on account of very high frequency of carrier (heterozygous) bulls (more than 50%) in the crossbred population, culling of such bulls would not be a feasible option.

Gir and Kankrej population confirmed to HW equilibrium while, crossbred populations were not in Hardy-Weinberg equilibrium implies that these animals were produced by AI where only a few elite bulls were used. The sequence data suggest that the exon 7 region of ß casein gene is highly conserved except a single base change leading to A1 and A2 alleles. The hypothesis regarding implications of A1 and A2 milk on human health is still under consideration and there is substantial uncertainty associated with this issue, however the present findings suggested high prevalence of favourable allele in Gir and Kankrej breed like other indigenous breeds and thus, less prone to the possible risk of ß-casein associated health issues.

In sequencing of CSN2 gene insert, the size of bovine ß-casein gene was found 8498 bp in length and consists of nine exons and eight introns. The complete nucleotide sequence of bovine ß-casein gene of *Bos taurus* is reported and uploaded at the Database of National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov/nuccore/M55158.1) by Bonsing *et al*. (1988). The SNP at 67th position was

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confirmed by cloning and sequencing of CSN2 insert. For cloning of CSN2 segment, two representative samples each from three different RFLP patterns were cloned in PTZ57R/T vector. Ligated products showed expected band size i.e. 275 bp for CSN2 (121 bp CSN2 + 154 bp M13 fragment) on 2% agarose indicating successful ligation reaction. Sequences obtained by sequence analyser were curated and vector sequences were removed using Vecscreen programme. The genotypes with their respective nucleotide at 86th position A, C and A/C=M for A1A1, A2A2 and A1A2 genotypes respectively are shown in Fig. 1 and Fig. 2.

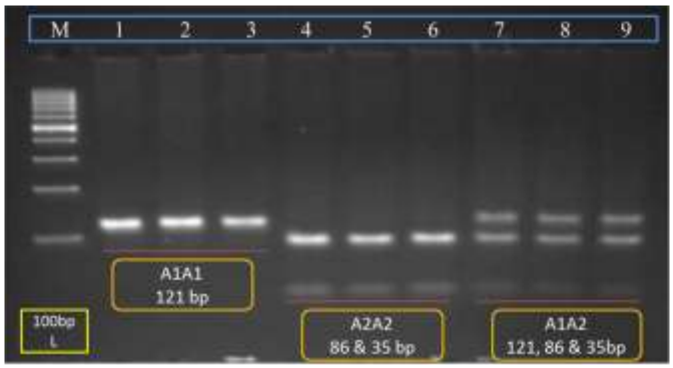

Fig. 1: Representative Ddel PCR-RFLP of CSN2 gene from Gir, Kankrej & HF crossbred bulls resolved on 2.0% agarose gel showing A1A1, A2A2 and A1A2

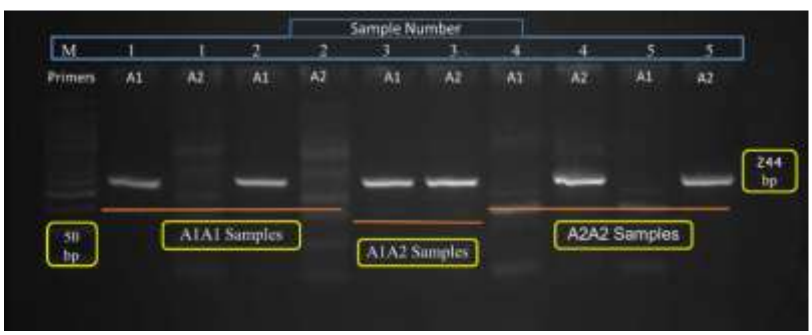

Fig. 2: Genotyping for ß casein using allele specific

These filtered sequences were then subjected to local alignment with Clone Manager. Results of alignment of all samples sequenced for CSN2 gene polymorphism viz., A1A1, A1A2 and A2A2 genotypes are shown in Fig. 3. The sequence data was subjected to NCBI BLASTn and was compared with data available in the public domain for bovine species and also for other species. It was confirmed that the amplified product was that of CSN2 gene fragment and BLASTn analysis revealed 99 per cent identity with that of *Bos indicus* and *Bos taurus* gene sequence data available in public domain (Table 2).

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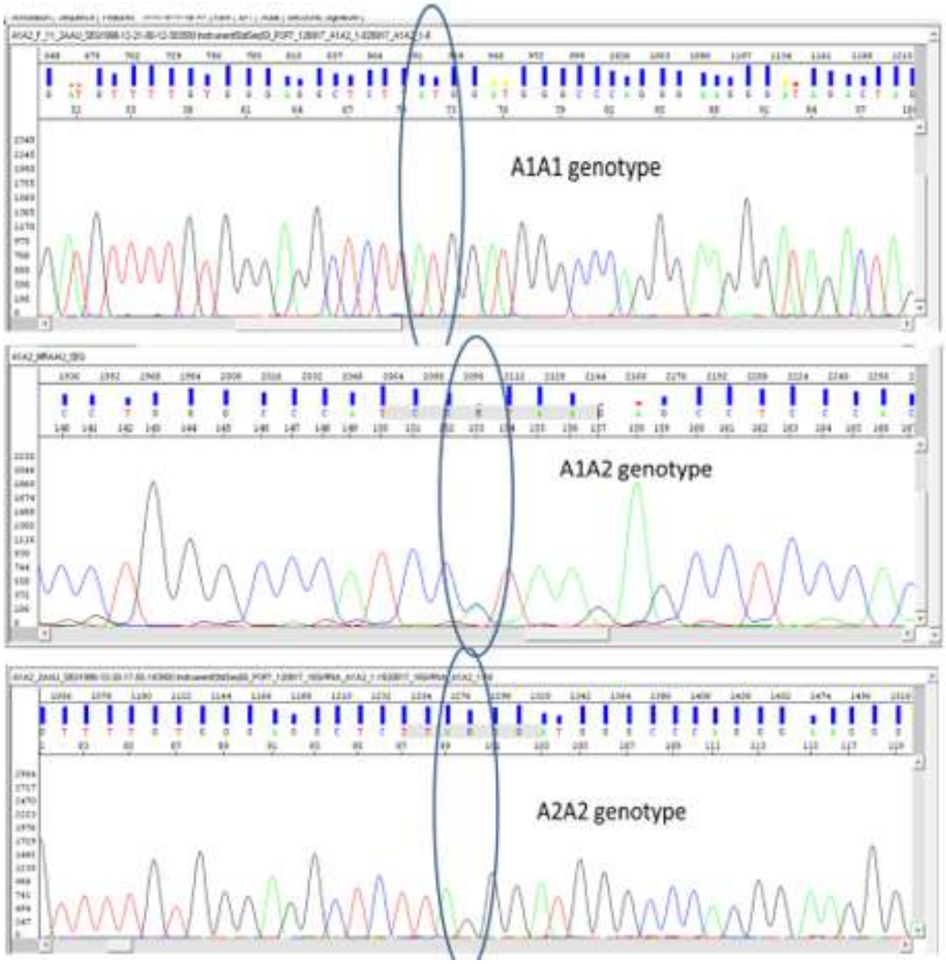

Fig. 3: Electropherogram showing single nucleotide change at 67th position in CSN2 gene. (a) A in A1A1 genotype, (b) C in A2A2 genotype, (c) A/C in A1A2 genotype

Table 2: BLASTn showing the percentage of identity of the exon 7 region of the CSN2 gene of indigenous cattle breeds with other species

Accession	Description	Maximum Score	Total Score	Query Cover	Identity
JN051276.1	Bos indicus breed Gyr ß-casein (csn2) gene, csn2-A2 allele, partial cds	219	219	100%	99%
X14711.1	Bovine ß-casein gene	207	207	100%	98%
KC993858.1	Bos taurus ß-casein (csn2) mRNA, complete cds	200	200	91%	99%
FN424088.1	Bubalus bubalis csn2 gene for ß casein, exons 1-9	219	219	100%	99%
AH001195.2	Capra hircus strain Saanen ß-casein (ß-casein) gene, complete cds	196	196	100%	96%
X79703.1	Ovis aries gene for ß-casein	196	196	100%	96%

The BLAST analysis also showed 99 per cent identity with that of *Bubalus bubalis*, 96 per cent with *Ovis aries* and *Capra hircus* (Table 2). The pair wise comparison of nucleotide sequence of CSN2 exon 7 regions

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of Gir, Kankrej cattle and crossbred cattle revealed a single base difference between A1 (A) and A2 (C) alleles (Fig. 4).

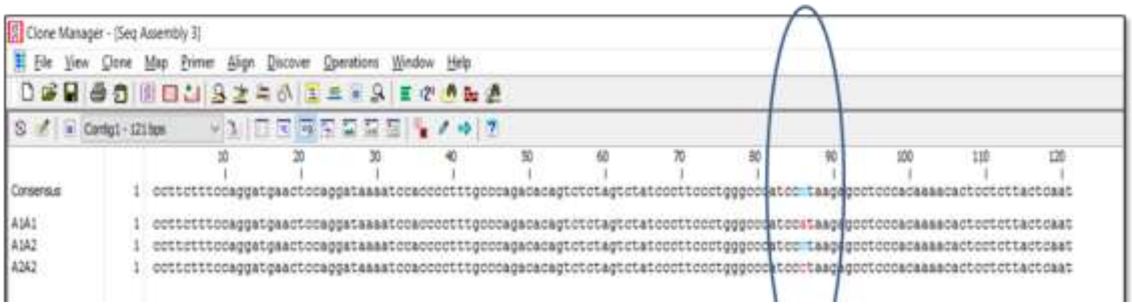

Fig. 4: Multiple sequence alignment of CSN2 sequence of A1A1, A1A2 and A2A2 genotypes of representative samples using clone manager.

1. **A1 ß Casein:**

ATTAGTAAGAGAGGGATGTTTTGTGGGAGGCT**CTTATG**GATGGGCCC
AGGGAAGGGATAGACTAGAGACTGTGTCTGGGCAAAGGGGTGGATTT
TATCCTGGAGTTCATCCTGGAAAGAAGG

PSFQDELQDKIHPFAQTQSLVYPFPGPI**H**KSLPQNIPLLL

2. **A2 ß Casein:**

ATTAGTAAGAGAGGGATGTTTTGTGGGAGGCT**CTTAGG**GATGGGCCC
AGGGAAGGGATAGACTAGAGACTGTGTCTGGGCAAAGGGGTGGATTT
TATCCTGGAGTTCATCCTGGAAAGAAGG

PSFQDELQDKIHPFAQTQSLVYPFPGPI**P**KSLPQNIPLLL

Fig. 5: DNA and protein sequence of A1 and A2 CSN2 gene

The result of BLASTp analysis of protein sequence of the partial $seven^{th}$ exon of CSN2 gene is presented in Fig. 5. The amino acid proline is substituted by histidine in A1 allele due to mutation at the 86^{th} position.

Conclusion

The results revealed predominance of allele A2 in Gir, Kankrej breed and their HF crossbreds (HF x Gir & HF x Sahiwal). The A1 allele was particularly rare in indigenous breeds. However, a Gir bull was found to A1A1. This is probably the first report of A1A1 animal in any of the indigenous breeds. Genotyping bulls for ß casein gene variants and culling of carrier bulls could be employed as a breeding strategy to enhance A2 milk production in indigenous breeds. However, this strategy would not be a feasible option for crossbred bulls considering its large proportion.

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