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DNA Polymorphisms in 3′UTR and Intron-9 Region of PPARGC1A Gene and its Association with Milk Production Traits in Gaolao Cattle

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ABSTRACT

Background: PPARGC1A gene mediates expression of genes involved in gluconeogenesis and is regarded as a prominent candidate gene governing the effect of milk fat in dairy bovines. The study was planned to identify DNA polymorphisms in the PPARGC1A gene in Gaolao Cattle and to find their association with milk production traits in Gaolao cattle.

Methods: The blood was collected from 224 purebred Gaolao cattle from farm and field along with test day milk from each cow for estimating milk components. Genomic DNA was assessed using phenol-chloroform extraction method and Kits. PCR-RFLP, PCR-SSCP and direct DNA sequencing methods were used to identify polymorphism.

Result: PPARGC1AG1- *Nhe*I locus of 3′UTR region were polymorphic with allele frequency for A alleles as 0.84 and for allele B as 0.16. In studied animals, significant differences were not observed for milk traits between genotypes at PPARGC1A-*NheI* locus. The analysis at PPARGC1AG4-SSCP revealed no significant differences in milk traits; however sequencing revealed SNP. PPARGC1AG1-*Nhe*I genotype-based sequencing revealed T>C SNP at 263rd position 02 computational SNPs *viz*; C-T at 263rd position and A-T at 268th position. The identified variation at the PPARGC1A gene may aid in identifying associations for development of markers for selection and genetic improvement.

Key words: Gaolao cattle, Milk traits, PCR-RFLP, PPARGC1A, Sequencing, SSCP.

INTRODUCTION

Traditional and advanced principles and tools in animal genetics and breeding will revolutionize future dairy cattle breeding for productivity enhancement. Molecular marker technology has paved the way for identifying the candidate gene regions underlying economic traits like milk production in dairy animals. The imputation of these identified variants information in genetic evaluation increases selection accuracy and improves genetically essential traits like milk yield and milk composition. Various researchers are currently working on genomic research to enhance milk productivity by conducting candidate gene polymorphism and association studies (Rahayu *et al.*, 2019; Liu *et al.*, 2020; Korkuc *et al.,* 2021).

Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PPARGC1A) gene mediates the expression of genes involved in gluconeogenesis. It is a prominent functional and positional candidate governing the potential quantitative trait locus effect for milk fat in dairy bovines. PPARGC1A gene is located on the 6th bovine autosome and to date, many SNPs related to milk production traits have been identified. Various studies have reported that SNPs like PPARGC1Ac.1892T>C and PPARGC1A-c.3359 A>C affect milk yield traits (Weikard *et al.,* 2005; Khatib *et al.,* 2007; Komisarek *et al.*, 2009; Viale *et al.,*2017)).

Gaolao cattle is a relatively fair milk yielder indigenous cattle breed well adapted to harsh, adverse and tropical climatic conditions of the Vidarbha region of Maharashtra state of India. Gaolao Breed is known by her prominent frontal bone and short stumpy horns. Candidate genes have been consistently reported from different studies for their association with the milk production traits (Strucken *et al.,* 2015). PPARGC1A was analyzed using a genome-wide association study approach to identify significant SNPs strongly linked with milk traits. Because of this, it is needed to study PPARGC1A gene to develop a database of variations linked with milk traits for their possible further use in improving productive and healthy Gaolao population.

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MATERIALS AND METHODS

Experimental animals and data collection

The adult milking Gaolao cows were selected for the current study from Gaolao farmers of the breeding tract. Around 05 ml blood was collected from 224 purebred adults milking Gaolao cattle using sterile needle and anticoagulant. The collected blood was stored at 4°C temperature until DNA isolation. Milk in the quantity of around 20 ml was collected from 135 adult milking cows to estimate milk components like fat%, protein%, SNF% and Lactose%, *etc.*

Nucleic acid extraction, quality and quantity estimation

The nucleic acid extraction was carried out using phenol, chloroform-isoamyl alcohol extraction protocol (Sambrook and Russel, 2001) with minor modifications and also using Kits. The quality and quantity were checked using a spectrophotometer and agarose gel electrophoresis. The genomic DNA was stored at -20°C in a freezer which was later used to prepare a working solution for PCR reactions.

Polymerase chain reaction

The primers were custom synthesized from a company for their use in amplification of 3′UTR and Intron-9 regions of PPARGC1A gene. The information about the gene region, amplicon product size, annealing temperature, primer sequence is given in Table 1. The PCR amplification of 3′UTR and intron-9 regions of PPARGC1A gene was carried out using PPARGC1AG1 and PPARGC1AG4 primers. The PCR amplification conditions for PCR components and thermal cycling conditions were optimized in the laboratory. PCR amplification was performed in a volume of 25 µl containing 12.5 µl 1X PCR buffer, 10 pM of each primer, 4% DMSO and 50-100 ng genomic DNA. The cycling protocol was as follows: 5 min at 95°C, 32 cycles of denaturing at 94°C for 45 seconds, annealing at Tm°C for 45 seconds (Table 1) and extension at 72°C for 45 seconds, with a final extension at 72°C for 7 min. The PCR amplifications were assessed in 1.5% agarose gel electrophoresis by loading 4µl of PCR product mixed with ethidium bromide (10 mg/ml). The stained PCR gels were visualized using UV fluorescence and documented in a gel documentation system (BR Biochem).

PCR-RFLP analysis

The quality PCR amplicons were used for PPARGC1AG1-*Nhe*I PCR-RFLP analysis in 224 animals of Gaolao cattle. The restriction digestion of the regions of the PPARGC1A gene was carried out using 5U/ µl of *Nhe*I restriction enzyme and 0.1- 0.5 µg PCR product of each primer and incubated at 37°C temperature for 14 hours. Later restriction enzyme digested PCR products were resolved in 3.5% agarose gel electrophoresis using ethidium bromide staining and later documented under GelDoc system.

PCR-SSCP analysis

SSCP analysis was carried out in 43 animals with the vertical electrophoresis apparatus (Bio-rad). The 8 % polyacrylamide gel was prepared using acrylamide: bisacrylamide (49:1), 10X TBE buffer, glycerol and autoclaved distilled water and electrophoresis was run at 150 volts for 5-6 hrs at 25°C temperature. After completing the electrophoresis run, the SSCP gel was stained using the standard 0.01% silver nitrate staining procedure with some modifications in the laboratory.

SNP analysis using bioinformatics tools

The obtained DNA sequences were analyzed using various bioinformatics tools (BioEdit BLAST, Clustal Omega) to detect nucleotide substitutions.

Statistical analysis

Genotype and allele frequencies were estimated using POPGENE Ver-sion 1.31 (Yeh and Boyle, 1997). The obtained gene and genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium using a Chi-square test (Devlin and Risch, 1995). The relationship between the milk component and genotype was tested using one-way ANOVA. The mathematical model is as below,

$$GT_{ijklm} = \mu + MY_i + F_j + S_k + L_l + P_m + + e_{ijklm}$$

Where,

GT_{ijklm} is the observed genotype.

μ = Overall mean.

MY_i= Fixed effect of Milk Yield.

F_j = Fixed effect of Fat %.

S_k = Fixed effect of the SNF %.

L_l = Fixed effect of Lactose %.

P_m = Fixed effect of the Protein %.

e_{ijklm} = Residual(error) effect of each observation.

All the analysis was done by SPSS Version 20 (IBM, USA).

Table 1: Name, region, nucleotide sequences, product sizes and annealing temperature of primers used for amplification of PPARGC1A gene in Gaolao cattle.

Names of primer	Region of gene	Primer nucleotide sequence	Product size (bp) / Tm (°C)
PPARGC1AG1	3'UTR	F:5′GCGAGCACGGTGTTACATTACTAAGGAGAGTTGGCTAG3′ R:5′GTTGTGTTGCACTCAATGGAC3′	357 bp/60°C
PPARGCA1G4	Intron-9	F:5′CATAGCCGGCG**C**CCCAGGTAAGATGCACGTTGGC-3′ R: 5′-CTGGTACTCCTCGTAGCTGTC-3′	195 bp/50°C

Where, F- Forward primer, R- Reverse primer.

Tm- Annealing temperature.

RESULTS AND DISCUSSION

Information about particular candidate genes like PPARGC1A due to their relevant biological functions, the polymorphism details and their effects on economic traits will help understand a molecular pathway that leads to productivity variation between different individuals of the established breed.

PPARGC1AG1- *Nhe*I PCR-RFLP results

The 357 bp fragment of 3′UTR region of PPARGC1A gene was amplified using PPARGC1AG1 primer (Fig 1) and digested using *Nhe*I enzyme (Khatib *et al*., 2007). The PPARGC1AG1- *Nhe*I PCR-RFLP analysis in 224 animals revealed three different genotypes, *i.e*., AA with 357 bp band size, AB with 357 bp, 317 bp and 40 bp band sizes and BB with 317 bp and 40 bp band sizes (Fig 2). PPARGC1AG1-*Nhe*I locus was found polymorphic for SNP (C.3359 A>C) with genotype frequency for AA genotype as 0.69, AB =0.30 and BB= 0.01. The allele frequency for the A alleles was found at 0.84 and for the allele, B was 0.16 at the PPARGC1AG1- *Nhe*I locus (Table 2). The non-significant chi-square value indicated that all the genotypic frequencies in the population were not in Hardy-Weinberg equilibrium (P>0.01). The current study's findings are in line with the results reported by Basak *et al.* (2019) in 146 Deoni breeds of cattle for AA, AC and CC genotypes with frequencies as 0.75, 0.21 and 0.04, respectively. For this locus, Kowalewska-Łuczak *et al.* (2010) also found two genotypes *i.e.* AA and AC as 0.76 and 0.24 respectively in 181 Jersey cows.

However, for the University of Wisconsin (UW) dairy cattle population at this SNP (C.3359 A>C), Khatib *et al.* (2007) reported genotype frequencies as 0.163, 0.506 and 0.331 for AA, AC and CC, respectively and as 0.123, 0.430 and 0.447, for the Cooperative Dairy DNA Repository (CDDR) population respectively.Weikard *et al.* (2005) identified genotype frequencies of AA, AC and CC genotype as 0.15, 0.58 and 0.27, respectively in German Holstein cattle. Similarly, in South Anatolian Red (SAR) indigenous cattle breeds of Turkey, Atila *et al.* (2014) reported two genotypes, *i.e*., AA and AC, with 0.23 and 0.77 frequencies,

Table 2: Allelic and genotypic frequencies at PPARGC1AG1- *Nhe*I locus in Gaolao cattle population.

Loci	N	Genotypes	No. of genotypes		Frequency		χ^2	P
			Observed	Expected	Genotypic	Allelic		
PPARGC1A G1- Nhel	224	AA	154	156.8792	0.69	0.84 (A)	2.004305	0.156853
		AB	67	61.2416	0.30	-		
		BB	03	5.8792	0.01	0.16 (B)		

**P<0.01.

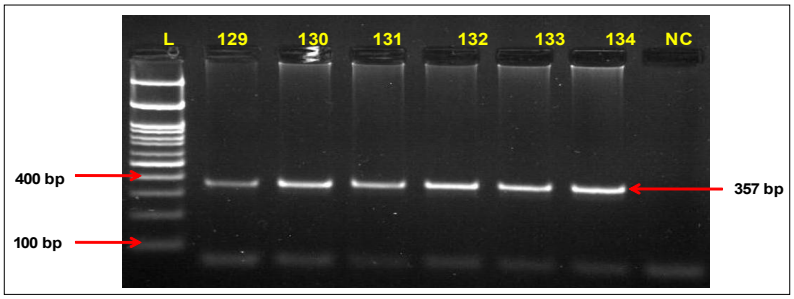

Fig 1: PCR amplification of 357 bp PPARGC1A 3' UTR gene fragment (PPARGC1A G1 primers) resolved in 1.5% agarose gel electrophoresis in Gaolao cattle.
Where, GB 129-134= Gaolao cattle number, NC- Negetive control, L= 100 bp DNA ladder (Himedia).

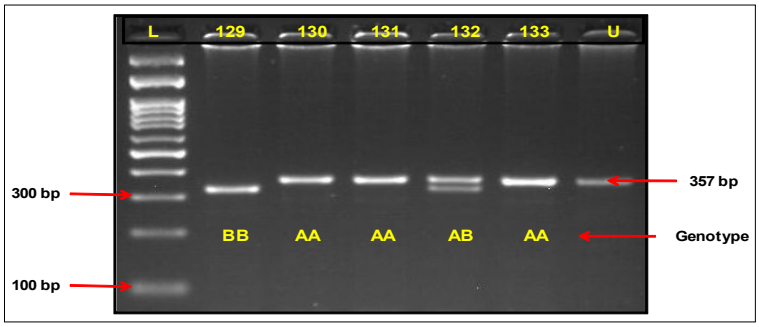

Fig 2: PCR-RFLP polymorphism (PPARGC1A G1-*Nhel*) 3' UTR region of PPARGC1A gene resolved in 3.5% agarose gel electrophoresis in Gaolao cattle.
Where, L= 100 bp DNA ladder (Himedia), GB 129-133= Gaolao cattle number, U- Uncut, AA, AB, BB= Genotype.

Table 3: Average effect of milk production traits at polymorphic PPARGC1AG1- *Nhe*I locus in Gaolao cattle.

Genotype	N	Fat % ± SE	SNF % ± SE	Lactose% ± SE	Protein% ± SE	MY ± SE	LN± SE
AA	94	4.34±0.45	8.57±0.05	4.39±0.04	3.15±0.03	4.16±0.18	2.63±0.14
AB	39	4.09±0.10	8.61±0.08	4.46±0.06	3.09±0.05	3.64±0.26	2.90±0.26
BB	02	3.85±0.15	8.65±0.25	4.23±0.12	2.94±0.05	4.00±2.00	2.00±0.00
Total	135	4.26±0.32	8.58±0.04	4.40±0.03	3.13±0.03	4.01±0.15	2.70±0.12

respectively. In East Anatolian Red (EAR) breeds, they found two genotypes, *i.e*., AA and AC, with frequencies as 0.40 and 0.60, respectively. However, Pasandideh *et al.* (2015) identified AA, AC and CC genotypes with frequency as 0.38, 0.52 and 0.10, respectively, in 398 Iranian Holstein cows. In the current study, no significant differences were found in milk production traits between all genotypes (Table 3) in 135 experimental animals. However, Khatib *et al.* (2007) found that A allele was associated with protein percentage in both populations for SNP A/C at position 3359 in the 3′UTR and milk traits in UW and CDDR populations. Similarly, in other studies (Weikard *et al.*,2005), the PPARGC1A- c.3359A>C SNP, have been reported to affect milk yield traits.

SNP detection at polymorphic PPARGC1AG1- *Nhe*I PCR-RFLP Locus

Three polymorphic genotypes were sequenced which revealed T-C SNP at 263rd position in the PPARGC1A gene sequence (Fig 3). The alignment of 279 bp PPARGC1AG1 sequence with *Bos taurus* reference sequence (KM111251.1) revealed 02 computational SNPs *viz*; C-T at 263rd position and A-T at 268th position. BLAST analysis of 279 bp sequence revealed sequence identity of 98.93% with cattle, buffalo and 98.21% with sheep and 97.96% with goat respectively. Distance tree was generated using pairwise alignments between obtained 279 bp sequence in Gaolao cattle with related database sequences (Fig 4). Researchers have performed the alignment analysis of bovine PPARGC1A which exhibited a similarity of 94% for humans and 91.1% for mice and rats (Weikard *et al.*, 2005).

PPARGC1AG4-SSCP and SNP detection

The PCR-SSCP analysis of 195 bp amplified fragment of PPARGC1A gene (Fig 5) was carried out in 43 samples using 8% PAGE. The silver staining of PPARGC1AG4 gene fragments revealed polymorphism with two SSCP patterns (A and B) at PPARGC1AG4-SSCP locus (Fig 6). The frequency of pattern A was 0.35 and of pattern B was 0.65 in 43 Gaolao animals. The analysis of SSCP Patterns

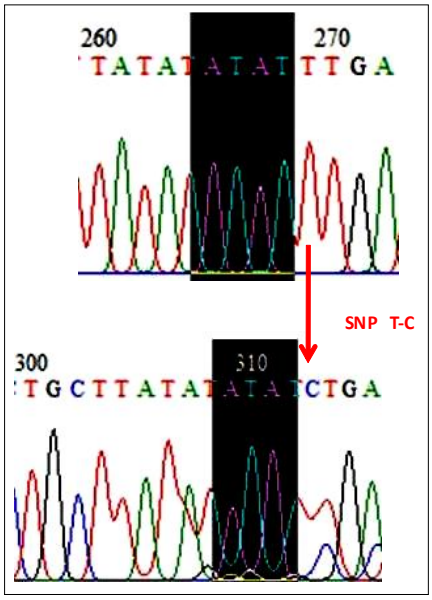

Fig 3: SNP T-C at 263rd position in 279 bp sequence of PPARGC1A G1 amplified PCR product in Gaolao cattle population.

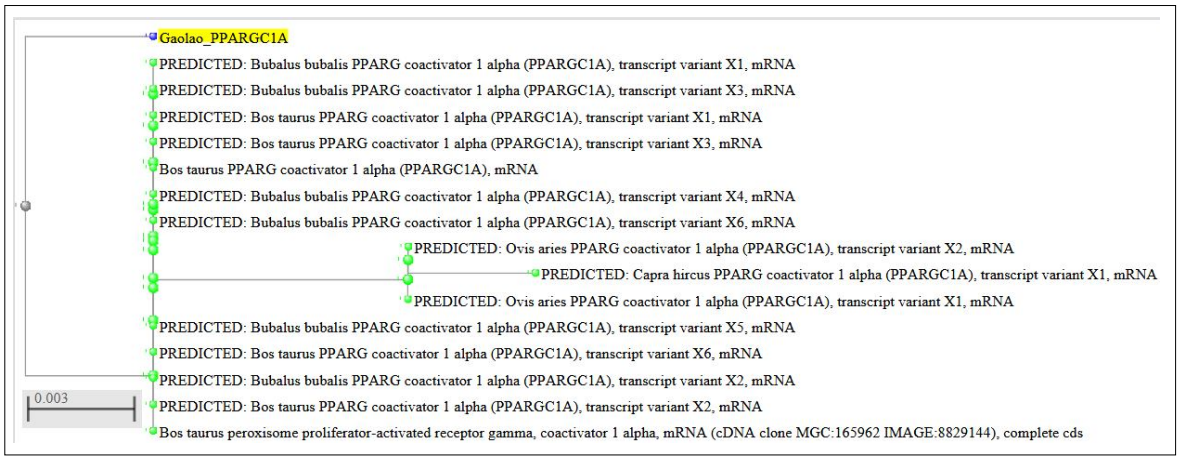

Fig 4: BLAST tree based on pairwise alignment between 279 bp query sequence obtained using PPARGC1A G1 primers and relevant data sequence.

Table 4: Average effect of milk traits at PPARGC1AG4-SSCP locus in Gaolao cattle.

SSCP pattern	N	Fat%± SE	SNF%±SE	Lactose%± SE	Protein %± SE	Milk yield±SE	LN± SE
A	15	3.97±0.12	8.46±0.10	4.38±0.08	3.09±0.05	3.90±0.33	3.27±0.33
B	28	4.24±0.11	8.66±0.09	4.37±0.06	3.15±0.06	3.95±0.32	2.50±0.32
Total	43	4.15±0.08	8.86±0.07	4.37±0.05	3.13±0.03	3.93±0.24	2.76±0.24

* indicates statistically significant at 0.05 ($p<0.05$) level.

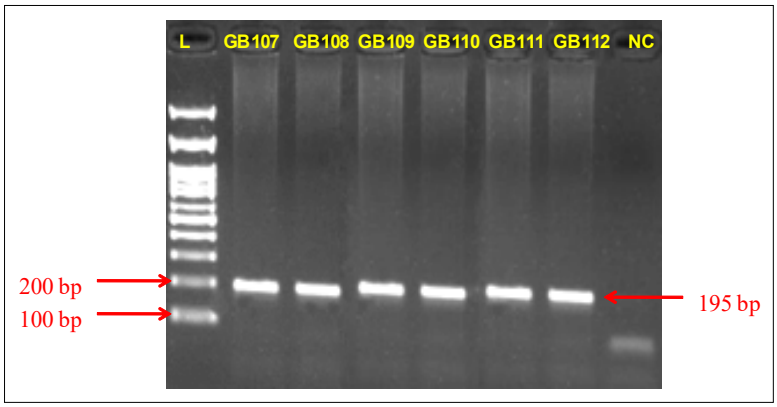

Fig 5: PCR amplification of 195 bp fragment of Intron-9 region of PPARGC1A gene (PPARGC1A G4 primers) resolved in 1.5% agarose gel electrophoresis in Gaolao cattle.
Where, GB 107-112= Gaolao cattle number, NC- Negetive control, L= 100 bp DNA ladder (Himedia).

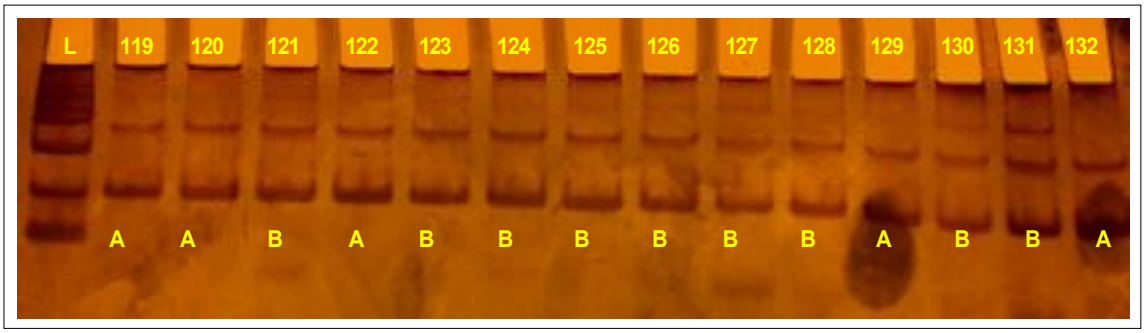

Fig 6: Polymorphic PPARGC1A G4-SSCP pattern in intron 9 region of 195 bp gene fragment of PPARGC1A gene (PPARGC1A G4 primers) in Gaolao cattle and visualised using 8% non-denaturing PAGE stained by silver staining.
Where, A- SSCP pattern-1, B- SSCP pattern-2 and L- Ladder. GB 119-132= Gaolao cattle sample numbers.

revealed no significant differences in milk production traits (Table 4). However, it was found that individuals with SSCP patterns B exhibited relatively more fat%, SNF%, lactose%, protein % and milk yield than patterns A. Two SSCP patterns A and B were sequenced which revealed nucleotide substitution indel SNP N-T at 116th position in Gaolao Cattle. However, Weikard *et al.* (2005) revealed SNP c.1892+19T>C in intron 9 of PPARGC1A gene exhibiting three genotypes TT, TC and CC with frequencies as 0.01,0.30 and 0.68 respectively in German Holstein. Khatib *et al.* (2007) reported three genotypes TT, TC and CC with frequencies as 0.019,0.65 and 0.331 respectively and a significant association between milk yield and milk fat traits. Schennink *et al.* (2009) screened Holstein Friesian Cattle for SNP c.1892+19T>C and reported frequency of C allele as 0.75 and T allele as 0.25, respectively. Boleckova *et al.* (2012) also reported the frequency of C allele as 0.80 and T allele as 0.20, respectively, in Czech Fleckvieh cattle. Kowalewska-Łuczak *et al.* (2010) also reported frequency of C allele as 0.37 and T allele as 0.63 respectively in 181 Jersey cows and revealed no significant association. Komisarek *et al.* (2009) also reported the frequency of C allele as 0.73 and T allele as 0.27 respectively in Polish HF cows and revealed no relation of T allele and milk yield traits. Alim *et al.* (2012) also reported the frequency of C allele as 0.69 and T allele as 0.31 respectively in Chinese HF cows and revealed the relation of TT genotype with milk higher protein level. Basak *et al.* (2019) also reported the frequency of the C allele as 0.635 and T allele as 0.365, respectively, in Deoni cattle. Atila *et al.* (2014) also reported the frequency of C allele as 0.65 and T allele as 0.35 respectively in South Anatolian Red cattle. Atila *et al.* (2014) also reported the frequency of C allele as 0.80 and T allele as 0.20, respectively, in East Anatolian Red cattle. They revealed the relation of CC genotype with high

milk fat%. Pasandideh *et al.* (2015) also reported the frequency of C alleles as 0.56 and T alleles as 0.44 respectively in Iranian Holstein cows. They revealed the relation of TT genotype with high-fat percentage and TC genotype was associated with milk protein.

CONCLUSION

PPARGC1AG1- *Nhe*I locus PCR-RFLP locus at 32 UTR region of PPARGC1A gene was polymorphic for SNP (C.3359 A>C) with frequency for the A alleles as 0.84 and allele B allele as 0.16 in 224 Gaolao cattle population; however, no significant differences were observed in milk traits between in all genotypes at PPARGC1A-*Nhe*I polymorphism. The comparison in PPARGC1AG4-SSCP Patterns revealed no significant differences in milk production traits. PPARGC1AG1- *Nhe*I PCR-RFLP genotype and PPARGC1AG4-SSCP pattern-based sequencing revealed SNPs in the PPARGC1A gene sequence in Gaolao cattle population. The identified genetic variation information at these loci in the PPARGC1A candidate gene of Gaolao cattle will aid in future studies for identification of significant associations for the development of markers for selection and improvement in milk production traits.

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Conflict of interest: None.

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