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Identification of single nucleotide variations in the AGPAT6 gene and its association with milk production traits in Murrah buffalo

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ABSTRACT

The present study investigated the association of genetic variations within the 5′UTR exons 1-1511, 3′UTR exons 1512-1723 of the sn-1-acylglycerol-3-phosphate-O-acyltransferase 6 (AGPAT6) gene of buffalo with milk production traits, using the PCR-SSCP and Sanger sequencing method. The study revealed two unique conformational patterns: '1' and '2' in 100 Murrah buffaloes. Ten primer pairs were used for amplification of the AGPAT6 gene and in total nine single nucleotide polymorphisms (SNPs) were identified: *viz*., two in Exon 4 and seven in Exon8. The frequency of SSCP pattern 1 was found to be consistently high in the buffalo population under study. The preliminary results showed substantial variations in the distribution of SSCP variant frequencies of pattern 1 and 2 within Murrah buffaloes. However, these variants of exon4 and exon 8 had non-significant association with milk yield, whereas the same variants in terms of SNPs in the coding region were found to have a significant ($P \leq 0.01$) association with fat percentage. This suggests that AGPAT6 affects the milk fat percentage, which is an economically important trait, and that further study of this gene is warranted in Murrah buffaloes. The polymorphisms observed can be used as molecular markers to assist selection.

Key words: AGPAT6 gene; allele substitution effect; Murrah buffalo; milk fat content; SNP; (PCR-SSCP)

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Introduction

India is the largest producer of milk in the world with 221.06 million tons of total milk production during 2022 (DAHD, 2022). Buffaloes comprise about half of the cattle population (109.85 million), but contribute around 49.2% whereas indigenous cattle contribute around 20% of the total milk production in the country (BAHS, 2022). The Murrah buffalo is the most popular and well established dairy breed of buffalo in the world, and has high milk producing ability with a high fat percentage and growth potential. Murrah buffaloes produce around 2000 kg of milk in a lactation of 305 days. This breed is being used for upgrading local buffaloes in many parts of Asia and other parts of the World. Milk fat is considered to be a major determinant of milk quality, in addition to consumer demand for milk. Moreover, in India, buffalo milk, being rich in fat and for its unique taste, is preferred to cow milk.

The gene sn-1-acylglycerol-3-phosphate-O-acyltransferase 6 (AGPAT6) is a 48-kDa protein having 456 amino acids in length, and plays an important role in the catalytic biosynthesis of glycerol lipids. There are nine known members of the AGPAT gene family (AGPAT1- AGPAT9) which are deferentially expressed in different tissues. AGPAT6 has been mapped to BTA 27 in a location (27:36522605-36539773) that is rich in quantitative trait loci (QTL) for milk production traits. This suggests it may be a candidate gene or marker for milk production. AGPAT6 is a microsomal enzyme that catalyses the final step of triglyceride synthesis, and has been annotated for its impending effects for performance in terms of milk yield and fat content in bovines (AGARWAL et al., 2006; CHEN et al., 2008). It has been found that AGPAT6 appears to be the most abundant AGPAT isoform in the mammary glands, with its expression strongly upregulated during lactation in cows, and it regulates the mechanism of milk fat synthesis of the bovine mammary glands (BEIGNEUX et al., 2006; BIONAZ and LOOR, 2008a). Most of these research findings in relation to AGPAT6, also known as the LPAAT-zeeta gene polymorphism, and association with milk composition traits, showed that they had a significant effect on milk production traits (BIONAZ and LOOR, 2008b; LITTLEJOHN et al., 2014).

AGPAT6 has been implicated in lipid metabolism, the expression of fatty acid-responsive genes and the maintenance of cell membrane fatty acid levels (LI et al., 2003; COLEMAN and LEE, 2004). Various effects of AGPAT6 on milk production and quality have recently been described. AGPAT6 influenced the milk fat synthesis, along with SLC27A6, ACSL1, FABP3 and LPIN1, and it was suggested that AGPAT6, along with DGAT, is one of the most important genes in bovine mammary glands that in co-ordination regulate the channeling of fatty acids toward copious milk fat synthesis (BIONAZ and LOOR, 2008a; 2008b; UPADHYAY et al., 2019). AGPAT6 variants have been mapped to the 5' UTR exons and intron 1 was found (LITTLEJOHN et al., 2014) to be significantly associated with high milk fat genotypes, and also additively associated with higher expression of the AGPAT6 gene but no differences in milk yield that would be suitable for polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis. AGPAT6 is comprised of 12 coding exons separated by 12 introns. Variations in bovine AGPAT6 have been investigated in both dairy cattle and buffaloes (SONG et al., 2013; LITTLEJOHN et al., 2014; VIALE et al., 2017; NANAEI et al., 2020). The purpose of this investigation was to guage the advantage of the PCR-SSCP technique in genotyping the variable region of AGPAT6 in a herd of 100 lactating Murrah buffaloes, in an Indian outdoor grass-fed dairy system, and to investigate whether there is any association between variations in the AGAPT6 gene and major milk production traits.

Materials and methods

All research involving animals was carried out in accordance with the guidelines of the Institute's Animal Ethics Committee (IAEC) of ICAR-NDRI, Karnal, India.

Experimental Animals and DNA samples. Genetic variations in *Bubalus bubalis* AGPAT6 were investigated in 100 Murrah cows with known

pedigree and data, available for a period of ten years (2003 to 2014), collected from the Murrah buffalo herd maintained at Buffalo Research Station, Venkataramannagudem, Sri Venkateswara Veterinary University (SVVU), Tirupati, Andhra Pradesh. Records, such as sire, dam, season of calving, year of calving, 305 days milk yield, total milk yield, 305 days fat yield, and total fat yield were also collected. The buffaloes were between three to nine years old (*i.e.* 2nd to 8th parity) and grazed during their lactation on mixed ryegrass/ white clover pasture. All buffaloes were maintained under standard management conditions, adhering to the Minimum Standard Protocols recommended by the Department of Dairying, Animal Husbandry and Fisheries, Government of India.

Milk sampling and phenotype measurement. All the cows were milked twice daily and the daily milk yield was recorded in kilograms wet weight using Lacto Star apparatus (Funke-Gerber, Germany). About 30 ml of milk samples were collected to estimate the fat percentage by Gerber's method. For calibration of the Lacto Star apparatus, the fat percentage of the milk was tested by the Gerber method (BIS, 1977).

Blood collection and isolation of DNA About ten milliliters of venous blood from non-consanguineous individuals was collected aseptically from the jugular vein in a vacutainer tube containing 0.5% ethylene diamine tetra acetic acid (EDTA). After collection, the samples were brought back to the laboratory under low-temperature conditions and stored at 4°C. Genomic DNA was extracted from the blood within 24 hours of collection by the high salt method (MILLER et al., 1988). A working solution of DNA was prepared by diluting the stock to 100 ng/µL for use as the DNA template in the polymerase chain reaction (PCR).

Primer design and polymerase chain reaction amplification of the AGPAT6 gene. To screen polymorphisms in the AGPAT6 gene from 5′UTR exons 1-1511 to 3′UTR exons 1512-1723 of buffalo, ten sets of oligonucleotide primers were designed (Table 1) from the *Bos taurus* AGPAT6 gene sequence AC_000184.1(63198042..36229006) using NCBI primer BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast), procured from Xcelris Lab Ltd., Bengaluru, India. PCR was performed in a reaction volume of 25 µl containing approximately 100 ng of genomic DNA, 20 pM of each primer, 200 µM of dNTPs (Bioron GmBH, Germany), one unit of Taq DNA polymerase (Bangalore Genei, India) and 2.5 µl of 10X Pol assay reaction buffer, containing 1.5 mM $MgCl_2$ and supplied with the enzyme. buffer. In the process of PCR amplification, the annealing temperature (T^A) was optimised for each primer sequence. The thermocycler (Bio-Rad, USA) conditions included: an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, with varying annealing temperatures based on the primer set (Table 1), extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR amplification was verified by electrophoresis of the PCR products (5 µl) at 100 V in 1.5% (w/v) agarose gel using 1 µl of loading dye (95% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol) as a stop dye, electrophoresed in 1×tris borate EDTA (TBE) (Sigma Aldrich, USA) buffer containing 0.5 µg/mL ethidium bromide (Sigma Aldrich, USA) along with a 100 bp DNA ladder (Sigma Aldrich, USA) as a marker for confirmation of the length of the PCR products. The gels were visualized and documented using UV light in the gel documentation system after ethidium bromide staining (Gel doc 1000, BioRad, Hercules, CA, USA).

Single-strand conformation analysis and sequencing. Amplified PCR products (10 µL) were resolved by running in 10% native PAGE gel [10% acry1amide: bis-acrylamide (29:l, Sigma Aldrich, USA)] using 10 µL denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA Sigma Aldrich, USA) and heat denatured at 95°C for ten minutes. After denaturation, the PCR products were immediately transferred to a chilled ice pack and kept at -20°C for 10 minutes. Vertical gel electrophoresis was carried out in a Bio-Rad Protean® II Xi Cell electrophoresis unit, using 1X TBE (Sigma Aldrich, USA) buffer at 200 V for 12 h at 4°C. Gels were silver-stained (SAMBROOK and RUSSELL, 2001) and photographed using a digital camera for SSCP pattern analysis. Band

patterns were characterized by the number of bands and mobility shifts, and each pattern was scored manually for recording SSCP variants. DNA samples showing different patterns on SSCP gels were selected and sent to Eurofins Pvt. Ltd., Bengaluru for sequencing in both directions. Two representative DNA samples for each SSCP variant were subjected to custom sequencing using an automated ABI DNA Sequencer 3730 XL (Thermo Fisher Scientific, Meridian Road, Rockford, IL, USA) for detection of single nucleotide polymorphisms (SNPs). Multiple sequence alignments were performed with a DNA Baser (Heracle BioSof SRL, Pitesti, Romania) and Clustal W multiple sequence alignment software (developed by Des Higgins) for detecting SNPs (HALL, 1999) by comparing the observed sequence of the AGPAT6 gene in Murrah buffaloes with the bovine AGPAT6 gene reference sequence (Ensemble Ref Seq:ENSBTAG00000005730). Differences between PCR products, as well as between PCR products and reference sequences available in the GeneBank, were classified as experimental and computational SNPs, respectively.

Table 1. Details of primer sequences (5'to 3' sequences) used for amplification: Primers sequence, annealing temperature and size of the amplified fragments of sn-1-acylglycerol-3-phosphate-O-acyltransferase 6 (AGPAT6) gene in buffalo, base pairs (bp)

Exon	Sequence	Amplicon length (bp)	Annealing Temperature (ºC)
Exon 1	F- CGAACTTGGGAATGGAGTTG R- GTCCCTCTGAGAGCTCACAATC	384bp	54.3
Exon 2	F- CTGTCACAGACTGGAAGTGGAG R- GCAACAGAACAGTCTCATCAGG	275bp	55.8
Exon 3	F-CCTGATGAGACTGTTCTGTTGC R- ACCACCCTCCTCTGAAGTGAG	372bp	55.5
Exon 4	F- ACAGGATAGCTCTCGCTTTCAC R- ATAAGATCAGTGAGGGGGAACA	221bp	54
Exons 5 and 6	F- GTGCGTGTAGGTAACAGCAGAG R- CAGGTCTGAAAGCACACTTGC	449bp	58
Exon7	F- TGGTTACCAGTAAAGCGTAGGG R- CGACTTGCCCTAAAATCAGACT	256bp	54
Exon8	F- ACAGATGTATGGATGGTGATGG R- ACTCGCAGTCCACACTGAGTC	263bp	55
Exon9	F- AAGAGCTGTCAGAGGGCTTTTA R- AGAGGAACACGTGGGGAGAT	304bp	53.4
Exons 10 and 11	F- GCACACACTGACACACACTCTT R- AGCAACCGCCTCAGAAATC	419bp	52.9
Exon 12	F- TGCCATGCTCTTGTCAGG R- CACACGGGCATCAGACAG	338bp	51.5

Statistical analysis. Performance data pertaining to 100 Murrah buffaloes were collected and classified according to different herds, years and seasons. The effects of different non-genetic factors, such as season (November-February; winter, March-June; summer, July-October; rainy), year (2003-2014), parity, the influence of different management practices on the farm, and field conditions were included in the analysis. Statistical analyses were carried out using SPSS 22 statistical software (IBM SPSS Inc., Chicago, IL, USA). To ensure normal distribution, the outliers were removed and only data within the range of mean ± 2 SD were considered. For genetic studies, a total of 17 sires with two or more daughters were considered. The effect of non-genetic factors was adjusted by least squares analysis (LSA) implemented in SAS software (Version 9.2). The models were used with the assumption that the different components being fitted into the model were linear, independent and additive. The following linear models were used for milk yield and composition traits, respectively:

$$Y_{ijlmn} = \mu + S_i + POC_j + SOC_l + AFC_m + G_n + e_{ijlmn}$$

where, Y_{ijlmn} = observed value of milk yield at j^{th} period of calving, at l^{th} season of calving and m^{th} group of AFC, G_n = fixed e-ect of n^{th} SSCP pattern set of twelve exons with μ = overall mean, Si = ith sire (i = 1, 2, 3, . .. , 17), POC = j^{th} period of calving (l = 1, 2, 3 and 4), SOC = l^{th} season of calving (November-February (winter)=1, March-June (summer) = 2, July-October (rainy) = 3), AFC = m^{th} age at first calving (n = 1, 2, 3, … , 5), e_{ijklmn} = random error which is normally and independently distributed with mean 0 and variance σ^2_e and

$$Y_{ijklmn} = \mu + a_i + b_j + c_k + d_l + f_m + e_{ijklmn}$$

where: Y_{ijklmn} = observed value of milk composition trait; m= trait mean; a_i= genotype effect (i= 1,2); b_j=lactation number effect (j = 1,.,4); c_k = calving age effect (k = 1,.,4); d_l = calving season effect (k = 1,.,4), f_m = lactation month effect (m = 1,.,4); e_{ijklmn} = error.

Results and discussion

PCR-SSCP analysis of the AGPAT6 gene in 100 Murrah buffalo cows revealed two different banding patterns (Fig.1). Exon 4 and exon 8 of the AGPAT6 gene showed polymorphism within the population. The SSCP pattern '1' was found to be most common among the individuals investigated in the present study, with frequency equal to 0.64, and the pattern '2' was less frequent, with overall frequency of 0.36 in exon 4. In Exon 8, the pattern '2' was found to be most common with a frequency of 0.59 among the studied population of Murrah buffalo, and pattern '1' was less frequent with an overall frequency of 0.41 (Table 2). Sequencing of PCR amplicons representative of the different SSCP patterns revealed two different DNA sequences. Nine SNPs were identified among these sequences, with two found in exon 4 and seven found in exon 8 of the AGPAT6 gene (Table 3).

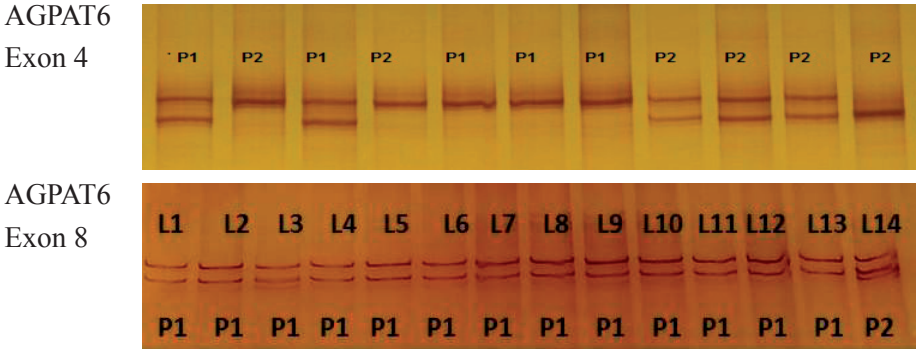

Fig. 1. PCR- SSCP pattern of the AGPAT6 gene in Murrah buffaloes

Polymorphism in exon 4 and exon 8 of the AGPAT6 gene in Murrah buffaloes; different SSCP patterns (P1 and P2) in a 12% polyacrylamide gel. Lanes 1, 3, 8, 9, 10 and 11, heterozygous animals with low and intermediate (P2 pattern) bands. Lanes 2, 4, 5 and 6 homozygous animals with low bands (P1 pattern) in exon 4. In AGPAT6 exon 8 Lanes 1, 2, 3, 4, 5, 6, 7, 9, 10 and 11 homozygous animals with low and high bands (P1 pattern) and Lane 12 heterozygous animals with low bands (P2 pattern).

Table 2. Frequency of SSCP variants of the AGPAT6 gene : Genotypic groups for the AGPAT6 gene pattern (locus) in Murrah buffaloes and the number of representatives per exons as the number of observations /pattern from the encoding AGPAT6 gene (AGPAT6 locus), presented in pairs as the frequency of SSCP variants

Region	Pattern/genotype	Number of observations	Frequency of SSCP variants	Chi square value
Exon 4	Pattern 1	64	0.640	0.574
	Pattern 2	36	0.360	
Exon 8	Pattern 1	41	0.410	0.730
	Pattern 2	59	0.590	

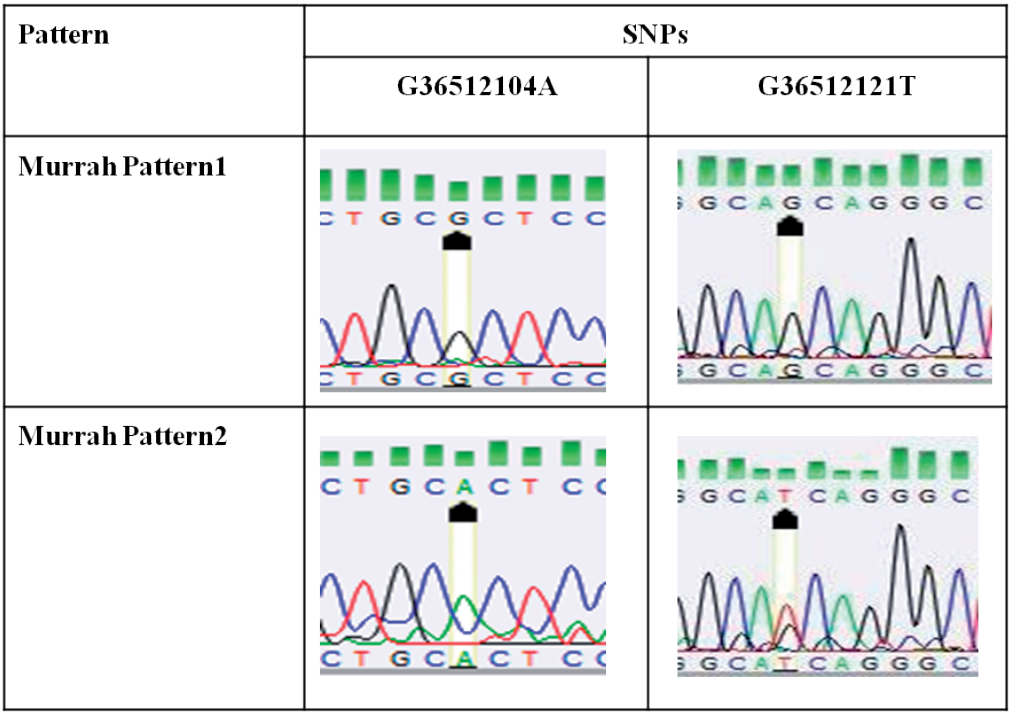

Fig. 2. 1. Sanger Trace Figures of SSCP variant sites of AGPAT6 Exon 4 in Murrah buffaloes

Detection of polymorphic sites in Exon 4 of the AGPAT6 gene in Murrah buffaloes. The black arrows indicate the change in nucleotide position found in different types of SSCP patterns when compared to the reference gene

Eleven PCR fragments indicating the chief functional domains of the AGPAT6 gene were screened for polymorphism. Sequencing of representative SSCP variants confirmed the nucleotide changes responsible for exhibiting different patterns in the gel. Of the nine exon SNPs, two were non-synonymous (g 36534971 G/C, g 36534989A/T), while the others were synonymous SNPs at the last nucleotide position of intron 4 and inton 8. Using the SSCP method, two new SNPs (G/C, A/T) were found, located in exon 8 at position g G36534971C, and g A36534989T (GenBank MK861921.1). The SNPs identified in the AGPAT6 gene of Murrah buffaloes were compared with other *Bos taurus* with transcript AC_000184.1. The sequences of Murrah buffaloes were found to be similar to the reference sequence with nine unique SNPs. In exon 4, one transition (G36512104A) and one transversion (G36512121T) were present in the coding region of the gene, causing a silent mutation (Fig.2.1). In exon 8, four transversions (G36534971C, G36535050C, G36535057T, G36535061C) were exhibited (Fig.2.2) in the coding region, whereas, another two transversions, i.e., T36533896A and G36533894T were observed in the non-coding 3'UTR and one transition, i.e., G36535041A was also found in the coding region. Out of these seven SNPs in the exonic region of Murrah buffaloes, G36534971C was found to cause an amino acid change from Aspartic Acid (D) to Histidine (H) (Fig. 4) and g A36534989T was found to cause an amino acid change from Isoleucine (I) to Phenylalanine (F) (Table 3).

Association of SSCP pattern set with milk production traits. SSCP variants of pattern 1 of exon4+pattern 2 of exon 4+pattern 1 of exon 8 had a significant ($P<0.05$) effect on milk production, whereas the combined effect of all the patterns was found to have a highly significant effect on the average fat percentage ($P<0.01$). Cows with pattern 2 of exon 4+pattern 1 of exon 8+pattern 2 of exon 8 were found to have the highest 305 day milk yield (1285.49±43.94) and the highest average fat percentage (8.16±0.03), compared to pattern 1 of exon 4+pattern 2 of exon 4+pattern 2 of exon 8 (1205.35±28.83 and 7.58±0.03) (Table 4).

Table 3. Summary of single nucleotide polymorphisms observed in the AGPAT6 gene in Murrah buffaloes: Changes in amino acid caused by nucleotide change with type of variation caused in exon4 and exon8 observed in the AGPAT6 gene in Murrah buffaloes

Region	Locus	Murrah	Type of variation	Amino acid change
Exon4	G36512104A	G/A	Transition	No change
	G36512121T	G/T	Transversion	No change
Exon8	G36534971C	G/C	Transversion	Aspartic Acid (D) to Histidine (H)
	G36535050C	G/C	Transversion	No change
	G36535057T	G/T	Transversion	No change
	G36535061C	G/C	Transversion	No change
	A36534989T	A/T	Transversion	Isoleucine (I) to Phenylalanine
	G36533894T	G/T	Transversion	No change
	G36535041A	G/A	Transition	No change

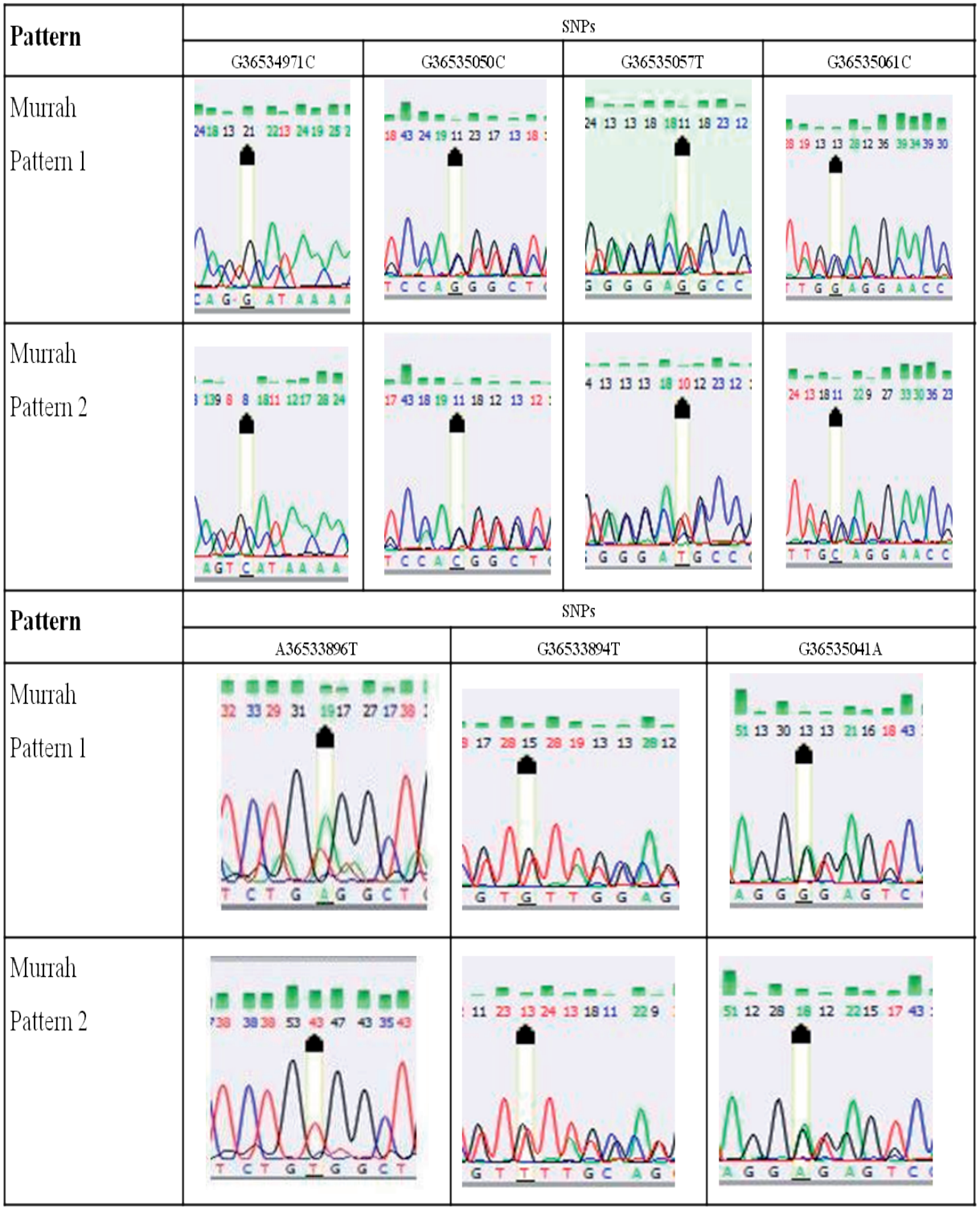

Fig. 2. 2. Sanger Trace Figures of SSCP variant sites of AGPAT6 Exon 8 in Murrah buffaloes

Detection of polymorphic site in Exon 8 of the AGPAT6 gene in Murrah buffaloes. The black arrows indicate the change in nucleotide position found in different types of SSCP patterns when compared to the reference gene

Phylogenetic analysis. A comprehensive phylogenetic tree was constructed, in which the observed AGPAT6 variants were compared with each other and with the AGPAT6-based DNA sequences of other livestock animals, using the NCBI-blastn server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). An inclusive tree was made using the neighbor joining tree option of the Clustal Omega server (https://www.ebi.ac.uk/ Tools/msa/clustalo/). The analysis revealed that the Murrah AGPAT6 gene is highly conserved among different species and has very important effects on cell function. Analysis of the phylogenetic tree showed that the Murrah (*Bubalus bubalis*) AGPAT6 gene has a close genetic relationship with the AGPAT6 gene in cattle. This implies that the AGPAT6 gene in river buffalo is functionally more similar to cattle.

The present findings reveal that the polymorphisms observed in 3′UTR of the AGPAT6 gene have a significant effect on various traits, such as 305 day milk yield and average fat percentage. The quantitative trait nucleotide (QTN) of AGPAT6 exon 4 and exon 8 in Murrah buffaloes had no significant effect (P≤0.01) on milk yield, whereas the same variants in terms of SNPs in the coding region, were found to have a significant (P≤0.01) association with fat percentage. Previous studies have also shown SNPs in intron2, intron1 and promoter regions of buffalo AGPAT6, and DGAT genes, without any significant association with milk traits (LITTLEJOHN et al., 2014; NAFIKOV et al., 2014; VIALE et al., 2017). None of the SNPs identified was similar to other bovine species, thereby signifying their specificity to the AGPAT6 gene of Murrah buffaloes.

AGPAT6 variation and milk production traits. Least squares analysis revealed a significant association between milk fat percentage and different SSCP patterns, whereas there was a non-significant association between milk yield and different SSCP patterns. The least square means for 305 day average lactation milk yield and fat percentage in the present study were found to be 1189.73±22.03 and 7.88±0.03, respectively. The least squares means of total milk yield was observed to be highest for SSCP pattern '2', with a value of 1238.46±40.37 kg, while it was lower in pattern '1' at 1185.49±32.94 kg. The least square means for fat percentage in SSCP pattern '1' within 3′UTR of the AGPAT6 gene was observed 80. 8±0.02% while for patterns '2' the least squares means were 75.8±0.03% (Table 4). Season of calving, period of calving and sire were found to have a significant effect on both the considered traits, that is, 305 day milk yield and average fat percentage, whereas age at first calving was only found to have a significant influence (<0.01) on 305 day lactation milk yield (Table 5). Similar feeding and management conditions were the reasons for the non-significant effect of age at first calving on average fat percentage in Murrah buffaloes. The present findings agree with earlier reports (DUBEY et al., 1997; NAFIKOV et al., 2014; STRILLACCI et al., 2014).

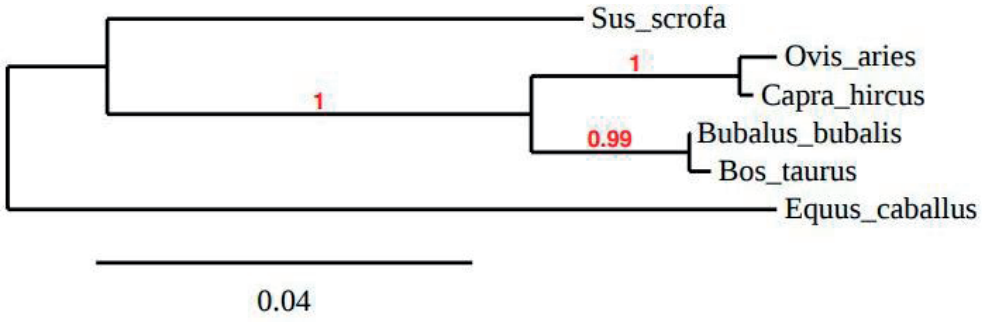

Fig. 3. Neighbor-joining phylogenetic tree based on the AGPAT6 gene among certain species

Phylogenetic tree revealing the evolutionary relationship (Neighbor-joining) between a set of homologous characters of several organisms based on the AGPAT6 gene among certain species

Table 4. The effect of combined patterns of both the exons of the AGPAT6 gene on milk yield and fat percentage in Murrah buffaloes (LSM ± S.E)

Effects	305 days Milk yield	Average Fat%
P1E4+P2E4+P1E8	$1217^{a} \pm 71.26$	$8.08^{b} \pm 0.02$
P2E4+P1E8+P2E8	$1285.49^{ab} \pm 43.94$	$8.16^{b} \pm 0.03$
P1E4+P2E4+P2E8	$1205.35^{ab} \pm 28.83$	$7.58^{ac} \pm 0.03$
P-Value	0.271(NS)	0.002**

P1E4, pattern 1 of exon 4; P2E4, pattern 2 of exon 4; P1E8, pattern 1 of exon 8; P2E8, pattern 2 of exon 8. Values bearing NS shows non-significance and P values bearing ** between rows differ significantly at P≤0.01. Means with different superscripts (a,b,c) in the same row differ significantly (P<0.05). Pattern sets with frequency less than 10 were removed from analysis.

Table 5. Least squares analysis of variance for non-genetic effects on 305 day milk yield and average fat percentage**:** Comparison among the least-squares means of 305 day milk yield and average fat percentage for the non-genetic effects with different patterns of AGPAT6 gene

Trait	Source	DF	Mean squares
305 day milk yield	Sire	16	221262.79*
	Period	4	155344.98*
	Season	3	43884.45**
	AFC	5	354845.25*
	Error	72	26849.06
Average fat percentage (%)	Sire	16	5466.43*
	Period	4	1976.36**
	Season	3	755.29**
	AFC	5	4023.09
	Error	72	2770.38

Means squares value bearing different superscripts ** and * between rows differ significantly at P≤0.01 and P≤0.05 level

In the present study, a significant association was found between milk production traits and SSCP different patterns. However, we attempted to associate the AGPAT6 gene polymorphism in 3′UTR with milk yield and fat percentage in buffaloes (*Bubalus bubalis*), where not much information on QTLs is available. The present investigation is the first study carried out in the Indian Murrah buffalo breed with the aim of identifying genomic regions putatively associated with milk production traits using the SSCP technique. Nine SNPs were identified associated with one of the analyzed traits (milk composition). Using the PCR-SSCP method, nine SNPs were found, located in exon 4 and exon 8. In exon 4, one transition (G36512104A) and one transversion (G36512121T) were present in the coding region of the gene, causing a silent mutation. In exon 8, four transversions (G36534971C, G36535050C, G36535057T, G36535061C) were exhibited in the

coding region, whereas two other transversions, i.e., T36533896A and G36533894T, were observed in the non-coding 3'UTR, and one transition, i.e. G36535041A, was also found in the coding region. Out of these seven SNPs in the exonic region of Murrah buffaloes, G36534971C was found to cause an amino acid change from Aspartic Acid (D) to Histidine (H), and g A36534989T was found to cause an amino acid change from Isoleucine (I) to Phenylalanine (F). The SNPs were deposited in the GenBank database under acc. no MK861921.1 for Murrah buffaloes (Fig.4).

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1  MFLLLPFDSLIVSLLGISLTVLFTLLLVFIIVPAVFGVSFGIRKLYMKTLLKIFAWATLR  60
2  MFLLLPFDSLIVSLLGISLTVLFTLLLVFIIVPAVFGVSFGIRKLYMKTLLKIFAWATLR  60
   ************************************************************

1  MERGAKEKNHQLYKPYTNGIIAKDPTSLEEEIKEIRRSGSSKALDNTPEFELSDIFYFCR  120
2  MERGAKEKNHQLYKPYTNGIIAKDPTSLEEEIKEIRRSGSSKALDNTPEFELSDIFYFCR  120
   ************************************************************

1  KGMETIMDDEVTKRFSAEELESWNLLSRTNYNFQYISLRLTVLWGLGVLIRYCLLLPLRI  180
2  KGMETIMDDEVTKRFSAEELESWNLLSRTNYNFQYISLRLTVLWGLGVLIRYCLLLPLRI  180
   ************************************************************

1  ALAFTGISLLVVGTTMVGYLPNGRFKEFLSKHVHLMCYRICVRALTAIITYHDRKNRPRN  240
2  ALAFTGISLLVVGTTMVGYLPNGRFKEFLSKHVHLMCYRICVRALTAIITYHDRKNRPRN  240
   ************************************************************

1  GGICVANHTSPIDVIILASDGYYAMVGQVHGGLMGVIQRAMVKACPHVWFERSEVKDRHL  300
2  GGICVANHTSPIDVIILASDGYYAMVGQVHGGLMGVIQRAMVKACPHVWFERSEVKDRHL  300
   ************************************************************

1  VARRLTEHVQDKSKLPILIFPEGTCINNTSVMMFKKGSFEIGATVYPVAIKYDPQFGDAF  360
2  VARRLTEHVQHKSKLPFLIFPEGTCINNTSVMMFKKGSFEIGATVYPVAIKYDPQFGDAF  360
   ********** ***** **********************************************

1  WNSSKYGMVTYLLRMMTSWAIVCSVWYLPPMTRQAEEDAVQFANRVKSAIARQGGLVDLL  420
2  WNSSKYGMVTYLLRMMTSWAIVCSVWYLPPMTRQAEEDAVQFANRVKSAIARQGGLVDLL  420
   ************************************************************

1  WDGGLKREKVKDTFKEEQQKLYSKMIVGNHEDRSRS  456
2  WDGGLKREKVKDTFKEEQQKLYSKMIVGNHEDRSRS  456
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Fig. 4. CLUSTAL Omega multiple sequence alignment

Comparative alignment of conceptualised protein sequences (AGPAT6 gene), 456 amino acids) on the basis of polymerase chain reaction–single-stranded conformation polymorphism (PCR–SSCP) haplotype sequence in Murrah buffaloes and the NCBI Reference sequence AC_000184.1, drawn on the basis of the MegAlign module of DNAstar software version 7.1. CLUSTAL Omega multiple sequence alignment showed G36534971C was found to cause an amino acid change from Aspartic Acid (D) to Histidine (H) and g A36534989T was found to cause an amino acid change from Isoleucine (I) to Phenylalanine (F).

A recent report on buffaloes revealed that the involvement of the AGPAT1 and AGPAT6 genes in regulating milk fat synthesis extends beyond their influence on the lipogenic genes involved in the de novo pathway, encompassing their impact on fatty acid synthesis as well (MA et al., 2022). Similarly, previous findings on the Holstein-Friesian breed of cattle indicated the high association of AGPAT6 gene polymorphism with fat yield and rennet coagulation time (RCT) (VIALE et al., 2017). However, NAFIKOV et al. (2014), STRILLACCI et al. (2014) and VIALE et al. (2017) reported a significant association between AGPAT6 polymorphisms and milk fat composition in water buffaloes and *Bos taurus* cattle. LITTLEJOHN et al. (2014) reported the strong association of novel AGPAT6 sequence variants with milk fat percentage, highlighting a subset of candidate causal polymorphisms in the 5' UTR exons and intron 1 of the AGPAT6 gene in *Bos taurus* cattle. Although further experimentation is required to validate the identified SNP loci in other populations and breeds, the results of the present study can be considered as a preliminary foundation for future research on gene-assisted selection programs in the Murrah breed. It will also be interesting to explore the other regions of the gene for possible polymorphic nucleotide sites that have a significant effect on buffalo milk quality traits.

Conclusions

We found that variations in bovine AGPAT6 have an effect on milk production traits. An association study of different variants revealed a significant association with one of the milk production traits, although there were considerable differences among the least squares means of different SSCP variants. However, a significant association was observed between SSCP variants' milk fat percentages in Murrah buffalo. Further investigations are needed on a larger population of Murrah buffaloes, or other breeds of buffaloes, to confirm these findings before they can be recommended to breeding programs to improve milk production traits in cattle and buffaloes.

Declare of competing interest

No potential conflicting interest was reported by the authors.

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NATH, S., D. N. DAS, S. KUMAR ILLA, A. KUMAR NAGALEEKAR, D. REVANASIDDU: Identifikacija pojedinačnih nukleotidnih varijacija gena AGPAT6 i njihova povezanost sa svojstvima proizvodnje mlijeka u vodenih bivola pasmine Murrah. Vet. arhiv 94, 449-462, 2024.

SAŽETAK

U radu je istraživana povezanost genskih varijacija unutar 5′UTR eksona 1-1511, 3′UTR eksona 1512-1723 gena *sn-1-acylglycerol-3-phosphate-O-acyltransferase 6* (*AGPAT6*) sa svojstvima proizvodnje mlijeka u vodenih bivola. U 100 jedinki pasmine Murrah su uporabom PCR:SSCP i Sangerove metode sekvenciranja otkrivena dva jedinstvena konformacijska uzorka, 1 i 2. Za amplifikaciju gena *AGPAT6* upotrijebljeno je deset početnica, a ukupno je identificirano devet pojedinačnih nukleotidnih polimorfizama (SNPs): dva u eksonu 4 i sedam u eksonu 8. Ustanovljeno je da je u populaciji istraživanih bivola učestalost SSCP uzorka 1 konzistentno visoka. Preliminarni su rezultati pokazali znatne varijacije u raspodjeli učestalosti SSCP varijanti obrazaca 1 i 2 u vodenih bivola pasmine Murrah. Te varijante eksona 4 i eksona 8 nisu bile znakovito povezane s prinosom mlijeka, dok je za iste varijante u smislu SNP-ova u kodirajućoj regiji utvrđeno da su znakovito povezane ($P \leq 0,01$) s postotkom mliječne masti. Navedeno upućuje na zaključak da gen *AGPAT6* utječe na postotak mliječne masti. Budući da se radi o ekonomski važnom svojstvu, analizirani polimorfizmi mogli bi se upotrijebiti kao molekularni markeri u uzgojno-selekcijskom radu što istraživanja AGPAT6 gena u vodenih Murrah bivola čini dodatno opravdanim.

Ključne riječi: gen *AGPAT6*; učinak supstitucije alela; vodeni bivoli Murrah; sadržaj mliječne masti; SNP; (PCR-SSCP)