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Genotypic profiling of A1 and A2 *β-casein* alleles in Punganur cattle using PCR-RFLP

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ABSTRACT

Milk is regarded as nature's perfect food due to the presence of essential nutrients required for the growth and development of mammals. The consumption of milk with a specific *β casein* variant either A1 or A2 had a relationship with the risk of diseases has been identified. Considering the importance of *β casein* variants with the health significance, the present study was undertaken in Punganur cattle (N=175) by using PCR-RFLP with *Dde I* restriction enzyme that showed the distribution of A1A2 (121 bp, 86 bp, and 35 bp) in 5 animals, A2A2 (86 bp and 35 bp) in 170 animals with no evidence of A1A1 genotype. The genotypic frequency of the A1A2 heterozygous β-casein variant of exon7 of the *CSN2* gene was 0.029, whereas, for the A2A2 homozygous *β-casein* variant was 0.971 and the A1A1 homozygous *β-casein* variant is absent in Punganur cattle. The A1 allelic frequency of exon7 of the *CSN2* gene was 0.014, whereas, the A2 gene frequency is 0.986 in Punganur cattle. The study indicates that there is a minimum genetic variability in the tested population of the Punganur cattle breed suggesting less or lower level of crossbreeding activities with exotic animals.

Keywords: Allelic frequency, A1 and A2 alleles, *β-casein* gene, *Dde I*, Genetic frequency, Punganur cattle, PCR-RFLP

Cattle milk is a rich source of nutrients, proteins, and energy. Caseins and whey proteins are the two major types of milk proteins, of which caseins account for 80% of cow's milk proteins. Four different types of caseins are present in milk namely alpha S1 (CSN1S1, 39-46%), alpha S2 (CSN1S2, 8–11%), beta (CSN2, 25–35%), and kappa (CSN3, 8–15%) casein (Eigel *et al*. 1984, Roginsky 2003). *β-casein* is the degradation product of gamma casein (Ostersen *et al*. 1997) with 209 amino acids (Hanusova *et al.* 2010), which is more abundant and polymorphic with 13 known variants (Farrel *et al*. 2004). The A1 and A2 *β-casein* variants are prevalent in dairy cattle breeds. The domestication of cows, dating back thousands of years, witnessed a spontaneous mutation in the *β-casein* gene. Consequently, the A1 variant of *β-casein* became established in European and American cattle populations, and through breeding strategies, it may subsequently disseminated to other regions, including India.

A significant issue associated with the consumption of milk from exotic and crossbred cattle lies in the presence of the A1 *β-casein* variant. Upon digestion by the proteolytic enzymes in the gastrointestinal tract, A1 *β-casein* releases beta-casomorphin 7 (BCM7), a bioactive peptide with potent opioid-like activity. This activity is linked to adverse health effects including human ischemic heart diseases, insulin-dependent diabetes, atherosclerosis, etc (McLachlan 2001, Tailford *et al.* 2003, Sun *et al.* 2003, Laugesen and Elliott 2003, Kaminski *et al*. 2006, Mishra *et al*. 2009, Pal *et al*. 2015). The hypothesis that the consumption of A1 β-casein elevates the risk of various human diseases underscores the urgent necessity to prioritize dairy cows with the A2 variant of *β-casein*. Numerous studies affirm that indigenous breeds exclusively carry the A2 variant, but cross-breeding strategies aimed at augmenting milk production have introduced a blending of indigenous germplasm with the A1 variant. Therefore, efforts geared toward the conservation of native germplasm in indigenous breeds demand serious attention to mitigate potential health risks associated with A1 *β-casein* consumption.

India has well diversified cattle genetic resources consisting of 50 recognized cattle breeds as of 22 September 2021, as per the data available with ICAR-National Bureau of Animal Genetic Resources . As there is no known records on the *CSN2* polymorphism, present study was undertaken to identify the variants (A1/A2), and determine the possible genotypes (A1A1, A1A2, and A2A2) and gene frequency in the Punganur cattle breed.

MATERIALS AND METHODS

Experimental animal: Punganur cattle (NBAGR accession no. INDIA_CATTLE_0100_PUNGANUR_

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03022) is a miniature cattle breed from the Chittoor district of Andhra Pradesh. A total of one hundred and seventy-five (175) Punganur cattle including 52 cows, 35 bulls, 27 heifers, 24 young bulls, 17 female calves and 20 male calves maintained at Livestock Research Station, SVVU, Palamaner, Chittoor district were selected for the present study.

Sample: Whole blood of about 5 mL was collected aseptically from the jugular vein of all the animals in between 6.00 AM and 7.30 AM, using sterile 10 mL K3E (EDTA) BD Vacutainer tubes and stored at –20°C for subsequent DNA extraction. Genomic DNA isolation from the blood samples was carried out by using the modified phenol-chloroform method of Sambrook *et al.* (1989) and Michael and Sambrook, (2012). The extracted DNA was then stored at –20°C until further analysis. The quantification and purity of the DNA samples were measured by using gel electrophoresis (0.8% agarose) and nanodrop (Thermo Fisher Scientific, US). An average DNA concentration of 449.9 ng/µl was obtained for all the samples. The pure DNA of $OD_{260/A280}$ ratio 1.7 to 1.9 was considered and used for further analysis.

Polymerase chain reaction: A Polymerase chain reaction (PCR) was employed to amplify a 121 bp segment of exon 7 from the *CSN2* gene, utilizing specific primers (Table 1) as described by McLachlan (2006). While the forward primer sequence remained uniform, the reverse primer sequence featured a deliberate one-base mismatch at the penultimate position from the 3' end. This intentional mismatch disrupts hydrogen bonding with the complementary strand during PCR. The A1 and A2 *β-casein* genes differ by a single base, resulting in the creation of a restriction site exclusive to the A2. An endonuclease enzyme (DdeI), recognizing this specific site, selectively cleaves the A2 genotype DNA. Consequently, the A2A2 genotype exhibits two bands due to cleavage, the A1A1 genotype displays a single band (uncleaved), and the A1A2 genotype shows three bands, reflecting a combination of cleaved and uncleaved DNA fragments.

Polymerase Chain Reaction (PCR) was conducted in thin-walled PCR tubes, employing a total reaction mixture of 25 µl. The composition included 3µl of DNA, 12.5µl of 2X PCR Master mix (Promega, USA), 1µl each of forward and reverse primers, and 7.5µl of nuclease-free Water. A consistent final template DNA concentration of 100 ng in a 25 µl PCR reaction mix was maintained across all samples. The PCR procedures were executed in a Thermal Cycler from Eppendorf, Germany, with the cyclic conditions set as follows: Initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, extension at 72°C for 45 s, and a final extension at 72°C for 4 min. Subsequently, each PCR amplicon was analyzed on a 1.5% (w/v) agarose gel containing ethidium bromide (0.5g/ml) in 1X TAE buffer at 80 volts for 45 min. Visualization was achieved using a UV transilluminator.

The digestion of PCR amplicons was accomplished by incubating a 20 µl reaction mixture, comprising 2.0 µl of PCR product, 1 µl (10 units/µl) of *Dde I* restriction enzyme (New England Biolabs), 5µl of cutsmart buffer (New England Biolabs), and the remaining 12 µl of nuclease free water, for 15 min at 37°C. Subsequently, for genotyping, the digested PCR fragments were subjected to electrophoresis on a 3% agarose gel in 1X TAE buffer at 110 V for 90 min. Gel visualization was carried out under a UV transilluminator, and the sizes of PCR-RFLP products were compared with a 50 bp DNA ladder. The outcomes were recorded using a gel documentation system (Biorad).

Statistical analysis: The genotypes were determined based on the banding pattern in the gel observed with 100 bp and 50 bp DNA ladders as homozygous (A1A1 or A2A2)/ heterozygous (A1A2). Based on the number of heterozygotes and homozygotes. A chi-square test was carried out to test the populations for Hardy-Weinberg equilibrium (Snedecor and Cochran 1976). The genotype frequencies were calculated directly by counting the bands appearing in the gels. The gene and genotype frequencies were calculated by using the following formulae.

$$\text{Genotype frequency} = \frac{\text{Proportion of animals with particular genotype}}{\text{Total number of animals}}$$

$$\text{Gene frequency} = \frac{(2 \times \text{no .of homozygotes}) + (\text{no.of heterozygote})}{2 \times \text{total no. of individuals}}$$

Parameters: Experimental heterozygosity (Nei 1978), theoretical heterozygosity (Ho), polymorphism information content (PIC, Boltstein *el al.* 1908), effective number of alleles (ENA), level of possible variability realization (V%), expected homozygosity (E) (Crow and Kimura 1970) were calculated to evaluate the incidence of effectiveness of allele using the following formulas.

$$\text{He exp} = 1 - \sum (p^2+q^2)\ \text{He exp} = 1 - \sum (p^2+q^2),$$

$$\text{PIC} = 1 - \sum (p^2+q^2) \sum_{(i=1)}^{(n-1)} X \sum_{(j=i+1)}^{n} X\ 2P^2Pj^2,$$

$$E = \sum Pi^2,$$

$$\text{ENA} = \frac{1}{p^2+q^2},$$

$$V = \frac{1 - E}{1 - 1/N} \times 100$$

RESULTS AND DISCUSSION

In this study, the PCR-RFLP technique was applied

Table 1. Sequence of primers used for PCR

Primer	Primer sequence	Amplication product size
A1A2 Forward primer	5'CCTTCTTTCCAGGATGAACTCCAGG	121 bp
A1A2 Reverse primer	5'GAGTAAGAGGAGGGATGTTTTGTGGGAGGCTCT	

for sequencing the A1 and A2 *β-casein* variants in the Punganur cattle population. Fig. 1 provides an illustrative image of the PCR-RFLP profile of the *β-casein* gene (*CSN2*) using *Dde I* on a 3.0% gel. A total of 175 *β-casein* amplified PCR products were subjected to *Dde I* restriction enzyme digestion. Out of which 170 samples revealed two bands of 86 bp and 35 bp corresponding to the A2A2 homozygous *β-casein* variant and 5 samples revealed three bands namely an undigested 121 bp, two bands of 86 bp and 35 bp corresponding to A1A2 heterozygous *β-casein* variants and notably, no evidence of the A1A1 homozygous *β-casein* genotype was observed in the study population.

The frequencies of genes and genotypes for PCR-RFLP products were calculated and are outlined in Table 1. The genotype frequencies of the A1A2 heterozygous *β-casein* variant of exon 7 of the *CSN2* gene were the highest for cows (0.058) followed by young bulls (0.042) and male calves (0.05). Heifers, bulls, and female calves showed zero (0.0) A1A2 genotype frequencies, whereas, A2A2 homozygous variant was highest for heifers, bulls and female calves (1.0) followed by young bulls (0.96) and male calves (0.95).

The A1 gene frequency was highest in cows (0.029) followed by male calves (0.025) and young bulls (0.02) and the A1 gene frequency was null in heifers, bulls and female calves. A2 gene frequency was highest in heifers, bulls and female calves (1.0) followed by young bulls (0.98), male calves (0.975) and cows (0.971).

The gene frequency of A1 variant is 0.014, whereas the A2 gene frequency is 0.986 in the sample population. The genotypic frequency of A1A2 heterozygous *β-casein* variant of the herd was 0.029. In contrast, the genotypic frequencies of A2A2 homozygous was 0.971 and A1A1 homozygous *β-casein* variant was absent in the sample population of Punganur cattle (herd). The chi-square test revealed the genotypic frequencies are under Hardy-Weinberg equilibrium.

PCR-RFLP of exon7 of the *β-casein* gene with *Dde I* DNA polymerase restriction enzyme yielded two bands of 86 bp and 35 bp suggesting the presence of the A2 allele and A1 allele was not digested and a band of 121 bp remained intact. Three bands of 121 bp, 86 bp, and 35 bp (Fig. 1) were observed in the samples corresponding to the A1A2 heterozygous genotype, and two bands of 86 bp and 35 bp were observed in the case of the A2A2 homozygous genotype, the A1A1 genotype, which is expected to show the product size of 121 bp, were absent in the present study. The observations of Shende *et al.* (2017) in HF crossbred cattle, Miluchova *et al.* (2014) in the Slovak Spotted breed, and Kumar *et al.,* (2018) in Vrindavani crossbred and Sahiwal cattle were in agreement with present findings. *β-casein* gene polymorphism by PCR-RFLP with specific restriction enzyme *Taqα I* DNA polymerase revealed a single band of 251 bp for the A2 allele and two bands corresponding to 251 bp and 213 bp in samples of A1A1 homozygous and A1A2 heterozygous genotype by Ramesha *et al*. (2015) and Srinivas (2018).

The genotypic frequencies observed in the present study were higher than the findings of Srinivas (2018) whose observed genotypic frequencies were 0.17 (A1A2) and 0.83 (A2A2) and allelic frequencies of 0.083 (A1) and 0.92 (A2) in Punganur cattle. However, the findings of this study in the indigenous Punganur cattle with higher A2A2 homozygous genotypic frequency and very lower A1A2 heterozygous genotypic frequency indicated the purity of indigenous germplasm among the animals suggesting less or lower level of crossbreeding activities with exotic animals.

Shende *et al.* (2017) reported genotypic frequencies of 0.28 (A1A1), 0.72 (A1A2) and allelic frequencies of 0.6383 (A1) and 0.3617 (A2) using PCR-RFLP with *Dde I* in HF crossbred cattle. The mean genotypic frequencies reported by Miluchova *et al.* (2014) using PCR-RFLP observed the genotypic frequencies were 0.1261 (A1A1), 0.3333 (A1A2) and 0.5405 (A2A2) in Simmental breed, 0.1379 (A1A1), 0.4598 (A1A2) and 0.4023 (A2A2) in Holstein breed and 0.3034 (A1A1), 0.5168 (A1A2) and 0.1798 (A2A2) in Pinzgau breed and they reported that Simmental breed and Holstein breed had higher frequency of allele A2 (0.7072 and 0.6322) and Pinzgau breed had higher frequency of the allele A1 (0.5618). Kumar *et al.* (2018) reported genotypic frequency of 0.11 (A1A1), 0.47 (A1A2) and 0.42 (A2A2) and allelic frequencies of 0.35 (A1) and 0.65 (A2) in Vrindavani crossbred cattle and the genotypic frequencies of 0.13 (A1A2) and 0.87 (A2A2) and allelic frequencies 0.06 (A1) and 0.94 (A2) in Sahiwal cattle using ACRS-PCR with *Dde I.*

The calculated values for gene homozygosity (Ho), gene heterozygosity (He), and polymorphism information content (PIC) were determined to be 0.971, 0.029, and 0.0272, respectively. These findings suggest that the Punganur cattle population exhibits elevated homozygosity, diminished heterozygosity, and low PIC values, as summarized in Table 2.

Table 2. Gene and Genotype frequencies of the *β-casein (CSN2)* gene in Punganur Cattle

Animal no.	Frequency of Gene		Frequency of Genotype			$\chi 2$ value d.f=1
	A1	A2	A1A1	A1A2	A2A2	
175	0.014	0.986	0	0.029	0.971	0.036^{NS}

Table 3. Alleles effectiveness for *β-casein* gene in Punganur cattle

Alleles	Ho	He	E	PIC	Ne	V%
CSN2 A1;A2	0.971	0.029	0.972	0.0272	1.028	2.74

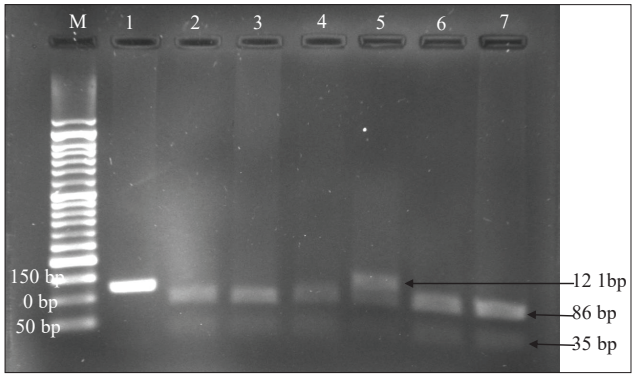

Fig.1. Representative image of 3.0% agarose gel for PCR-RFLP of *β-casein* (*CSN2*) gene by *Dde I.* M: Marker. Lane 2,3,4,6,7: A2A2 Genotype; Lane 5: A1A2 Genotype.

The level of possible variability realization (V%) was 2.74 which is relatively low, this suggests that only a small proportion of the genetic diversity is expressed in the population. The effective number of alleles (Ne) was 1.028, indicating some level of polymorphism in the population. The combination of a relatively low V% and a slightly higher Ne suggests that there might be some genetic diversity in the population, but not all potential variability is being realized.

The genotypic characterization of A1/A2 *β-casein* alleles in Punganur cattle has provided valuable insights into the genetic composition of this indigenous breed. The predominance of the A2A2 homozygous genotype in 97% of animals and the scarcity of the A1A2 heterozygous genotype in 03% of animals signify the preservation of the native germplasm and suggest limited crossbreeding with exotic breeds. The observed genotypic and allelic frequencies underscore the importance of conserving the unique genetic heritage of Punganur cattle to maintain the health benefits associated with A2 milk consumption. As consumer awareness grows regarding the potential health implications of A1 milk, the genetic integrity of indigenous cattle breeds gains even greater significance. Future research endeavours can further explore the implications of *β-casein* variants on milk quality, human health, and breeding strategies, ensuring the sustainable preservation of valuable genetic resources for generations to come.

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