

Original Research Article

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SNPs within Exon VIII of DGAT1 Gene in Gaolao Cattle

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ABSTRACT

Keywords

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DGAT1 gene is major candidate gene and K232A polymorphism revealed pronounced influence on fat percentage in cattle milk. It was planned to reveal polymorphisms within regions of DGAT1 gene in Gaolao cattle using PCR-RFLP and DNA Sequencing and to find association between polymorphisms and milk production traits. DNA was extracted from purebred Gaolao cattle. DGAT1G1-*Cfr*I locus in Exon-VIII region exhibited monomorphic pattern; however DGAT1G2-*Bgl*II locus was polymorphic with frequency for the K alleles as 0.9875 and for allele A as 0.0125 in Gaolao population. The animals exhibiting PCR-RFLP as AA (K) & GC (A) genotype were sequenced which revealed one SNP T-G at 231st position. DNA sequencing of HRM analysed DGAT1G3-212 bp amplicons revealed 03 polymorphic sites as computational SNPs at 52nd position as SNP G-A, at 51st position as SNP G -A and at 67th position as SNP A-G. DGAT1G4 amplified sequence revealed SNP, C-A at 23rd position. In various studies, the allele (K) encoding the lysine variant has proved to be associated with regard to the milk fat synthesis, and which can be a useful marker for milk fat traits in selection and breeding.

Introduction

The genetically uniform systems are vulnerable to extreme and adverse weather conditions, emerging diseases and pathogens. Livestock biodiversity is crucial for sustaining long term sustainable productivity (Frison *et al.*, 2010). Declining Gaolao cattle exhibits relatively low milk yield and better adaptability, disease-resistance and feed efficiency. To stop declining trend in breeding population of Gaolao Cattle; conservation and

genetic improvement is need of hour. The average milk yield per lactation is 604 Kg with average 4.32% Fat (Kargirwar, 2004). The breeding tract of Gaolao cattle is spread in Wardha and Nagpur districts of Maharashtra, Balaghat and Chindwara districts of Madhya Pradesh and Rajnandgaon district of Chhattisgarh.

Diacylglycerol O-Acyltransferase 1 (DGAT1) gene is a candidate gene (Kennedy *et al.*, 1957; Weiss *et al.*, 1956) and is a lipogenic

enzyme which plays key role in the synthesis of triacylglycerols. It is located on bovine chromosome 14 which is of size 14,117 bp consisting of 17 exons. The substitution named ApA to GpC dinucleotide is located in exon VIII of bovine DGAT1 gene which replaces lysine (K) by alanine (A) in encoded protein (K232A polymorphism) has pronounced influence on milk yield and composition, especially on fat percentage in milk (Grisart *et al.*, 2002; Winter *et al.*, 2002; Kaupé *et al.*, 2004; Lacorte *et al.*, 2006; Fangyu *et al.*, 2020). The DGAT1 lysine variant (K) is associated with increasing fat yield, fat and protein percentage and the alanine variant (A) is associated with increasing protein and milk yield (Bennewitz *et al.*, 2004; Patel *et al.*, 2009; Berry *et al.*, 2010).

A range of molecular markers, which reflect breed evolution, recent demographics and economic potential, needs to be applied in combination to provide optimal data for livestock management and conservation (Bruford *et al.*, 2003). The SNPs are genetically stable selection markers and abundant in genome used for genotyping and amenable to high-throughput automated analysis (Heaton *et al.*, 2002). Current advances in molecular genetic techniques has provided opportunity to reveal significant candidate gene variants in Gaolao cattle related with milking traits. In view of this current study was planned to study the polymorphisms within regions of DGAT1 gene in Gaolao cattle to detect SNPs within DGAT1 gene in Gaolao Cattle.

Materials and Methods

Experimental Animals and Data

The present study consisted of purebred Gaolao cows from Farmer's herds & Breeding Farm. The information sheet was

generated to record individual cow records as milk traits related phenotypic data. Milk components like Fat %, Protein %, SNF % & Lactose % were estimated using milk analyzer.

Blood collection & DNA extraction

Blood in the quantity of around 05 ml was collected from jugular vein in a vacutainer tube containing EDTA from 40 experimental cows. DNA extraction procedure was carried out using Blood DNA Isolation Kit (HiPurA SPP, Himedia) as per the manufacturer's guidelines. The quality and quantity of the genomic DNA was assessed using agarose and Nanodrop before its use in PCR amplification.

Primers and primer designing

The primers reported by other researchers were used for screening Gaolao cattle population at DGAT1 locus and some primers for other regions (exon VIII and intron I) of DGAT1 were designed (www.primer3.com) based on the reference sequence (AJ318490) (Table 1).

Polymerase Chain Reaction (PCR)

PCR reaction of 25 µl volume were carried out using 50ng of genomic DNA, 10 pM concentration for the primers DGAT1G1, DGAT1G3 and DGAT1G4 and 1^µM concentration for the primer DGAT1G2 (Forward and Reverse), 12.5ul of PCR 10X Go taq Master mix (Promega). The PCR thermal cycling conditions for DGATG1, DGAT1G3 and DGAT1G4 were set on thermal cycler (Himedia eco96) as below; initial denaturation for 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 61.5°C for 30 seconds, extension at 72°C for 30 seconds (35 cycles) and final

extension at 72°C for 10 minutes. However for DGAT1G2 thermal cycling conditions were as; initial denaturation for 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 58.5°C for 30 seconds, extension at 72°C for 30 seconds (30 cycles) and final extension at 72°C for 10 minutes. Four regions 411 bp of exon VIII, 378 bp of exon VIII, 212 bp of intron 1 and 198 bp of exon VIII of DGAT1 gene were amplified using DGAT1G1, DGAT1G2, DGAT1G3 & DGAT1G4 primers using 10 pM, 1µM, 10pM and 10 pM concentrations respectively. The PCR products of DGAT1 gene were resolved in 2.5% agarose gel electrophoresis at 50V for 45 minutes. The PCR amplified products were visualised for compact and sharp amplification with desired product size under Gel Documentation System.

PCR-RFLP

The restriction enzymes were used for digestion of the amplified PCR products (Table 2) using specific concentration and quantity at recognition site which needs to be polymorphic for variation in the population. The RE/ *Cfr*I treated PCR products were incubated for complete digestion at 37°C for 6 hours. The mixture of PCR Product and restriction enzyme/ *Bgl*II was kept for RE digestion at 37°C for 3 hours.

High resolution melting (HRM) analysis & protocol

HRM is a mutation scanning method (Wittwer *et al.*, 2003) used for analysis of genetic variation as well as mutation. HRM is based on thermodynamic differences between DNA fragments. The amplicons of 212 bp DGAT1 gene representing intron 1 part of gene obtained using DGAT1G3 primer were selected for HRM analyses. The following were the components required to be processed

for HRM analysis;

- a. Genomic DNA (50ng/µl) – 40 samples
- b. 212 bp PCR product obtained using DGAT1G3 primer
- c. Real time PCR and HRM

The PCR products were first carefully optimized to get sharp and precise amplification before its HRM analysis. The same products were again set at optimized conditions for HRM analysis as bellow;

96 well plate was taken & 40 wells were used, in each 20µl Master mix was added PCR was set at the following thermal cycling conditions; 95°C for 15 minutes, 94°C for 60 seconds, 61°C for 60 seconds, 40 cycles, 72°C for 60 seconds and 72°C for 10 minutes.

A melt curve graphs were generated based on slowly melting the DNA sample through a range of temperatures in the presence of a dsDNA-binding dye.

Direct DNA sequencing & Sequence analysis using Bioinformatics Tools

Based on PCR-RFLP and HRM analysis results, PCR products amplified were selected for confirmation of mutation by sequencing. The obtained DNA sequences were edited and analyzed using various bioinformatics tools for detection of nucleotide substitutions.

Statistical analysis

Genotype and allele frequencies were estimated using POPGENE Version 1.31 (Yeh and Boyle, 1997). The obtained gene & genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium (HWE) by using a Chi-square test (Devlin and Risch, 1995; Nielsen *et al.*, 1998). The relationship between the milk component and

genotype was tested using one-way ANOVA. The mathematical equation was given by,

$$Y_{ij} = \mu + \tau_i + \epsilon_{ij},$$

Where,

Y_{ij} = the milk component of i^{th} cow for the j^{th} genotype

μ = the average milk component

τ_i = average effect of the j^{th} genotype

ϵ_{ij} = error effect, All the analysis was done by SPSS Version 20 (IBM, USA).

Results and Discussion

PCR-RFLP DGAT1G1-*CfrI* locus

Restriction digestion analysis of 411 bp PCR product of the exon VIII region of DGAT1 gene with *CfrI* enzyme exhibited monomorphic pattern by showing only KK genotype pattern (Figure 1) in current experimental Gaolao cattle population. Similar with the findings in current study, indigenous cattle exhibited fixation of K allele was found in Ongole cattle (Krovvidi *et al.*, 2013) and Haryana Cattle. The predominance of average K allele frequency (0.915) in Gir and Kankrej indigenous zebu cattle as compared to A allele frequency (0.085) was found while genotyping at *CfrI*-RFLP locus (Patel *et al.*, 2017). Ganguly *et al.*, (2013) reported the frequency of A allele in Frieswal and Sahiwal cattle was 0.36 and 0.04, respectively.

However, in Tarurine cattle exhibited lower frequency of K allele & higher frequency of A allele. Krovvidi *et al.*, (2013) reported frequencies of K and A alleles as 0.74 and 0.26 in Jersey crossbred and 0.60 and 0.40 in Friesian crossbred cows for DGAT1/*CfrI* polymorphism. PCR-RFLP using *CfrI* restriction enzyme in 100 Iranian Holstein Cattle revealed KK, KA and AA genotype

frequencies as 0.31, 0.41 and 0.28, respectively. The allele frequencies of K and A alleles were estimated as 0.515 and 0.485, respectively (Ahani *et al.*, 2015). In Polish Holstein-Friesian bulls the frequencies of K and A alleles were 0.54 and 0.46, respectively at DGAT1-*CfrI* locus (Woszuk *et al.*, 2008).

PCR-RFLP DGAT1G2-*BgII* locus

Restriction digestion analysis of 378 bp PCR product of the Exon-VIII region of DGAT1 gene with *BgII* enzyme revealed two genotype patterns in studied Gaolao cattle population. The first pattern with two fragments of 282 and 96 bp was designated as AA (K) genotype and second pattern with three fragments was obtained (254,96&28 bp) was referred as GC (A) heterozygous genotype (Figure 2).

DGAT1G2-*BgII* locus was found polymorphic with genotype frequency for homozygous AA (K) genotype as 0.975 and for GC(A) genotype the frequency was found as 0.025. The allele frequency for the K alleles was found 0.9875 and for allele A was 0.0125 at DGAT1G2- *BgII* locus. (Table 3). The non significant ($P > 0.05$) Chi-square value for all the genotypes showed Hardy-Weinberg law in equilibrium. Higher K allele frequency was reported by various other researchers. Komisarek *et al.*, (2011) studied the effects of polymorphisms in *DGAT1* genes using *BgII* restriction enzyme to find out its relation with reproduction and production traits in 209 Jersey cows & reported that allelic frequencies for K and A alleles as 0.79 and 0.21 respectively at DGAT1-*BgII* locus. The average and standard error of Fat %, Protein %, SNF% & Lactose% exhibited non-significant effect of all genotypes. The Karl Pearson correlation analysis indicated the relationship between genotypes with milk components was non-significant ($P > 0.05$).

Table.1 Name, region, primer nucleotide sequence and restriction enzymes used to screen polymorphism in DGAT1 gene of Gaolao cattle

Sr. No.	Name of Primer & Region	Primer Sequence	Product Size (bp)	Reference/RE
1.	DGAT1G1 Exon VIII	FP 5'-GCACCATCCTCTTCCTCAAG-3' RP5'-GGAAGCGCTTTCGGATG-3'	411 bp	(Winter <i>et al.</i> , 2002) / <i>CfrI</i>
2.	DGAT1G2 Exon VIII	FP5'-TGCCGCTTGCTCGTAGCTTTGGCC-3' RP5'-ACCTGGAGCTGGGTGAGGAACAGC-3'	378 bp	(Komisarek <i>et al.</i> , 2011) / <i>BglI</i>
3.	DGAT1G3 Intron I	FP 5' -TGGCTTCTGCAGTGGACTC-3' RP 5' - ACAGGAGCTGGTGTATGCAA-3'	212 bp	Designed
4.	DGAT1G4 Exon VIII	FP5'-CGTAGCTTTGGCAGGTAAGG-3' RP 5' - AGTTGAGCTCGTAGCACAGG -3'	198 bp	Designed

Table.2 Restriction digestion reaction of DGAT1G1 and DGAT1G2 PCR amplified Products of DGAT1 gene in Gaolao Cattle

Sr. No.	RE Components	DGAT1G1- <i>CfrI</i>			DGAT1G2- <i>BglI</i>		
		Initial Concentration	Volume	Final concentration	Initial Concentration	Volume	Final concentration
1.	Cut smart Buffer	10X	2.5µl	1X	10X	2.5µl	1X
2.	Amplified PCR product	-	10µl	500ng	-	10µl	500ng
3.	Restriction Enzymes	5U/µl	0.4µl	2U	10U/µl	0.5µl	5U
4.	MBG water	-	Upto 25µl	-	-	Upto 25µl	-

Table.3 Allele and genotype frequency at DGAT1G2- *BglI* locus in Gaolao cattle

Name of Locus	Sample No. (N)	Allele frequencies		Genotype frequencies		
		K	A	KK	KA	AA
DGAT1G2- <i>BglI</i>	40 ^{NS}	0.9875	0.0125	0.975	0.025	0.000

NS- Non Significant at P > 0.05

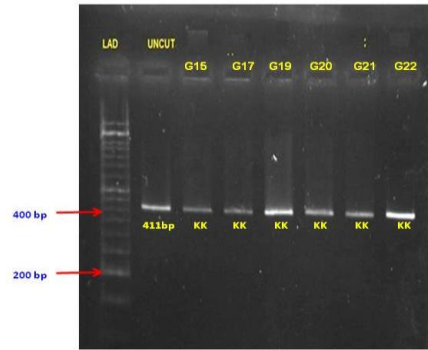


Figure 1: DGAT1 gene polymorphism (DGAT1G1-Cfr1) in Gaolao cattle using *Cfr1* restriction enzyme digestion of 411 bp PCR product (DGAT1G1) visualized in 3.0 % agarose gel electrophoresis
Where,
L = 100bp DNA Ladder, G15-22 = Gaolao Cattle
KK Genotype = 411 bp

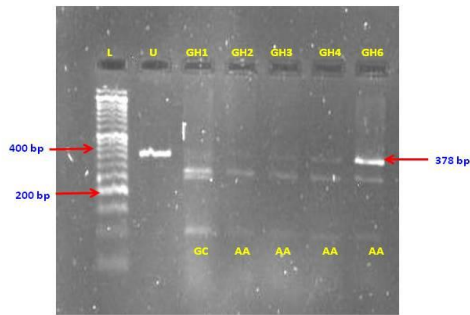


Figure 2: DGAT1 gene polymorphism (DGAT1G2-Bgl1) in Gaolao cattle using *Bgl1* restriction enzyme digestion of 378 bp PCR product (DGAT1G2) visualized in 2.5% agarose gel
Where,
L = 50 bp DNA Ladder
AA Genotype = 282 bp & 96 bp
GC Genotype = 254 bp, 96 & 28 bp

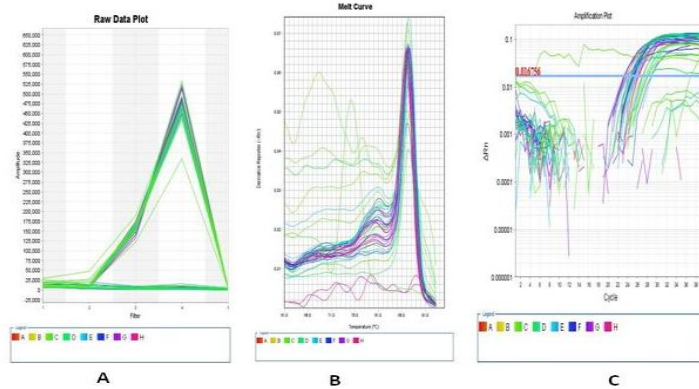
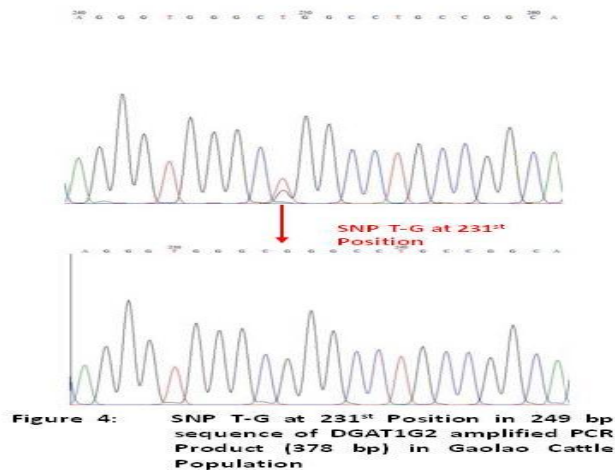


Figure 3: HRM Analysis (A) Raw data plot of HRM of DGAT1G3, (B) Melting Curve of HRM using DGAT1G3 amplicons & (C) Amplification plot



HRM analysis

The amplicons of 212 bp DGAT1 gene fragments of intron 1 were processed for HRM analyses in which the aligned plot didn't exhibit differences in their melt curve behaviour indicating the absence of variation (Figure 3). However, random samples were selected for sequencing to know variation sites in the region of gene in Gaolao Cattle.

DNA Sequence Analysis of polymorphic patterns at DGAT1G2- *Bgl*I Locus

The PCR products (378 bp) of the Exon-VIII region of DGAT1 gene exhibited polymorphism in PCR-RFLP analysis using *Bgl*I enzyme which revealed KK & KA genotypes approximately of 378 bp for KK & 282 bp, 254 bp and 96 bp for KA genotypes respectively. In view of detecting exact nucleotide change for the polymorphic restriction site, we selected 02 PCR products representing KK & KA genotypes for forward and reverse sequencing (04 reactions) with purification. These obtained sequences were first edited for getting consensus sequence for genotype KK & KA by removing misamplified sequences after validating them with their chromatographic peaks using

BioEdit software. The forward sequence and reverse primer sequence was aligned using T-Coffee, multiple sequence alignment programme to find out the regions of similarity as the sequence of the KK Genotype (GB-38) and for KA genotype (GH-1) DGAT1G2-*Bgl*I locus in Gaolao Cattle. The Final edited sequences for both the genotypes were aligned using ClustalW Omega tool which revealed one Transversion SNP T-G at 231st position in DGAT1G2 amplified PCR product query sequence of DGAT1 gene in Gaolao Cattle. The same SNP was validated using sequence chromatograms of two different genotypes (Figure 4). The sequence alignment of 249 bp DGAT1G2 amplified DNA sequence of DGAT1 gene of Gaolao cattle with *Bos taurus* reference sequence (AJ318490) revealed total of 02 SNPs viz; T-C at 217th position & G-T at 232nd position. In order to know the sequence homology of DGAT1G2 amplified 249 bp sequence of DGAT1 gene of Gaolao cattle with other species BLAST analysis was performed & developed BLAST tree view revealed sequence identity of 99.20 with European cattle, Yak and 98.80 with Buffalo, 97.99 with Sheep, 97.59 with Goat respectively.

DNA Sequence analysis of DGAT1G3 Amplified 212 bp DGAT1 Amplicon in Gaolao Cattle

The obtained edited DGAT1G3 amplified sequence of 219 bp size of Gaolao cattle was aligned with reference sequence (Accession No. AJ318490) using CLUSTAL alignment tool which revealed 03 polymorphic sites as computational SNPs i.e. SNP G -A at 51st position, at 52nd position as SNP G-A, and at 67th position as SNP A-G. In order to know the sequence homology of Gaolao DGAT1G3 amplified region of DGAT1 gene with other species BLAST analysis was performed & developed BLAST tree view which revealed sequence identity of 97.66%, 97.18%, 96.71%, 88.29%, 87.22% and 86.44% with *Bos indicus*, *Bos taurus*, *Bos mutus*, *Bubalus bubalis*, *Capra hircus* & *Ovis aries* respectively. The query sequence was closely related with *Bos indicus* and *Bos taurus* DGAT1 sequences as compared to *Bubalus bubalis*, *Capra hircus* and *Ovis aries* sequences.

DNA Sequence analysis of DGAT1G4 amplified 198 bp DGAT1 Amplicon in Gaolao Cattle

The obtained edited DGAT1G4 amplified sequence of 140 bp size of Gaolao cattle was aligned with reference sequence (Accession No. AJ318490) using CLUSTAL alignment tool which revealed 01 polymorphic sites as computational SNPs at 23rd position as SNP C-A. In order to know the sequence homology of Gaolao region gene with other species BLAST analysis was performed & developed BLAST tree view which revealed sequence identity of 99.28% with Zebu, European cattle, Buffalo, Yak and 98.56% with Goat, 97.84% with Camel and Sheep and Pig 90% respectively.

In conclusion, it is to state that restriction

digestion analysis at DGAT1-*CfrI* locus exhibited monomorphic pattern and DGAT1G2-*BgII* locus was found polymorphic pattern in experimental Gaolao cattle. DGAT1G2-*BgII* locus was found polymorphic with allele frequency for the K alleles as 0.9875 and for allele A as 0.0125. The result of analysis variance showed non-significant ($P>0.05$) effect of all genotypes on milk components which may be due to small sample size resulting in higher error variance. The DGAT1G2 amplified PCR products revealed one SNP T-G at 231st position in of AA (K) & GC (A) genotypes in Gaolao Cattle. The DGAT1G3 amplified sequence revealed 03 polymorphic sites as computational SNPs at 52nd position as SNP G-A, at 51st position as SNP G -A and at 67th position as SNP A-G. DGAT1G4 amplified sequence revealed 01 polymorphic sites as computational SNPs at 23rd position as SNP C-A. In present study, it was found that the allele K at DGAT1-*CfrI* locus was fixed in experimental Gaolao Cattle population. The polymorphisms at regions DGAT1 gene in Gaolao Cattle were revealed which after validation may aid in development of candidate gene markers for selection and genetic improvement of indigenous cattle.

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