



Genetic Association of *DGAT1* and *ESR1* Gene Polymorphism with Performance Traits in Murrah Buffaloes

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ABSTRACT

Background: Diacylglycerol O-acyltransferase 1 (*DGAT1*) gene plays a pivotal role in milk fat synthesis whereas Estrogen receptor gene (*ESR1*) gene helps in regulating reproductive performance of dairy animals. The present study was designed to identify genetic association of *DGAT1* and *ESR1* gene with performance traits in Murrah buffaloes.

Methods: Genomic DNA was isolated from 100 Murrah buffaloes maintained at Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. One set of primer for each gene has been custom designed and was amplified.

Result: The amplicon size of 517 bp and 514 bp was obtained for *DGAT1* and *ESR1* gene respectively. Upon custom sequencing, the *ESR1* gene was found to be monomorphic in the studied population; whereas, in *DGAT1* gene, three SNPs were detected as 290A>G, 306G>A and 317T>C. The genetic variant 306G>A among all, showed significant association with average fat% in Murrah buffaloes ($p<0.01$). The findings indicated that inclusion of association of *DGAT1* gene related information could be helpful for selection of dairy buffaloes in order to improve the fat yield after validating it in a very large buffalo population.

Key words: *DGAT1*, *ESR1*, Gene polymorphism, Murrah buffaloes, Performance traits.

INTRODUCTION

The buffalo is regarded as a vital livestock of the animal husbandry sector in India, because of its substantial role in national milk and meat production. Notably, India is responsible for contributing two-thirds of the world's buffalo milk (FAOSTAT, 2022). Since milk production and reproduction are two major traits deciding the economical worth of a buffalo, they have been a prime target for culling and selection decisions. The superiority of buffalo milk lies in its higher quality, containing twice the amount of fat and other components compared to cow milk (Liu *et al.*, 2020). In India, the pricing system for milk is based on its fat content, leading to buffalo milk commanding a higher price per volume. Milk fat not only enhances the nutritional value of milk and dairy products but also plays a crucial role in determining their flavour and physical characteristics (Kathiravan *et al.*, 2021). *DGAT1* has been observed to be expressed in various tissues, including the small intestine, liver, adipose tissue and mammary gland (Cases *et al.*, 1998). Due to their significant involvement in fat metabolism, *DGAT1* enzymes are considered to be one of the ideal candidate gene in animal production (Coleman *et al.*, 2000). The synthesis of triglycerides (TGs) is facilitated by enzymes known as *DGAT1*, which covalently links diacylglycerol with fatty acyl CoA which further forms triacyl glycerol and eventually gets store into the adipose tissues (Fig 1). The *DGAT1* gene has been observed on chromosomes 14, 15 and 9 in cattle, buffalo and goat respectively and reported its potential role in fat metabolism (Khan *et al.*, 2021). Therefore, the *DGAT1* gene has garnered substantial attention in recent past to explore its possible role and association with important performance

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traits in genome-wide studies conducted in buffaloes (Liu *et al.*, 2020). Molecular methods enable the identification of genetic variations or polymorphisms present among individuals/population (Saikia *et al.*, 2019). These polymorphisms aid in assessing differences in the specific traits, thereby influencing genetic determination of the performance traits (Jeevana Sai *et al.*, 2025).

The role of hormones and their respective receptors in the reproductive performance is noteworthy in buffaloes. Estrogen not only impact reproduction but also influence the growth, differentiation and functionality of various tissues, including the mammary gland, uterus, ovaries and testes (Rani *et al.*, 2016). Estrogen binds to its receptors, which fall under the nuclear receptors 'superfamily, consequently regulating gene expression. Due to its

involvement in reproduction, the estrogen receptor gene (*ESR1*) is considered as a potential candidate for exploring fertility traits in farm animals (Szatkowska *et al.*, 2011) and thus considered for present study to explore the variations exhibited by this gene. Therefore, the present study was executed to observe the influence of non-genetic factors on performance traits apart from the effect of SNPs wrt *DGAT1* and *ESR1* genes on the lactation traits in Murrah buffaloes.

MATERIALS AND METHODS

The present study was carried out on 100 Murrah buffaloes on the basis of lactation records maintained at Directorate of Livestock Farm (DLF), GADVASU, Ludhiana in order to study the polymorphisms in *DGAT1* and *ESR1* in Murrah buffaloes. Approximately 10 ml of blood was aseptically collected from each buffalo and transferred into sterile vacutainers containing 0.5% EDTA and stored at -20°C. Genomic DNA extraction from whole blood was performed using the Phenol-Chloroform-Isoamyl alcohol method (Sambrook and Russell, 2001). The quality and integrity of DNA was evaluated through agarose gel electrophoresis. The purity and concentration of the DNA were examined using Nanodrop One (UV-Spectrophotometer). The forward and reverse pair of primers for both the genes namely *DGAT1* and *ESR1* were designed based on the reference sequence of *Bubalus bubalis* available at NCBI, using

Primer 3 software. The map location of *DGAT1* gene is on chromosome number 15 in buffaloes, spanning over 8822 base pairs and consists of 17 and 16 exons and introns respectively and *ESR1* gene is located on chromosome 10 consisting of 9 exons and 8 introns in *Bubalus bubalis*. These primers were designed to amplify the complete coding region as well as exon-intron boundaries. Two sets of forward and reverse gene specific oligo-nucleotide primers for *DGAT1* and *ESR1* gene were synthesized from Barcode Biosciences Pvt. Ltd, Bangalore, India (Table 1).

The PCR reaction mixture was prepared using PCR Master Mix. The contents were thoroughly mixed by vortexing, followed by a spin. PCR amplification was carried out using a Thermal Cycler (Bio-Rad T100). The PCR protocol was followed which included the steps viz. initial denaturation and cyclic denaturation at 95°C for 2 minutes and 30 seconds respectively, annealing temperature for *DGAT1* and *ESR1* gene was 63°C and 61°C respectively for 15 seconds whereas for cyclic extension and final extension, the temperature was set up at 72°C for 15 and 45 seconds respectively. Each 0.2ml tube containing the PCR reaction mixture was placed in the thermal cycler for the amplification of the targeted region of both the genes. After PCR amplification, the agarose gel electrophoresis was done using 1.5% agarose gel and the power supply was set to 95V for 90 minutes. The gel visualized under UV transilluminator and photographed under G: Box gel

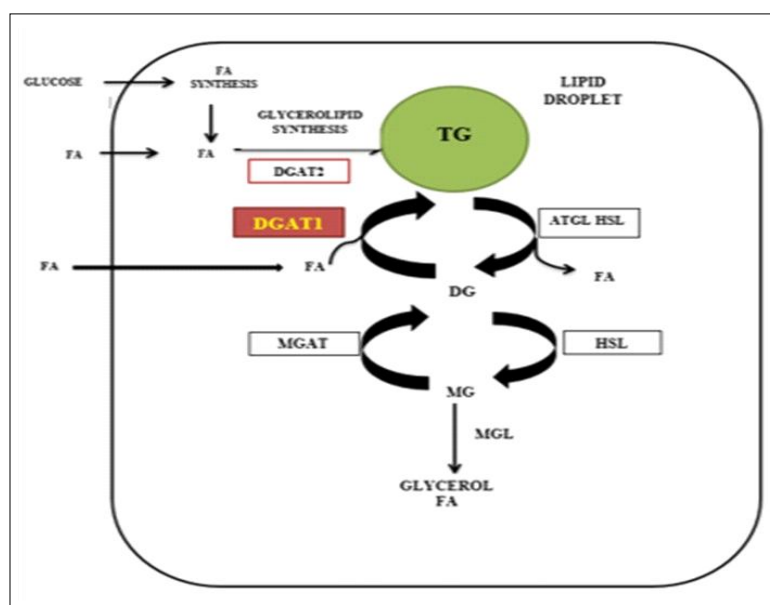


Fig 1: Mechanism of action of *DGAT1* gene in *Bubalus bubalis*.

Table 1: Primers used for *DGAT1* and *ESR1* gene amplification.

Primer	Primer sequence	T _m	GC%	Amplicon size
DGAT1-F	5'-AAAGCAGTCCAACACCCACG-3'	61.10	55.00	517 bp
DGAT1-R	5'-CTCCCCCGCAGACACTTCTA-3'	60.97	60.00	
ESR1-F	5'-ATTGTGTCCCACTATCTCTCCCA-3'	60.57	47.83	514 bp
ESR1-R	5'-AGGGCTGTTAGCATTAATAATGGTT-3'	59.75	36.00	

documentation system (Syngene). To purify and conduct custom sequencing from both ends (52 and 32), the amplified PCR products obtained from two sets of primers targeting the *DGAT1* and *ESR1* genes along with primer sets were sent to Barcode Biosciences Pvt. Ltd. (Bangalore). The chromatogram obtained after DNA sequencing were visualized using Chromas software and critically checked for any change in nucleotide position. The output sequences were aligned with corresponding reference sequences for both genes from NCBI using Clustal omega multiple sequence alignment software to identify the genetic variation.

The gene and genotypic frequencies were estimated and General Linear Model was used to estimate the effect of non-genetic factor (NGF) and SNPs on lactation traits in Murrah buffaloes. The non-genetic factors such as season of first calving was classified into five categories as Winter (1st Dec-31st Jan), Spring (1st Feb-15th Apr), Summer (16th Apr-30th Jun), Rainy (1st Jul-15th Sept), Autumn (16th Sept-30th Nov); the period of first calving was divided into three categories as POFC1 (2016-2018), POFC2 (2019-2021) and POFC3 (2022-2023) and the lactation number were categorised as 1, 2, 3 and ≥ 4 . The statistical model for association analysis of NGF and genetic factors with performance traits in Murrah buffaloes using Statistical Analysis System (SAS) is under:

$$Y_{ijklm} = m + S_i + P_j + L_k + SNP_l + e_{ijklm}$$

Where,

Y_{ijklm} = Observation of mth animal wrt lth SNP, kth lactation number, jth period of calving and ith season of calving.

μ = Overall mean.

S_i = Effect of season (1 to 5).

P_j = Effect of period (1 to 3).

L_k = Effect of lactation number (1 to 4).

SNP_l = Effect of genotype (1 to 3).

e_{ijklm} = Random error.

RESULTS AND DISCUSSION

PCR amplification and identification of SNPs

The genomic DNA from 100 Murrah buffaloes was isolated and primer were set on thermocycler for implication of target region of 517 bp (consisting part of intron 14, exon 15, intron 15, exon 16, intron 16, exon 17 and intron 17) and 514 bp (comprising partial intron 2, exon 3 and intron 3) of *DGAT1* and *ESR1* genes respectively; (Fig 2-3). The chromatograms were viewed using Chromas software suite and then aligned using Clustal Omega online tool to conduct comparative sequence analysis with *Bos taurus* and reference Buffalo sequence of both the genes having

accession number NC_059171.1 (*DGAT1*) and NC_059166.1 (*ESR1*). The aligned sequences showed transition at three positions i.e. 290A>G, 306G>A and 317C>T in *DGAT1* gene as shown in Table (2) and Fig (4-6). On comparing the corresponding region of *DGAT1* gene with *Bos taurus*, 15 inter-species nucleotide variations were identified in which 10 genetic variants were of transition type whereas 5 genetic variants were of transversion type. The targeted region of *ESR1* gene appeared to be monomorphic in the studied buffalo population; Therefore, no association studies was performed to determine the effect of genotypes on performance traits of Murrah buffaloes. However, 3 nucleotide variations

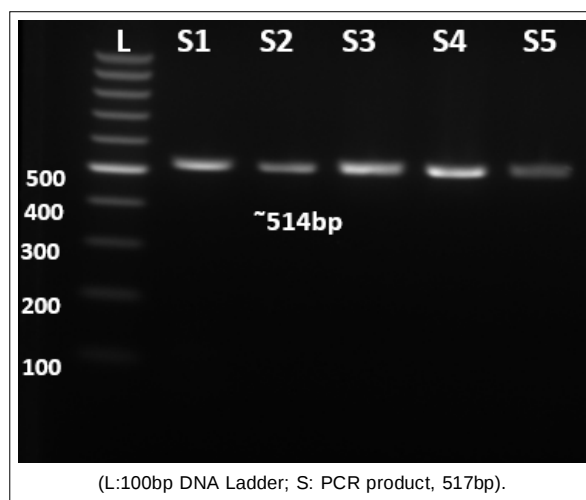


Fig 2: Agarose electrophoresis gel depicting PCR amplicon of *DGAT1* gene in Murrah buffalo.

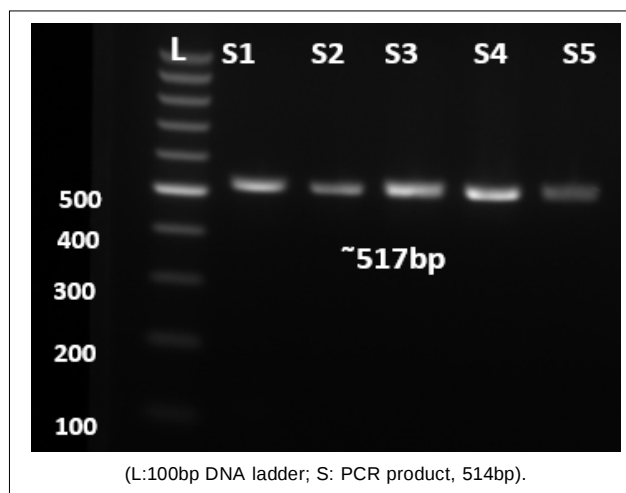


Fig 3: Agarose electrophoresis gel depicting PCR amplicon of *ESR1* gene in Murrah buffalo.

Table 2: Nucleotide changes in *DGAT1* gene of Murrah.

Position amplicon	Ref buffalo	Region	Murrah	Type of mutation
290	A	Intron 16	G	Transition
306	G	Intron 16	A	Transition
317	C	Intron 16	T	Transition

were observed upon comparison between *Bubalus bubalis* and *Bos taurus* reference sequence, in which, transition was found at A87G and C430T and transversion at T436G position.

Estimation of gene and genotypic frequencies

At locus 290A>G, the gene frequency of alleles A and G were estimated to be 0.698 and 0.301 with genotype frequencies for AA, AG and GG were found as 0.44, 0.52 and 0.04, respectively (Table 3). The observed genotypes for SNP 306G>A were AA, AG and GG with their gene frequency for A and G alleles as 0.45 and 0.55 respectively

and genotypic frequencies as 0.25, 0.40 and 0.35, respectively. The observed genotypes for SNP 317C>T were CC and CT with their gene frequencies for C and T allele as 0.98 and 0.02 respectively and genotypic frequencies as 0.95 and 0.05 respectively in the Murrah buffaloes; whereas, genotype TT was not observed in the screened population.

Association of SNPs with performance traits

The General Linear Model was used in SAS software to estimate the effect of non-genetic factors and genetic factors on production traits like milk fat% and milk production traits of 88 Murrah buffaloes up to their fourth

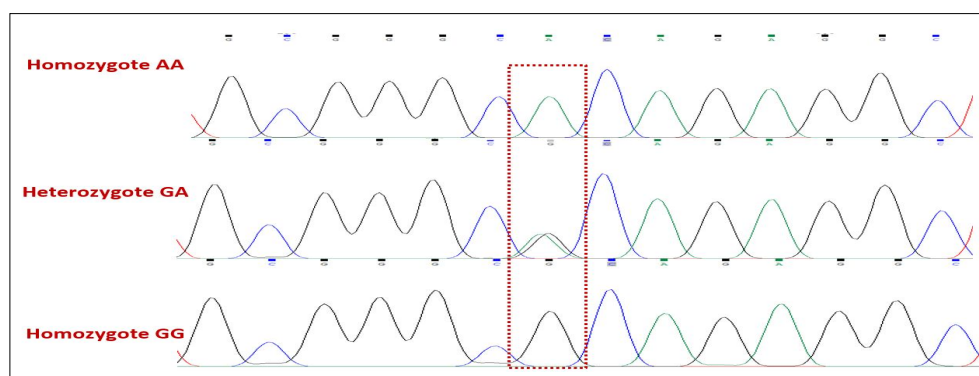


Fig 4: Chromatogram showing SNPs at position 290 A>G.

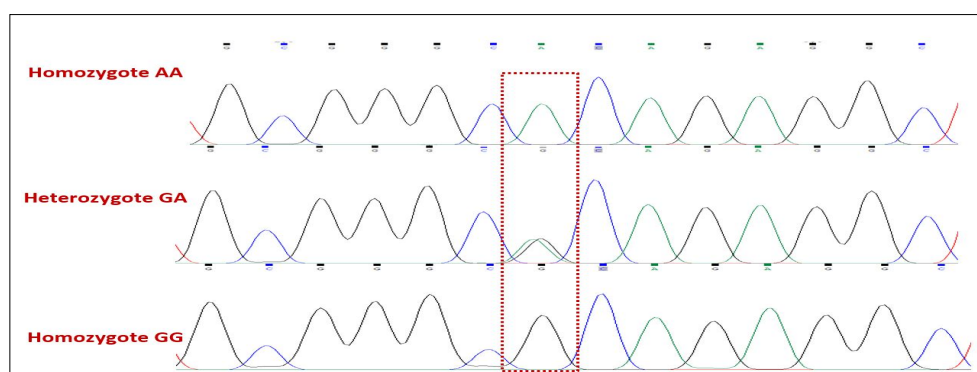


Fig 5: Chromatogram showing SNPs at position 306 G>A.

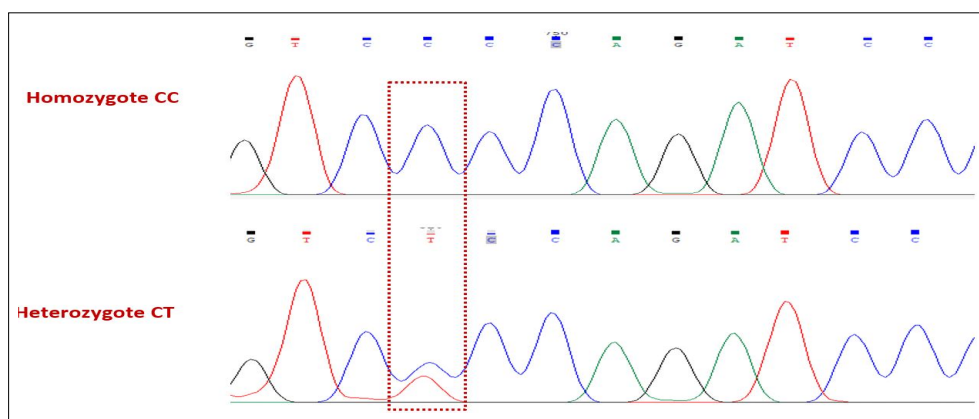


Fig 6: Chromatogram showing SNPs at position 317 T>C.

Table 3: Allelic and Genotypic frequency of *DGAT1* gene SNPs.

SNP	Allele	Allele frequency	Genotype	No. of individuals	Genotype frequency
A290G (N=88)	A	0.70	AA	39	0.44
	G	0.30	AG	45	0.52
			GG	4	0.04
G306A (N=83)	A	0.45	AA	21	0.25
	G	0.55	AG	33	0.40
			GG	29	0.35
C317T (N=88)	C	0.98	CC	84	0.95
	T	0.02	CT	4	0.05

Table 4: ANOVA for factors affecting lactation traits.

Variable	Effect	DF	F value	P value
Avg. Fat % (165)	SOFC	4	1.72	0.15
	POFC	2	4.76	0.01**
	LN	3	1.26	0.29
	A290G	2	2.81	0.06
	G306A	2	5.22	0.01**
305DMY (164)	SOFC	4	1.69	0.16
	POFC	2	7.99	<0.001**
	LN	3	0.84	0.48
	A290G	2	1.52	0.22
	G306A	2	0.52	0.60
LY (165)	SOFC	4	1.65	0.16
	POFC	2	10.06	<0.001**
	LN	3	0.63	0.60
	A290G	2	1.83	0.16
	G306A	2	0.77	0.47
PY (162)	SOFC	4	1.08	0.37
	POFC	2	2.97	0.05*
	LN	3	3.51	0.02*
	A290G	2	1.18	0.31
	G306A	2	0.57	0.57
LL (159)	SOFC	4	1.72	0.15
	POFC	2	8.72	<0.001**
	LN	3	2.78	0.04*
	A290G	2	1.07	0.34
	G306A	2	1.57	0.21

**Sign indicates 1% level of significance, * Sign indicates 5% level of significance.

lactation records. The overall least squares mean for milk production traits viz average fat%, 305 Days Milk Yield (305DMY), Lactation Yield (LY), Peak Yield (PY), Lactation Length (LL) were found as 7.697±0.055%, 2158.4±56.022 kg, 2198.32±59.284 kg, 13.302±0.248 kg and 276.497±5.277 days, respectively as represented in the Table (5).

Analysis of variance (ANOVA) of NGF on performance traits

The ANOVA for non-genetic factors on various lactation traits (Table 4) revealed that only period of first calving had significant effect on all lactation traits ($p \leq 0.01$) under study viz. average fat%, 305DMY, LY, PY and LL. The average

fat% was found to be higher in the period between 2022 and 2023 as 8.002±0.211% and lower estimate was in the duration from 2019 to 2021 as 7.542±0.186%; however, the fat percentage in both periods were not significantly different from the first period i.e. 2016-2018 (7.557±0.155). It might be due to progression in the nutritional interventions during the recent period in addition to the advanced managemental practices. In the current study, period of first calving significantly affected other lactation traits viz 305DMY, LY, PY and LL in which higher estimate was found during the period 2016-2018 and lower during 2022-2023 ($p \leq 0.01$); however some traits showed non-significant effects with other periods. The lactation number had significant effect on PY and LL ($p \leq 0.05$) and the maximum PY was obtained during fourth parity and minimum during first parity; whereas, LL was found highest during second parity and lowest during first parity with no significant differences among the classes for both the traits (Table 5).

ANOVA of SNPs on performance traits

The effect of SNP G306A was found to be significantly associated with average fat percentage ($p \leq 0.01$) in Murrah buffaloes. The average fat percentage was found to be higher in GG genotype as 7.974±0.164% and lower in GA genotype as 7.493±0.181%; and both of them were not significantly different from the AA genotype (Table 5). Other lactation traits were found to be non-significantly associated with any of the genotype at the same locus. Also, there was non-significant effect of genetic variant A290G on the lactation traits observed in the studied population. The SNP C317T was not considered for association analysis due to very low gene frequency of T allele as presented in Table (3).

In the present study, results revealed clear existence of 3 SNPs in *DGAT1* gene at A290G, G306A and T317C and it was also found that G306A was significantly associated with average fat%. Similar to our findings, Isik *et al.*, (2021) conducted polymorphism study in *DGAT1* in 150 Anatolian water buffaloes in which mutations were detected as G155C and C275T in the 422 bp amplified sequence containing partial regions of 7,8 and 9 exons. Also, Naserkheil *et al.*, (2019) performed gene polymorphism studies of *DGAT1* gene on 200 Iranian buffaloes by DNA sequencing and identified nine nucleotide variants at various positions. In line with our studies, de Freitas *et al.*, (2016) detected three

SNPs at 11644C>T, 11783G>T and 11785T>C positions in a study conducted on 196 Murrah buffaloes and it was also found that SNP at 11785T>C, located at exon 17, was significantly affecting milk fat and milk protein. The genotype TC had maximum fat% as 7.13% and genotype TT had minimum fat% as 6.72%; while, protein% was reported for TT genotype as 4.45% and CC genotype as 4.24% in buffaloes at 5% level of significance. Contrary to the present findings, Silva *et al.*, (2016) found no significant impact of *DGAT1* gene on milk production traits in Murrah buffaloes; however, four nucleotide variants were observed at different positions. Yuan *et al.*, (2013) investigated gene polymorphism studies of *DGAT1* gene in 118 Chinese buffaloes and observed seven polymorphic positions at 7299T>C, 7313C>T, 7474G>A, 10839T>C, 11192G>A, 11573G>A and 11785C>T and observed amino acid substitution (valine to alanine) at position 484 from SNP found at 11785C>T. However, no association studies were conducted to analyse the effect of genotypes on the performance traits. Ozdil and Ilhan (2012) conducted similar studies to detect gene polymorphism in exon 8 region of Anatolian buffaloes and found three SNPs at 43T>C, 154G>A and 373G>C positions. Several studies have also been performed in cattle such as Couto *et al.*, (2022) studied genetic polymorphism for *DGAT1*, *CAPN-9*, *CAPN-14* and *Leptin* gene in 95 Nellore cattle which revealed four nucleotide variations at 11730A>T, 11809G>C, 11858A>G, 11927G>A that altered the amino acids too and influenced the meat tenderness quality of beef cattle. Meng *et al.*, (2013) conducted similar studies in Dehong buffaloes to detect polymorphism in exon 17 region of *DGAT1* gene

and detected SNP at 1350C>G in exon 17 however it was not significantly affecting milk constituents.

Li *et al.*, (2018) performed genetic association studies in coding region of *DGAT1* gene in 971 buffaloes which revealed a non-synonymous mutation (SNP 9046T>C) from arginine to histidine in exon17 region. The reported SNP significantly affected the milk fat% and TT genotype had maximum fat% (8.26±0.04%) and CC genotype had minimum fat% (8.13±0.04%). There was also significant effect by SNP 8330T>C on peak milk yield, milk yield and milk protein% in which highest milk yield was obtained by CT genotype (2869.39±40.67 Kg) whereas lowest yield by TT genotype (2413.33±86.90 Kg). Gothwal *et al.* (2023) conducted genetic association of *DGAT1* gene mutations in indigenous cattle with their production and reproduction traits and revealed significant association of K232A located at exon 8 with milk production traits like total milk yield, 300 days milk yield. Mishra *et al.* (2007) conducted a comparative study of *DGAT1* gene polymorphism in Chinese buffaloes and Indian buffaloes and reported 19 novel SNPs among Indian buffaloes and also concluded that genetic sequences of *DGAT1* gene of Indian buffaloes were closely linked with Chinese buffaloes. Pareek *et al.*, (2005) also found similar observations in Polish black and white cattle in which significant effect of *DGAT1* (K232A) on milk fat and milk protein were detected.

In the present investigation, *ESR1* was found to be monomorphic in the studied population. In line with our results, Fouda *et al.* (2021) conducted study on 243 Egyptian water buffaloes targeting the promoter region of *ESR1* gene and observed monomorphic pattern in the studied

Table 5: Least squares means (±SE) for performance traits in Murrah buffaloes.

Factor	Categories	Avg. Fat (%)	305DMY (Kg)	LY (Kg)	PY (Kg)	LL (Days)
	Overall mean	7.697±0.055	2158.400±56.022	2198.320±59.284	13.302±0.248	276.497±5.277
SOFC	Autumn	7.61±0.203	2483.52±193.757	2542.89±203.466	13.94±0.892	300.46±18.082
	Rainy	7.58±0.204	2242.12±193.853	2282.84±203.561	13.87±0.902	283.97±18.127
	Spring	7.80±0.182	2057.42±173.238	2099.56±181.700	12.50±0.799	263.67±15.950
	Summer	7.55±0.216	2375.14±210.273	2412.77±216.280	13.64±0.988	297.55±19.867
	Winter	7.97±0.173	2327.60±164.989	2383.54±173.176	13.14±0.760	295.56±15.152
POFC	2016-2018	7.557 ^{ab} ±0.155	2730.622 ^a ±148.201	2855.482 ^a ±154.918	14.641 ^a ±0.686	330.027 ^a ±13.743
	2019-2021	7.542 ^b ±0.186	2193.287 ^b ±178.028	2221.524 ^b ±186.019	13.112 ^a ±0.822	278.917 ^b ±16.449
	2022-2023	8.002 ^a ±0.211	1967.569 ^b ±201.069	1955.953 ^b ±210.935	12.505 ^b ±0.930	255.776 ^b ±18.793
LN	L1	7.52±0.16	2207.11±148.11	2316.84 ^a ±155.59	11.98±0.69	313.58±13.88
	L2	7.71±0.18	2411.17±169.6	2466.83 ^b ±178.16	13.62±0.78	293.50±15.63
	L3	7.82±0.2	2240.36±195.8	2235.87 ^{ab} ±202.03	13.63±0.9	272.33±18.05
	L4	7.76±0.27	2329.99±255.64	2357.74 ^{ab} ±268.15	14.45±1.18	273.55±23.56
A290G	AA (39)	8.05±0.157	2057.24±150.793	2070.07±157.355	12.75±0.699	270.19±13.916
	AG (45)	7.75±0.129	2216.79±123.386	2241.93±129.089	13.85±0.571	280.44±11.479
	GG (04)	7.30±0.332	2617.45±316.068	2720.97±331.687	13.66±1.458	314.08±29.135
G306A	AA (21)	7.634 ^{ab} ±0.217	2420.06±208.157	2500.10±216.806	14.00±0.974	308.61±19.719
	GA (33)	7.493 ^b ±0.181	2257.27±172.679	2295.19±181.318	13.08±0.795	278.56±15.888
	GG (29)	7.974 ^a ±0.164	2214.15±156.912	2237.67±164.456	13.18±0.725	277.55±14.416

Values with different superscripts within each trait and factor differ significantly ($p \leq 0.05$).

population. Kathiravan *et al.* (2017) also revealed similar monomorphic results in a study on 204 Murrah buffaloes for detection of SNPs in *ESR1* gene in exon 13 region. Sarla *et al.* (2015) investigated genetic variation in the *ESR1* gene in buffaloes with the help of PCR-RFLP technique and found 870bp *ESR1* gene had monomorphic pattern in the studied population. Contrary to our results, polymorphism had been observed in various studies such as Moravcikova *et al.* (2015) performed a study on 100 Holstein cattle and found two SNPs at 5' non-coding region. Mohamadnejad *et al.* (2015) performed a study to detect genetic polymorphism at 5' flanking region of *ESR1* gene in Iranian cattle and found SNP at 1213G>A position with gene frequencies ranging from 0.55 to 0.96 (A allele) and 0.04 to 0.20 (G allele). Also, Szreder *et al.*, (2007) conducted similar studies in Black and White Polish cattle and found transition at 1213A>G at 5' non-coding region of *ESR1* gene. Similarly, Othman and Abdel-samad (2013) detected gene polymorphism *ESR1* genes in 100 Egyptian buffaloes and they found three SNPs in *ESR1* gene as 89G>A, 200A>G and 201A>G. Also, Zahmatkesh *et al.* (2011) conducted a study in 200 Holstein Friesian cattle to detect genetic association of *ESR1* gene with its performance traits and found that SNP 1213G>A possessed significant effect on average milk fat % at 5% level of significance whereas non-significant effect on milk yield, milk protein and SNF.

CONCLUSION

The present study revealed that *DGAT1* gene exhibited three polymorphic sites at A290G, G306A, T317C and significant association of G306A was observed on average fat% in the studied population. Therefore, interventions of molecular techniques can be utilised for detection of effect of genetic and non-genetic factors on the performance traits of the dairy animals thereby increasing the frequency of the favourable genes to augment productivity. However, further validation of *ESR1* gene for their association with performance traits and functional traits is required to bridge the gap in research for better selection of dairy animals along with breeding improvement and also for longevity of the herd.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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