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Frequencies evaluation of β-Casein gene polymorphisms and its association with milk yield, fat% and protein% in dairy cows reared in Madhya Pradesh, India

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Abstract
The present study was carried out for the determination of β-casein genetic variants (A1 and A2) in Gir and HF crossbred cows of Madhya Pradesh along with its association with milk production traits at β casein gene. The frequencies of A1A1, A1A2 and A2A2 genotypes were found to be 0.00, 0.00 and 1.00 in Gir breed of cows and 0.00, 0.60 and 0.40 in HF crossbred of cow, respectively. The respective gene frequency for A1 and A2 alleles were found to be 0.00 and 1.00 in Gir breed and 0.30 and 0.70 in HF crossbred of cow. Higher frequency of A2 allele was observed in Gir breed of cows as compared to HF crossbred of cows under the study. Chi - square values between observed and expected genotypic frequencies at this locus were found to be non-significant in Gir breed of cows, indicating that the populations is in Hardy-Weinberg Equilibrium for these group of animals, while Chi-square values was found to be significant for HF crossbred cows revealing that this populations is in Hardy-Weinberg disequilibrium. A1A2 genotype of HF crossbred showed significantly higher MY than A2A2 genotype among both breeds of cows. Significantly higher fat percent was recorded in A2A2 genotype of Gir as compared to HF crossbred cows. The mean protein per cent in the milk of Gir for A2A2 genotype was significantly higher than milk of both genotype (A1A2 &A2A2) of HF Crossbred.

Keywords: Casein gene, genotype, Gir, HF cross Breeds

Introduction
Bovine milk is composed of 87% water, 3.68% lipids, 3.51% proteins, 4.98% lactose, and 0.74% microelements, Jenness *et al.* (1979) [5]. The most important are caseins, which represent 82% of the total protein content, Tailford *et al.* (2003) [16] However, β-casein, which accounts for 36% of the total protein content, is also important for curd formation and determining the surface properties of micelles, which are useful features for cheese production, Pearse *et al.* (1986) [12] & Ng-Kway Hang (2006) [10] Dairy cattle have 12 β-casein variants (A1, A2, A3, B, C, D, E, F, H1, H2, I, and G. The most frequent variants are A1 and A2 where as the B variant is less common, Farrel *et al.* (2004) [2] whereas the E variant is detectable only in the Italian Piedmontese breed, Voglino *et al.* (1972) [19] and the F variant has been detected in the Emilia Romagna region (Northern Italy) at a very low frequency (0.006) Massella *et al.* (2017)The A2 variant is considered the oldest variant, from which the others originated via mutation. The difference between the A1 and A2 variants is due to a mutation in position 67, which causes an amino acid to change from histidine (in the A1, B, and C variants) to proline (in the A2, A3, E, and I variants). To summarize, the A1 and A3 variants originated from the A2 variant; subsequently, the I variant was derived from the A3 variant, and the B and C variants were derived from the A1 variant.

Material and Methods
The laboratory procedures *viz*., sample preservation, DNA extraction, polymerase chain reaction (PCR), quality and quantity check of DNA and restriction fragments length polymorphism (RFLP) were performed in the Department of Animal Genetics and Breeding and Central Laboratory, College of Veterinary Science and Animal Husbandry, Mhow.

Experimental animals
A total number of 100 animals were selected for the study 50 each of Gir and HF crossbred cows.

From 100 animals 06 lactating cows of Gir and 06 of HF crossbred were selected on the basis of presence of A1 and A2 variants of Beta Casein gene. The selected animals were used for trial of its milk on mice.

Collection of Blood
About 5 ml of blood was collected from jugular veins in EDTA coated vaccutainers (Akuret) from each animal included in the study. The collected blood samples were brought to the laboratory by maintaining the cold chain and stored at 4℃ till further processing.

DNA extraction
The extraction of genomic DNA from collected blood samples was done by John's method (John *et al*., 1991) [6] with minor modifications. The required solutions and reagents were prepared in the laboratory using molecular (Sigma) grade chemicals.

Required solutions and reagents
1.solution-1 –Prepared in the lab.
2. solution-2-–Prepared in the lab.
4. Phenol
5. Chloroform
6. Isoamyl alcohol
7. Sodium acetate (3M)
8. Isopropanol
9. Ethanol (70%)
10. Tris EDTA buffer

Procedure
1. 05 ml of blood was mixed with 5ml of solution-1 and 120µl of Nonidet P-40 to lyse the cells. It was mixed by inverting several times and centrifuged at 2000 rpm for 15 minutes at 24 ℃.
2. The supernatant was discarded and the pellet was gently resuspended in 800µl of solution-2 to lyse the nuclei and mixed well. Equal volume of saturated phenol (800µl) was added to the suspension and centrifuged at 11,000 rpm for 9 minutes at 4 ℃. Upper phase was transferred to the clean microcentrifuge tube and equal volume of saturated phenol: chloroform: isoamylalcohol (25:24:1) was added. 4.The tube was centrifuged at 11,000 rpm for 9 minutes, at 4 ℃ and the upper phase was transferred to another microcentrifuge.
3. 5. Equal volume of chloroform: isoamylalcohol (24:1) was added and centrifuged at 11,000 rpm for 9 minutes, at 4℃. The upper phase was transferred to another tube.
4. The DNA was precipitated by adding $1/10^{th}$ volume of sodium acetate (3M) (82.03, molecular weight of sodium acetate X 3 = 246.09 g in 1000 ml) and equal volume of isopropanol. After gentle mixing the tube was centrifuged at 11,000 rpm for 9 minutes, at 4 to 8℃ to form DNA pellet.
5. The isopropanol was carefully discarded and the pellet was washed 3 times with 70% ethanol and the pellet was air dried.
6. Pellet was resuspended in 100 µl of 0.3X TE buffer and was incubated at 65 ℃
7. for 1 hour in a dry bath. The sample obtained was stored at -20 ℃ till further use.

Assessment of quantity and quality DNA
The concentration, purity and quality of DNA were checked by UV spectrophotometer and agarose gel electrophoresis.

Spectrophotometry
The concentration and purity of DNA were checked by UV – spectrophotometer. Optical density (OD) value at 260 nm and 280 nm were measured using UV-spectrophotometer. DNA samples with an OD 260/280 ratio of 1.7 to 1.9 were considered relatively pure.

Agarose gel electrophoresis
Furthermore, the DNA samples were subjected to 0.8% agarose gel electrophoresis.

PCR- Restriction fragment length polymorphism (PCR-RFLP)
Template DNA
The samples with higher concentration of DNA were diluted to 30 ng/µl using nuclease free water (Sigma). Three microlitre (3 µl) of DNA was used as template for PCR reaction.

Primers
The primers used for the amplification of β-casein gene were selected on the basis of the previous reports (Miluchova *et al.*, 2013).
(F): 5´- CCT TCT TTC CAG GAT GAA CTCCAG G-3´
(R): 5´ - GAG TAA GAG GAG GGA TGT TTTGTG GGAGGC TCT- 3´

PCR reaction mixture
Amplification of the DNA samples extracted from the blood was done in a final volume of 25 µl reaction mixture in PCR tubes.

Setting of PCR reaction
The PCR tubes of 0.2 ml containing 25 µl reaction mixtures were kept in a preprogrammed thermo cycler (Applied Biosystems) and the standardized reaction program was set.

Agarose gel electrophoresis of PCR products
The PCR amplification was determined by 1.7% agarose gel electrophoresis. (1.7g) agarose in 0.5X TBE buffer (pH 8.0) was used. Agarose was melted in 0.5X TBE buffer.

Restriction digestion of the Polymerase chain reaction product
Restriction digestion of the PCR products was performed by using restriction enzyme (*DdeI)* which has its recognition site at G^AATTC.

Restriction endonuclease and its recognition site

S. No.	Restriction Endonuclease	Recognition site	Manufacturer
1.	*DdeI*	G^AATTC	Thermo Scientific, Lithuania

Determination of polymorphism by agarose gel electrophoresis
The polymorphism of β Casein gene was detected by 2% agarose gel electrophoresis.

Sequencing and analysis
Sequencing of amplicon will do for the confirmation of genotype of the cows. The sequences obtained from genotype

will align using Clustal W (Thompson *et al*., 1994) [18] and analysed by using MEGA 6 software (Tamura *et al.*, 2013) [17].

Milk collection and milk records

Information on each animal included in study such as identification number and lactation yield were recorded. About 50 ml milk was collected. The milk samples were brought to the laboratory maintaining cold chain and were processed for determination of various milk composition traits by automatic milk analyzer (Ultra mb).

Statistical Analysis:

Gene and genotype frequencies for different milk protein gene regions under study were estimated using Popgene 32(version1.32), Microsoft Windows-based freeware for population genetic analysis (Yeh *et al.*, 1999) [20]. The Chi-square test was use to test the populations of different breeds either in equilibrium at this locus or not.

Study the effect of various polymorphic variants of milk protein genes on milk yield, Fat % and Protein of the data were subjected to least squares analysis of variance employing linear model.

Testing Hardy-Weinberg (H-W) equilibrium:

For testing the population in Hardy-Weinberg (H-W) equilibrium 50 animals from each cattle breed were taken and chi-square test was performed for calculating expected genotype frequencies and comparing them with the observed ones.

Result and Discussion

1.Frequencies of gene and genotypes at β– casein gene locus: The frequencies of A1A1, A1A2 and A2A2 genotypes were found to be 0.00, 0.00 and 1.00 in Gir breed of cows and 0.00, 0.60 and 0.40 in HF crossbred of cow, respectively. Similar finding was reported by Sodhi *et al*., (2012) [15] in Holstein Friesian. The respective gene frequency for A1 and A2 alleles were found to be 0.00 and 1.00 in Gir breed and 0.30 and 0.70 in HF crossbred of cow. Higher frequency of A2 allele was observed in Gir breed of cows as compared to HF crossbred of cows under the study (Table No. 01). Pandey *et al.*(2018) [11] reported that the β- casein gene showed A2A2 and A1A2 genotypes were observed in Sahiwal and HF crossbred cattle. The genotypic frequencies of β-casein (CSN2)/ DdeI gene for A1A1, A1A2 and A2A2 are 0.00, 0.30 and 0.70 in Sahiwal and 0.00, 0.64 and 0.36 in HF crossbred cattle, respectively and the gene frequency A1 and A2 is 0.15 and 0.85 in Sahiwal and 0.32 and 0.68 in HF crossbred cattle. High frequency of A2 allele was observed in both the breeds of cattle under the study. Similar findings were reported by Hanusova *et al*., (2010) [3] in Slovakian bulls with frequencies of 0.20 and 0.80 for A1A1 and A1A2 genotypes, respectively while Malarmathi *et al*., (2014) [7] reported three genotypes *viz*, A2A2: 0.37, A1A1: 0.17 and A1A2: 0.46 in Kangayam and HF crossbred cattle. The pure Kangayam (*Bos indicus*) cattle breed had only A2 gene and showed only A2A2 genotype, which produce safer A2 milk for the human consumption. The Holstein Friesian crossbred animals also showed mostly of A2 gene with the frequency 0.59. Ramesha *et al.*, (2016) [13] reported that the frequency of A1 allele was very low in Malnad Gidda (0.01), Kasargod variety (0.04) and Jersey (0.08), while the frequency of A1 allele in Holstein Friesian and HF crossbred male was 0.17 and 0.29, respectively. Chi - square values between observed and expected genotypic frequencies at this locus were found to be non-significant in Gir breed of cows, indicating that the populations is in Hardy-Weinberg Equilibrium for these group of animals, while Chi-square values was found to be significant for HF crossbred cows revealing that this populations is in Hardy-Weinberg disequilibrium.

Table 1: Distribution of gene and genotype frequency of β-Casein gene variants of Gir and HF crossbred cows

Breeds	Genotype Frequencies			Chi- square (X^2) Value	Gene Frequencies	
	A1A1	A1A2	A2A2		A1	A2
Gir	0.00	0.00	1.00	0.00^{NS}	0.00	1.00
HF Crossbred	0.00	0.60	0.40	5.00*	0.30	0.70

NS- Non-significant, * significant (p<0.05)

1.1 Association of β-casein (CSN2)/ gene polymorphic variants with Milk yield and milk composition traits

The polymorphic variants of β-casein gene (CSN2)/ *Ddel* in different breeds of lactating cows and their association with milk yield per lactation (MY), Fat (%) and Protein (%),have been studied as below:

1.1.1 Milk yield (MY) of different variants at β-casein (CSN2) gene of Gir and HF crossbred cows

The results of analysis of variance have been presented in table no. 02. The effect of genotypes was found significant (P<0.01) for MY trait. The mean Milk Yield per lactation (MY) in Gir and HF crossbred cows has been presented in table no. 03.

Table 2: Least squares analysis of variance for milk yield in Gir and HF crossbred Cows

Source of Variance	DF	MS	F-Value
Genotypes	2	16470339.00	109.89**
Error	98	149879.00	
Total	100		

****** Highly significant (p<0.01)

Only A2A2 genotype were observed in all the animals of Gir breed but in HF crossbred both A1A1 and A1A2 genotypes were noticed. The mean MY (L) for A2A2 genotype of Gir was 1481.00±43.00, while the corresponding milk yield of A2A2 and A1A2 genotypes was 1793.00±132.00 and 2745.00±111.00 in HF crossbred cows respectively, as shown in Table no. 03. A1A2 genotype of HF crossbred showed significantly higher MY than A2A2 genotype among both breeds of cows. Table no. 03 shows Milk yield (MY) of different variants at β-casein (CSN2) gene in both breeds of cows.

Table 3: Means for Milk yield (MY) of different variants at β-casein (CSN2) gene of Gir and HF crossbred cows

Variants	Breeds	
	Gir	HF crossbred
A1A1	0.00±0.00	0.00±0.00
	(00)	(00)
A1A2	0.00±0.00	$2745.00^{a\pm}$111.00
	(00)	(30)
A2A2	1481.00^{b}±43.00	1793.00^{b}±132.00
	(50)	(20)
Overall	1481.00^{b}±43.00	2269^{a}±243.00
	(50)	(50)

Means bearing the different superscript differ significantly (p<0.05), Number of animals are depicted in parenthesis.

1.1.2 Fat (%) of different variants at β-casein (CSN2) gene of Gir and HF crossbred cows

The results of analysis of variance have been presented in (table no. 04). The effect of genotypes was found significant ($P< 0.01$) for Fat per cent trait. The means of fat per cent in Gir and HF crossbred cows has been presented in table no. 05.

Table 04: Least squares analysis of variance for Fat (%) at β-casein (CSN2) gene of Gir and HF Crossbred cows

Source of Variance	DF	MS	F-Value
Genotypes	2	7.967	7.93**
Error	98	1.004	
Total	100		

****** Highly significant ($p<0.01$)

The mean Fat per cent ranged from 1.86±0.12 (HF crossbred) to 3.03±0.94 (Gir). Significantly higher fat percent was recorded in A2A2 genotype of Gir as compared to HF crossbred cows. The highest fat per cent was noticed in Gir (3.03±0.94) for A2A2 genotype, whereas the lowest fat per cent was observed in HF crossbred for A2A2 genotype (Table no. 05).

Table 5: Means for Fat (%) of different variants at β-casein (CSN2) gene of Gir and HF Crossbred cows

Variants	Breeds	
	Gir	HF crossbred
A1A1	0.00±0.00	0.00±0.00
	(00)	(00)
A1A2	0.00±0.00	$2.19^{b\pm}$0.22
	(00)	(30)
A2A2	3.03^{a}±0.94	1.86^{b}±0.12
	(50)	(20)
Overall	3.03^{a}±0.94	2.02^{b}±0.17
	(50)	(50)

Means bearing the different superscript differ significantly ($p<0.05$),Number of animals are depicted in parenthesis.

Similar finding of highest Fat (%) in A2A2 genotype in Gir was reported by Shende *et al.,* (2017) [14], where indigenous cattle produced significantly more Fat compared to A1A2 and A1A1 genotype. Contrary to above findings Cardak (2005) [1] and Ikonen *et al*., (2001) [4] reported highest Fat (%) in A1A1 genotype.

1.1.3 Protein (%) of different variants at β-casein (CSN2) gene Gir and HF crossbred cows

Analysis of variance was performed on Protein % which is presented in table no. 06.

Table 6: Least square analysis of variance for protein (%) at β-casein (CSN2) gene of Gir and HF crossbred cows

Source of Variance	DF	MS	F-Value
Genotypes	2	0.9204	6.10**
Error	98	0.1508	
Total	100		

****** Highly significant ($p<0.01$)

The mean protein per cent in the milk of Gir for A2A2 genotype was 3.63±0.07. The mean protein per cent in A1A2 and A2A2 genotyped HF crossbred was found to be 3.50±0.06 and 3.57±0.08. There was a significant difference noticed in the mean protein per cent in the milk of Gir and HF crossbred of cows (Table no. 07).

Table 7: Means for Protein (%) of different variants at β-casein (CSN2) gene of Gir and HF crossbred cows

Variants	Breeds	
	Gir	HF crossbred
A1A1	0.00±0.00	0.00±0.00
	(00)	(00)
A1A2	0.00±0.00	3.50^{b}±0.06
	(00)	(30)
A2A2	3.63^{a}±0.07	3.57^{b}±0.08
	(50)	(20)
Overall	3.63^{b}±0.07	3.53^{a}±0.07
	(50)	(50)

Means bearing the different superscript differ significantly ($p<0.05$), Number of animals are depicted in parenthesis.

Conclusions

1. The amplification of PCR product of DNA samples of Gir and HF crossbred cow revealed 121 bp for beta β-casein gene and was visualized as a single compact band on agarose gel electrophoresis (2%) from the DNA samples of Gir and HF crossbred cows.
2. PCR-RFLP analysis revealed that the Gir cows have a single undivided band of 121 bp of A2A2 genotype whereas HF crossbred have three different bands of 121 bp 86 bp and 35 bp revealed that presence of two genotypes A1A2 and A2A2 and two alleles A1 and A2. The Gir cows showed only A2 allele and A2A2 genotype on the other hand HF crossbred cows showed both A1 and A2 alleles and A1A2 and A2A2 genotypes.
3. The frequencies of A1A1, A1A2 and A2A2 genotypes were found to be 0.00, 0.00 and 1.00 in Gir cows and 0.00, 0.60 and 0.40 in HF crossbred cow, respectively. The respective gene frequency for A1 and A2 alleles were found to be 0.00 and 1.00 in Gir and 0.30 and 0.70 in HF crossbred cows.
4. The population of Gir cows was found under Hardy-Weinberg equilibrium whereas the HF crossbred population was found under Hardy-Weinberg Disequilibrium.
5. Association study of genotype and milk production traits revealed that the A1A2 genotype of HF crossbred showed higher milk yield on other hand A2A2 genotype of Gir breed of cows showed that higher Fat % and Protein %.

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