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Polymorphic Variants Analysis in *Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha* (PPARGC1A) Gene of Indian and Egyptian Buffaloes

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ABSTRACT

Background: *Peroxisome proliferator-activated receptor gamma coactivator 1-alpha* (PPARGC1A) is a candidate gene for milk fat in livestock. The present study was carried out to investigate polymorphism in PPARGC1A and study its potential association.

Methods: Two hundred forty animals from Murrah and Bhadawari Indian buffalo breeds and Egyptian buffaloes were selected. The statistical analysis used was Tukey's honest significance test to investigate the association of different genotypes. The PCR-SSCP (polymerase chain reaction-single strand conformation polymorphism) assays and sequencing technologies were used to confirm the SNPs.

Results: Three Novel single nucleotide polymorphisms (SNP1- g.993A>T, SNP2- g.1237T>A and SNP3-g.1238G>C) and one insertion (g.1240_>G bp) were identified in this gene. Due to nucleotide mutations, four non-synonymous mutations were observed in amino acid sequences of buffalo breeds. Significant differences in protein and SNF output were discovered in Egyptian buffalo breeds. In both Murrah and Bhadawari buffaloes, fat, protein and SNF yield were substantially correlated with detected genotypes while there was non-significant association of protein and SNF percent in buffalo breeds. The information provided will be valuable in studying the involvement of PPARGC1A in regulating fat synthesis and enhancing milk quality in buffaloes.

Key words: *Bubalus bubalis*, PCR-SSCP, PPARGC1A, SNPs.

INTRODUCTION

Livestock farming, particularly buffalo farming in India and Egypt, is a crucial source of livelihood. Buffaloes are highly versatile animals, capable of producing milk, meat, fuel, draught power and manure, making them a valuable asset (Yang *et al*., 2022). They contribute approximately 13% of the global milk supply and buffalo milk is renowned for its superior processing qualities due to its higher protein and fat content compared to dairy cattle milk (Rehman *et al*., 2021 and Qiu *et al*., 2020). The quality and quantity of milk have long been recognized as vital attributes (Yang *et al*., 2022) and India should enhance the productivity of buffaloes since they are gradually replacing cattle in many regions that produce milk (Anil *et al*., 2012). Depending on genetic and environmental factors, different buffalo breeds may have variable milk fat percentages (Raut *et al*., 2012). The objective of the present study was to identify SNPs in PPARGC1A gene that impact essential milk characteristics including the quantity and quality of fat and protein.

PPARGC1A, also known as PGC1a, is a member of the PPAR family and plays a role in various tissues of buffalo. It is involved in milk fat production and acts as a transcriptional coactivator of the peroxisome proliferator-activated receptor (Chen *et al*., 2022). In different mammals, the PPARGC1A gene has been shown to regulate genes associated with fatty acid and glucose metabolism (Qiu *et al*., 2020). In buffalo, the PPARGC1A gene, located on chromosome 7's centromere, is a potential candidate gene for a milk fat yield QTL on BTA6 (Weikard *et al*., 2005). Its molecular weight in buffalo is approximately 90.49 kDa (Hosseini *et al*., 2021). PPARGC1A has been identified as a crucial gene influencing milk-related traits in cattle and milk fat in buffalo (Pasandideh 2020). The SNPs identified in this gene have a significant impact on milk yield and can serve as potential markers for gene-assisted selection in buffaloes (Qiu *et al*., 2020). Studies

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have shown that a SNP (T>C) in the PPARGC1A gene had a significant effect on milk fat production in the German Holstein population (Pecka-Kie b *et al*., 2021). Genomic breeding value assessment is gaining interest as a means to facilitate efficient and rapid selection of farm animals (Yang *et al*., 2022). Finding SNPs in the exon 3 region of the PPARGC1A gene, is crucial because there was minimal information on this genomic region. This study was conducted to understand better how various milk production traits in dairy buffalo are related to polymorphisms identified in PPARGC1A gene.

MATERIALS AND METHODS

The present study included 240 animals of Murrah (60) and Bhadawari (40) buffalo breeds of India and Egyptian buffalo (140) based on their geographical distribution. Present investigation was conducted in year 2021-2023 at the Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar. About 10 ml of blood was collected from each animal in a vacutainer tube containing anticoagulant ethylene diamine tetra acetic acid (EDTA) and stored at -20°C for DNA extraction. Blood was collected from the jugular vein of buffaloes and it did not harm the animals. So, the study didn't require ethical approval. With just a few modifications, the phenol-chloroform extraction procedure (Russel and Sambrook, 2001) was used to extract genomic DNA. The purity and concentration of DNA were determined with UV Spectrophotometer (DeNovix DS-11).

Based on the *Bubalus bubalis* (GU066311.1) reference sequence primers for PPARGC1A were designed using IDT oligo analyzer, *OligoCalc* and NCBI primer blast online software. Primer sequences of the PPARGC1A gene were as follows: forward 5′ TCTCCCAGTGTCAACTCAC-3′ and reverse 5′- TAGCCAGAGGCAACTCCAA-3′. Utilizing a Bio-Rad thermal cycler, PCR amplification was carried out (model C1000). The reaction mixture consisted of 5 µL of 5X Phusion HF buffer, 0.5 µL of 10 mM of dNTPs, 1.25 µL of 10 ìM each primer, 0.25 µL of Phusion DNA polymerase and the final volume was adjusted to 25 µL with nuclease-free water. PCR conditions included denaturation at 95°C for 3 min, followed by 35 cycles of denaturation for 40 sec at 95°C, annealing for 25 sec at 53.5°C and extension for 40 sec at 72°C, with the final extension phase taking place for 7 min at 72°C. 3ul of PCR amplicons with a standard 100 bp DNA ladder (Promega) as a marker were electrophoresed in 1.3% agarose gels using 1X TAE buffer at 6 volt/cm for 45 min. After that, ethidium bromide-stained gels were fluorescently visualized under UV light and photographed by a gel documentation system (Syngene).

The amplicons were subjected to detect novel SNPs. For SSCP analysis, several factors were tested to optimize the methodology. Amplicons were tested at 10%-16% polyacrylamide gel, 12-15 h electrophoresis at 100-150 V. 5 ul of each PCR amplicon was mixed with 5 ul denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene-cyanol and 0.025% bromophenol blue) and then the mixture was instantly snap-chilled on ice after being denatured at 95°C for five minutes. Denatured PCR products were loaded onto non-denaturing 14% polyacrylamide gel and electrophoresis was carried out for about 15 h at 150 V. The samples were run in 1X TBE buffer at 4°C in the refrigerator to prevent buffer heating. The composition of polyacrylamide gel was 30% acrylamide/bis-acrylamide in a 29:1 ratio of 5.6 ml, 5X TBE-2.4 ml 10% ammonium per sulphate-200 ul, TEMED-10 ul and 4.0 ml distilled water used to make up a total volume of 12 ml. Silver nitrate was used for staining the gels, as per the method described by Bassam, Caetano-Anolles and Gresshoff (1991) with minor modifications. SSCP band patterns were visualized directly by using a developer solution and observed to find the SNPs. PCR products that showed different patterns in PCR-SSCP stained gels were sent for Sequencing (Macrogen Korea).

Sequencing results of the PPARGC1A gene were then examined by different software. Observed sequences were aligned using SnapGene version 6.2.1, multiple sequence alignment software. Furthermore, using EMBL transseq (http://www.ebi.ac.uk/Tools/st/), all DNA sequences were converted into protein sequences. These protein sequences were then aligned using SnapGene.

An analysis of the relationship between individual genotypes and selected traits of buffaloes were analysed by using SPSS software (version 26.0). The genotype frequency and allele frequency of the PPARGC1A gene were calculated. The statistical analysis was carried out using Tukey's honest significance test to investigate the association of different genotypes with buffalo breeds' milk characteristics (George and Mallery 2019).

RESULTS AND DISCUSSION

The PPARGC1A gene's exon 3 region was amplified effectively from the genomic DNA of all breeds and the size of the amplicons was 295 bp, as shown in Fig 1. Khatib *et al*. (2007) genotyped the same gene included in present study in 2 independent populations with more than 1,400 individuals and 434 bulls.

PCR-SSCP analysis

The PCR-SSCP analysis of exon 3 of the PPARGC1A gene using primers resulted in three different SSCP patterns AA (represented by 2 band pattern upper and lower), BB (containing 2 band pattern middle and lower) and AB (having 3 band pattern upper, middle and lower) in Murrah, Bhadawari and Egyptian buffalo breeds (Fig 2). Samples with distinct PCR-SSCP patterns were sequenced by using ABI PRISM 3730XL Analyzer (Macrogen Korea).

Sequence analysis and identification of SNPs

The Genotype proportions and allelic frequencies were calculated for the PPARGC1A gene and the same have been described in Table 1. The allele A was found to have the highest frequency among Egyptian buffalo, while the allele

B exhibited the highest frequency among Bhadawari buffalo. The frequencies of A and T alleles were estimated to be 0.768 and 0.232, respectively in 8th exon of PPARGC1A in Anatolian water buffaloes (Alyoruk and Erdo an, 2018) and Kowalewska- uczak, Kulig and Kmie, (2010) observed the most common alleles were T (c.1892T>C) with a frequency of 0.63 and A (c.3359A>C) with a frequency of 0.88 in Jersey cows.

To confirm the SNPs, sequenced samples analysis was performed upon comparing with the reference (GU066311.1) available at NCBI; three SNPs were identified in the samples under study, namely SNP1- g.993A>T in Murrah (PRC1M, PRC2M) and Egyptian buffalo (PRC4E) in Fig 3a, SNP2- g.1237T>A in Egyptian buffalo (PRC4E) and SNP3- g.1238G>C in Murrah (PRC1M), Bhadawari (PRC3B) and Egyptian buffalo (PRC4E) in Fig 3b. There was the insertion (Fig 3b) of nucleotide (G) observed at position g.1240_>G bp in both Murrah (PRC1M) and Bhadawari (PRC3B). Obtained SNPs for this study have been deposited in the European Variation Archive (EVA) at EMBL-EBI under accession no. ERZ16273728. On the other hand, seven SNPs were detected in this gene in the Italian Mediterranean buffalo (Hosseini *et al*. 2021), of which one was found in the promotor, four in exons and two in introns. Two SNP polymorphisms in the Salers cow breed's PPARGC1A gene's intron 9 (1892T>C) and 3'UTR (3359A>C) regions were examined by Pecka-Kieb *et al*., (2021). Similar findings was reported by Qiu *et al*., (2020) in complete coding sequence (CDS) of PPARGC1A gene for both swamp and river buffalo, eight SNPs were found in buffalo, in which the c.778C>T, c.1257G>A and c.1311G>A were shared by two types of buffalo with similar allele frequencies, while the c.419C>T, c.759A>G, c.920C>A, c.926G>A and c.1509A>T were only observed in river buffalo.

Nucleotide sequences were translated to the corresponding peptide sequence using the EMBOSS Transeq program. Because of the SNP1, there was no amino acid change observed, SNP2 nucleotide mutations change

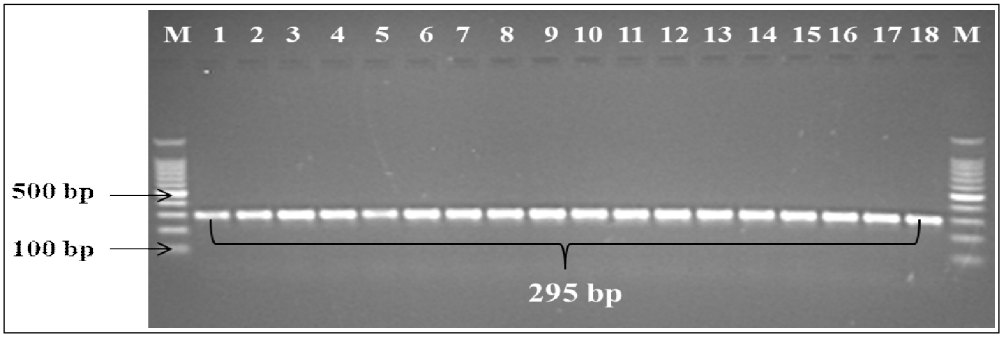

Fig 1: The PPARGC1A gene's amplicons M: 100 bp DNA Marker. Lanes 1–18: 295 bp PCR product.

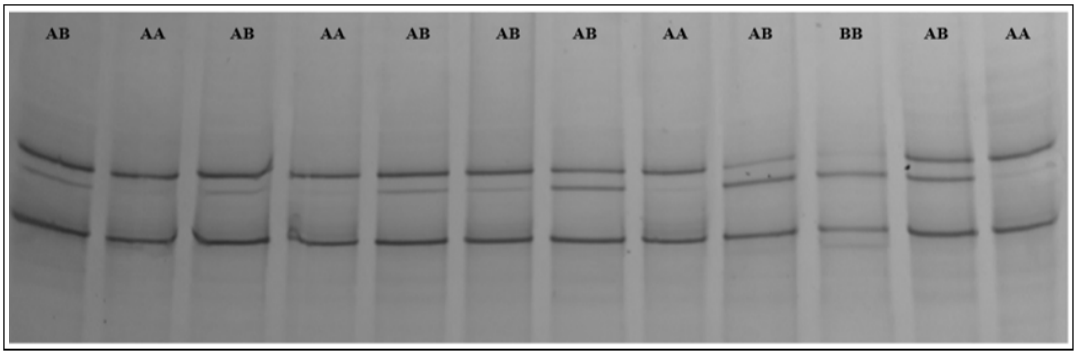

Fig 2: Amplified PPARGC1A gene from the genomic DNA was observable on a polyacrylamide gel stained with silver-nitrate and shows different patterns.

Table 1: Based on PCR-SSCP investigation of the PPARGC1A gene table shows the genotype and allele frequencies.

Breeds	n	Genotype frequency			Allele frequency	
		AA	AB	BB	A	B
E.B	140	0.71	0.26	0.03	0.62	0.38
M.B	60	0.75	0.18	0.07	0.56	0.44
B.B	40	0.10	0.10	0.40	0.35	0.65

EB: Egyptian breed; MB: Murrah breed; BB: Bhadawari breed; n: number of animals.

the cysteine to threonine, SNP3 showed a non-synonymous mutation change Leucine to Proline, and because of the insertion of G, two amino acid changes were observed (Tryptophan-Leucine and Leucine-Alanine) in Table 2. A previous study by Alyoruk and Erdo an, (2018) c.1598A>T polymorphism in exon 8 of PPARGC1A could affect the amino acid sequence and change the structure and function of the protein by altering the GAC codon that encodes Aspartic acid (Asp) to a GTC codon that encodes Valin (Val) amino acid. Similar to our results in CDS region of PPAGC1A gene three SNPs; SNP419, SNP920 and SNP926 were led to the non-synonymous amino acid changes of p.Ser140Phe, p.Pro307His and p.Arg309Lys in swamp and river buffaloes (Qiu *et al.*, 2020).

Association of observed genotypes with milk components

The associations between genotypes of buffalo breeds with the milk components were investigated. As shown in Table 3, significant differences were found in protein and SNF yield among AA, AB and BB genotypes in Egyptian buffalo breeds. Fat, protein and SNF yields were significantly associated with observed genotypes in both Murrah and Bhadawari buffaloes. Fat percent shows significant association only in Bhadawari breed and the protein and SNF percent in all genotypes were found to be non-significantly associated. Highest fat percent was observed in BB genotype of Bhadawari buffaloes as well as higher protein percent was also observed in BB genotype but in Murrah buffalo breed and high SNF percent in AB genotype of Egyptian buffaloes. Higher fat yield was observed in genotype BB of Murrah buffalo, AB genotype of Bhadawari buffalo have highest protein yield and BB genotype of Egyptian buffalo have higher SNF yield. According to Schennink *et al.* (2009) there was a substantial correlation between milk fat yield and the PPAGC1A gene variants c.1790+514G>A, c.1892+19T>C and c.1892+19G>A in dairy cows. Similar to our analysis significant association between an SNP in intron 9 of the PPARGC1A gene and milk fat yield in a large dairy cattle population, suggesting that the gene could contribute to the genetic variation underlying the QTL for milk fat synthesis on BTA6 (Weikard *et al.*, 2005). According to Kowalewska-uczak, Kulig and Kmie, (2010) study no significant association between individual genotypes of both SNPs and milk traits Study conducted by Hosseini *et al.*, (2021) aimed to identify polymorphisms in the PPARGC1A gene in Italian Mediterranean buffaloes shows g.304050G>A and g.325997G>A were associated with both milk yield and protein percentage. In contrast current study results confirm that protein and SNF percentage show non-significant association between individual genotypes of all three breeds.

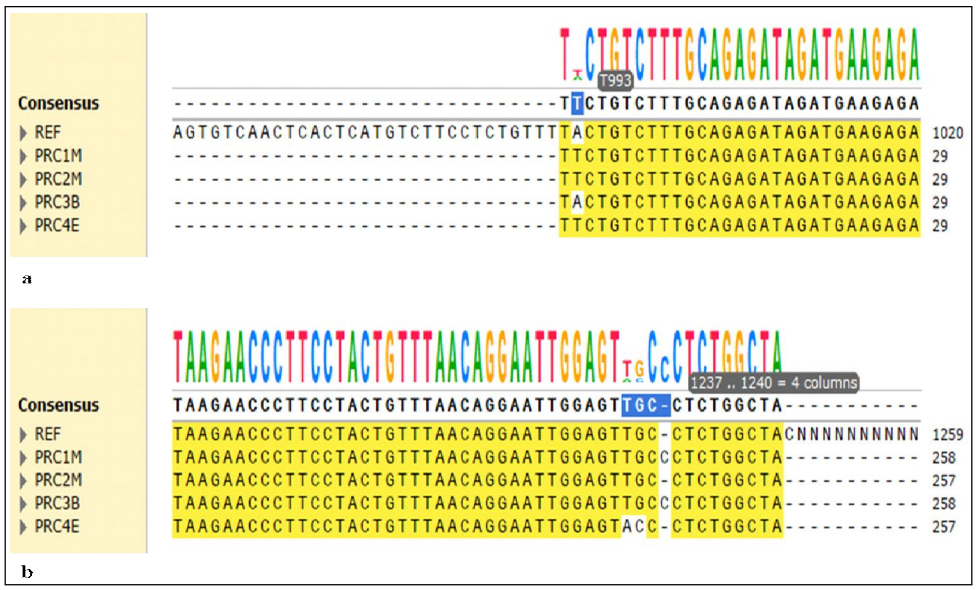

Fig 3: Depicts the identified nucleotide substitutions and insertion occurring in exon-3 of the PPARGC1A gene.

Table 2: Characteristics of single nucleotide polymorphisms.

SNPs	SNP localization	Nucleotide change	Amino acid change
SNP1	Chr7g.993	A>T Substitution	-
SNP2	Chr7g.1237	T>A Substitution	Cysteine/Threonine
SNP3	Chr7g.1238	G>C Substitution	Leucine/Proline
INS	Chr7g.1240	≥G (Insertion)	Tryptophan/Leucine, Leucine/Alanine

Table 3: Genotypes association with milk characteristics of buffalo breeds.

Buffalo breeds	Milk characteristics	Observed genotypes (Mean±SE)			Significantly
		AA	AB	BB	
Egyptian	Fat %	8.23±0.13^{a}	7.97±0.21^{a}	7.80±0.61^{a}	NS
	Protein %	3.34±0.05^{a}	3.39±0.69^{a}	3.12±0.28^{a}	NS
	SNF %	9.43±0.05^{a}	9.57±0.10^{a}	9.52±0.24^{a}	NS
	Fat yield (kg)	17.18±0.14^{a}	17.22±0.22^{a}	16.73±0.56^{a}	NS
	Protein yield (kg)	10.50±0.10^{a}	11.46±0.16^{b}	10.48±0.66^{a}	*
	SNF yield (kg)	25.88±0.22^{a}	26.04±0.36^{a}	29.26±0.15^{b}	*
Murrah	Fat %	7.85±0.12^{a}	7.50±0.34^{a}	7.80±0.15^{a}	NS
	Protein %	3.72±0.07^{a}	3.69±0.14^{a}	3.87±0.33^{a}	NS
	SNF %	9.38±0.08^{a}	9.41±0.19^{a}	9.49±0.12^{a}	NS
	Fat yield (kg)	16.94±0.19^{a}	17.28±0.49^{a}	19.37±0.33^{b}	*
	Protein yield (kg)	10.72±0.15^{ab}	11.17±0.31^{b}	9.77±0.412^{a}	**
	SNF yield (kg)	26.56±0.27^{b}	23.54±0.75^{a}	24.76±1.49^{ab}	**
Bhadawari	Fat %	8.96±0.28^{ab}	8.06±0.56^{a}	9.65±0.35^{b}	**
	Protein %	3.42±0.18^{a}	3.22±0.39^{a}	3.79±0.16^{a}	NS
	SNF %	9.26±0.18^{a}	9.11±0.17^{a}	9.21±0.17^{a}	NS
	Fat yield (kg)	17.88±0.24^{b}	16.63±0.40^{ab}	16.13±0.26^{a}	**
	Protein yield (kg)	10.24±0.24^{a}	11.55±0.76^{b}	10.60±0.24^{ab}	**
	SNF yield (kg)	26.08±0.40^{a}	29.13±0.25^{b}	25.05±0.56^{a}	*

S: significantly different ($p \leq 0.05$); NS: non-significantly different ($p \geq 0.05$); SE: standard error; SNF: Solid-not fat; Mean values followed by different lower-case letters are significantly different at $p \leq 0.05$(*****) and $p \leq 0.01$(******) according to Tukey's honest significance test.

CONCLUSION

The identified SSCP pattern within exon 3 of the PPARGC1A gene followed by Sequencing revealed three SNPs (SNP1-g.993A>T, SNP2- g.1237T>A and SNP3-g.1238G>C) and one insertion of nucleotide (g.1240_>G) in studied buffalo breeds. Because of the nucleotide mutations, the amino acids were substituted. Four amino acid substitutions were found because of SNPs. These SNPs may serve as indicators of milk output and quality in buffaloes. In Egyptian buffalo breeds, there were noticeable changes in the protein and SNF yield between the AA, AB and BB genotypes. Fat, protein and SNF yield in observed genotypes were substantially correlated in both Murrah and Bhadawari buffalo breeds. Only the Bhadawari breed exhibits a significant correlation with fat percentage. Finally, these scientific efforts in the present study will help to select dairy animals that are more sustainable regarding the quantity and quality of milk supply.

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Data statement

The data used to support the findings of this study have been deposited in the European Variation Archive (EVA) at EMBL-EBI under accession number ERZ16273728.

Competing Interests

The authors declare no conflict of interest.

Ethics approval

Not applicable.

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