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Association of prolactin gene polymorphism with milk production traits in Gaolao cattle

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ABSTRACT

The present study aimed to identify the DNA polymorphisms within prolactin gene regions of Gaolao cattle and to investigate their relation with milk production traits. The genomic DNA was isolated from 245 unrelated animals of Gaolao cows, and test-day milk traits data were recorded. PCR-RFLP, PCR-SSCP and direct sequencing methods for mutation confirmation were used to study polymorphism within the prolactin gene. The PCR-RFLP genotyping at the exon-3 region of the prolactin gene revealed polymorphism and found an association of the PRLG1-RsaI 'AA' genotype with milk yield (5.05±0.14*) in Gaolao cattle. The exon-4, intron-3,4, Exon-5, 5'flanking, and exon-1 regions of prolactin revealed SSCP polymorphisms and SNPs. The results of the study indicate the existence of a substantial amount of genetic variation within the above-studied regions of the prolactin candidate gene. The identified association at PRLG1-RsaI genotype with milk yield will aid in future gene-assisted selection and improvement strategies in Gaolao indigenous cattle.

Keywords: DNA marker, Milk production traits, Prolactin, Zebu cattle

INTRODUCTION

Milk production is a quantitative trait controlled by the cumulative additive effect of various candidate genes. Genome-wide association studies (GWAS) targeting candidate genes related to milk production traits are essential for identifying statistically significant SNPs, which might prove potentially crucial in the efforts for marker identification. Currently, various genomic research studies emphasizing the identification of markers for bovine milk production traits are being reported worldwide (Kim *et al.,* 2021; Atashi *et al.,* 2022). Genome-wide association studies use high-density SNP chip technology to locate genes related to traits like milk production, which has the advantage of detecting effective causal alterations compared with traditional strategies (Hirschhorn and Daly, 2005). The findings of these studies may be used for future implementation of genomic evaluation to improve the milk productivity of indigenous cattle breeds. The functional gene markers, exhibiting an effect on milk production traits in many breeds, could provide useful information for the characterization and evaluation of marker -assisted selection programs in indigenous dairy cattle.

Prolactin (PRL) is essential for initiating and maintaining lactation, being also mainly respon-

sible for the synthesis of milk proteins, lactose, lipids, and other major milk components (Le Provost *et al.,* 1994). Prolactin is a lactotropin and polypeptide hormone of approximately 22 kDa molecular weight, secreted from the anterior pituitary gland (Bole-Feysot *et al.,* 1998). The bovine PRL gene maps to chromosome 23 and encompasses five exons spanning a 10 kb genomic segment and encoding a 199 amino acid mature protein (Cao *et al.,* 2002). Several polymorphic sites have been detected within t he PRL gene, and significant associations betwe en PRL variants and milk production traits have been described in dairy cattle (Udina *etal.,* 2001; Brym *et al.,* 2005; Patel *et al.,* 2017; Thuy *et al.,* 2018; Semerci and Balcioğlu 2022). Several polymorphic sites have been detected within the PRL gene, and significant associations between PRL variants and milk production traits have been described in dairy cattle (Udina et al., 2001; Brym et al., 2005; Patel et al., 2017; Thuy et al., 2018; Semerci and Balcioğlu 2022). Gaolao is a unique and vital zebu cattle breed of the Vidarbha region of Maharashtra and is showing drastic decline. Gaolao cattle is a fair yielder, disease-resistant, thermotolerant one, but still well-adapted to adverse climatic conditions (Patil *et al.,* 2005). For genetic improvement of yielder small population cattle, it is essential to generate genomic variants related to economic traits for their utilization in traditional selection methods. The current study was planned to investigate prolactin gene polymorphisms and their association with milk production traits in Gaolao cattle.

MATERIALS AND METHODS

Experimental Animals

The present study was carried out on 245 adult purebred type unrelated milking Gaolao cattle reared by the farmer's from villages of the Wardha district. The test day phenotypic data for milk production traits was generated followed by survey in the villages of Wardha district. Blood samples were collected aseptically by jugular vein puncture in a sterile vacutainer (Merck). The experiment and research plan under the SERB-DST project (File No. EMR/2017/000323) were duly approved by the Institutional Animal Ethics Committee (No. NVC/IAEC/24/2019 Dt.12/04/2019).

DNA Extraction, PCR Amplification

DNA from each animal was isolated using a DNA extraction kit (HiPura DNA extraction Kit, Himedia). Based on the review and literature survey, the gene fragments of the prolactin gene were selected for molecular screening. For the present study of PRLG1, PRLG2,

Table 1. Details of Primer Sequences, Amplified Regions, Annealing Temperatures and Product Sizes for Prolactin Gene Polymorphism in Gaolao Cattle

Amplified Region	Primer Sequence	Product Size (bp)	Annealing Temperature	Techniques Used
PRLG1/ Exon-3	F:5′CGAGTCCTTATGAGCTTGATTCTT3′ R:5′GCCTTCCAGAAGTCGTTTGTTTTC3′	156	59°C (30 cycles for 30s)	PCR-RFLP
PRLG2/ Exon4, Intron3&4	F:5′CACATGTTACCAAATCCACTGAA3′ R:5′CTCACCTGGCCAAATATCATCTC3′	249	53 °C (35 cycles for 45s)	PCR-SSCP
PRLG4/ Exon-5	F:5'TGATACACTGGCTCCAAAATCC3' R:5'TCCTTAGTTTGCAGGGACGG3'	207	53 °C (35 cycles for 45s)	PCR-SSCP
PRLG5/5' Flanking region & Exon1	F:5'GGCAAAAGGGAAGGGAATGC3' R:5'ACCTTTCTGCGACGAACCTT3'	165	53 °C (35 cycles for 45s)	PCR-SSCP
PRLG6/ CDS	F:5'GCCAGGTATCCCTTCGAGAC3' R:5'AAATTGAAACAGGTATGTCACTGC3'	304	53°C (35 cycles for 45s)	PCR-SSCP

PRLG4, PRLG5 and PRLG6 fragments of prolactin gene, the primers were custom-synthesized from Eurofins Genomics Pvt. Ltd. India. The details about primer sequences, amplified regions, annealing temperatures, and product sizes are given in Table 1.

PCR reactions for selected regions were performed in the 25 µl reaction mixture containing 12.5 µl DreamTaq Green PCR Master Mix (Thermofisher Scientific), 1.0 µl forward and reverse primer (PRLG1, PRLG2, PRLG4, PRLG5 and PRLG6), 7.5 µl molecular biology grade water and 3.0 µl genomic DNA. The PCR products were separated on 1.5 to 2% agarose gel (w/v) stained with ethidium bromide. The PCR-RFLP analysis was carried out using 12 µl PCR products digested using 5 Units of *Rsa*I restriction enzyme along with molecular biology grade water and cut smart buffer and were incubated at 37°C for 17 h. The restriction digested products were PCR-RFLP marker technique analysis results using *Rsa*I enzyme for PRLG1 amplicon were separated on 4% horizontal agarose gel electrophoresis due to large fragment size difference. However; PRLG2, PRLG4, PRLG5 and PRLG6, regions of prolactin gene were analyzed using PCR-SSCP marker technique in which smaller base pair differences can only be detected in high resolution vertical gel of high percentage for high resolution of differences. . To detect polymorphism at PRLG2, PRLG4, PRLG5 and PRLG6, regions of prolactin gene 10,10, 12 and 8% PAGE were used and subjected to silver nitrate staining.

Genotyping and Data Analysis

The Popgene32 software (Yeh et al. 1999) was used for the estimation of gene and genotypic frequencies and to test Hardy-Weinberg equilibrium. The representative polymorphic genotypes were subjected to purification and Sanger sequencing. The effect of prolactin gene polymorphism on milk yield and components was tested by logistic regression model using SPSS Version20 (IBM, USA). The model used was as follows,

$$\ln(P_{ijk}/1\text{-}P_{ijk}) = \beta_0 + \beta_i G_i + \beta_j P_j + e_{ijk}$$

Where, P_{ijk} is the probability of the desired class of observation for several dependent variables such as milk yield, fat percentage, SNF percent, Lactose percent and protein percent. β_0 = the intercept; G_i is the effect of i^{th} Genotype with corresponding regression coefficient indicated by β_i. P_j is the j^{th} fixed effect of parity on the dependent observation with corresponding regression coefficient β_j. e_{ijk} is the residual error corresponding to the responding variable.

RESULTS AND DISCUSSION

The quantitative nature of economic traits is more challenging for identifying and developing genetic markers for these traits. Hence, exploring QTLs to identify SNPs and their effect

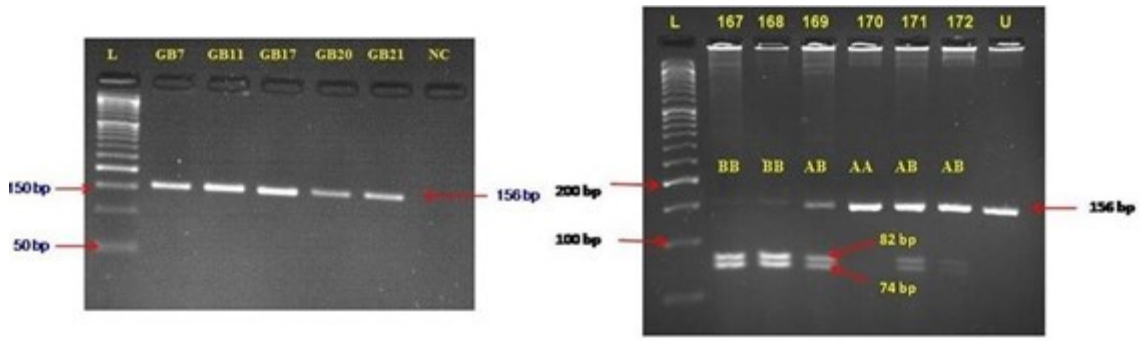

Figure 1. Electrophoresis profiles (a)PRLG1-PCR amplification of 156 bp exon-3 region of prolactin gene (b) PCR-RFLP polymorphism (PRLG1-Rsal) resolved in 4% agarose gel electrophoresis in Galao cattle. Where GB7-21 and 167-172=Galao cattle numbers, NC-negative control, U- uncut AA genotype = (156 bp), BB=genotypes (82 and 74 bp), AB=(156, 82 and 74 bp).

on economic traits will aid in gene-assisted selection.

PCR amplification and PRLG1 –RsaI genotyping

PCR amplification of 156 bp amplicon was generated using PRLG1 primers encompassing the exon-3 region in the experimental Gaolao cattle population. The *Rsa*I genotyped mutation resolved into three genotypes with fragment sizes; AA with 156 bp, AB with 156, 82 and 74 bp & BB with 82 and 74 bp and sizes (Figure1).

The PRLG1 –*Rsa*I locus was found to be polymorphic with the frequency for the A allele was found to be 0.625 and for allele B was 0.375 at this locus. (Table 2). The $\chi 2$ test value observed for the exon-3 region at the PRLG1-*Rsa*I locus of the prolactin gene showed significant deviation from H-W equilibrium ($p<0.05$). The association study of PRLG1-*Rsa*I polymorphism

Table 2. Genetic Diversity Measures at PRLG1-*Rsa*I Locus in Exon3 Region of Prolactin Gene in Gaolao Cattle

n	Genotypes	Frequency		$\chi 2$	p	n_e	H_e	* I
		Genotypic	Allelic					
245	AA (72)	0.294	0.625(A)	40.77	0.00*	1.88	0.47	0.6618
	AB (162)	0.661	0.375 (B)					
	BB (11)	0.045	-					

Significant ($p<0.05$), H_e = Expected Heterozygosity, Ne =Effective number of alleles

Table 3. Least Squares Means And Standard Errors (SE) For Milk Production Traits at Polymorphic PRLG1-*Rsa*I Locus Genotypes in Gaolao Cows

Genotypes	n	Fat % ± SE	SNF % ± SE	Lactose% ± SE	Protein% ± SE	MY ± SE	LN± SE
BB	10	4.09±0.24	8.66±0.11	4.35±0.06	3.12±0.04	3.80±0.50	2.6±0.34
AB	117	4.42±0.36	8.71±0.05	4.53±0.04	3.15±0.02	4.68±0.16	2.90±0.15
AA	92	4.13±0.10	8.56±0.08	4.62±0.04	3.16±0.03	**5.05±0.14***	2.72±0.11
Total	219	4.28±0.20	8.64±0.04	4.56±0.03	3.15±0.02	4.80±0.11	2.81±0.09
p value	-	0.76	0.30	0.08	0.92	0.03	0.58

LN= Lactation Number * Significant ($p<0.05$)

Table 4. Multinomial Logistic Regression Analysis for Effect of Genotypes on Milk Yield

Genotype[a]		B	Std. Error	Wald	Sig.	Exp(B)	95% Confidence Interval for Exp(B)	
							Lower Bound	Upper Bound
AA	Intercept	0.040	0.904	0.002	0.965			
	Milk Yield	0.491	0.212	5.389	0.020	1.634	1.079	2.474
AB	Intercept	1.016	0.868	1.368	0.242			
	Milk Yield	0.340	0.207	2.710	0.100	1.405	0.937	2.107

a= The reference category is: BB

with milk production traits revealed significantly ($p<0.05$) higher milk yield of 5.05±0.14 kg for the AA genotype compared to other genotypes (Table 3 and 4) in 219 Gaolao cows.

Data was analyzed by multinomial logistic regression which revealed that the model fit with a chi-square value of 6.834 which was significant ($p<0.05$). The chi-square goodness of fit indicated data was sufficient for the fitness of the model ($p>0.05$). The pseudo R^2 value of 0.038 indicated that the independent variables exhibited relatively less effect on dependent variables. Table 4 indicates that the genotype AA differed significantly for milk yield as compared to other genotypes.

In the present study, the PRLG1 –*RsaI* locus was polymorphic with an 'A' allele frequency of 0.625 and a 'B' allele frequency of 0.375, indicating polymorphism at the locus in 219 Gaolao cows. In the current population, at the PRLG1-*Rsa*I locus, the AA genotype was found associated with milk yield. Various other studies have reported similar results; a prolactin gene polymorphism study at the same locus in 390 animals of Turkey cattle reported the frequency of an 'A' allele as 0.61 and a B allele as 0.39 in agreement with our study (Akyuz *et al.,* 2014). Genetic polymorphism study at the Prolactin –*Rsa*I locus and reported the frequency of the 'A' allele as 0.62 and of the 'B' allele as 0.38 in 40 random samples of Gaolao cattle (Sodhi *et al.,* 2011). PRL gene polymorphism at the locus reported the frequency of A allele as 0.58 and of B allele as 0.42 in 720 Holstein –Russian cows (Wojdak *et al.,* 2008). The study reported a frequency of the A allele as 0.63 and the B allele as 0.37 in 54 Frieswal genotypes (Mahajan *et al.,* 2012) and found the association of AA genotype on milk yield.

In contrast to a current study, genotyping at same locus reported the frequency of AA genotype as 0.776 in American Swiss Cattle against 0.294 reported the association of AA genotype with favorable milk production during lactation compared to other genotypes (Alfonso *et al.,* 2012). Another study for detecting the relationship between PRL-*RsaI* polymorphism and milk traits in 120 animals of Montebeliard cattle reported AA genotype frequency as 0.81 and indicated a relationship with high milk yield (Barthez *et al.,* 2016). PRL polymorphism study in 200 Gir and 100 Kankrej animals revealed the frequency of the AA genotype as 0.25 and 0.13, respectively and they also reported the favorability of the AA genotype for milk yield (Patel *et al.,* 2017). The screening of 427 black and white Jersey cattle at the exon-3 region at the PRL-*Rsa*I locus revealed the frequency of AA genotype as 0.09 but indicated its statistical association with milk traits in Jersey cows (Dybus *et al.,* 2005). The association of prolactin gene variation with milk traits in 126 Frieswal cattle revealed the frequency of the AA genotype as 0.59 which indicated an association with milk traits (Singh *et al.,* 2015). The polymorphism in 105 animals of Sahiwal cattle revealed frequency of the AA genotype as 0.30 and indicated a relation with milk yield (Karuthadurai *et al.,* 2021). PRL gene polymorphism in 150 animals of Holstein black -and -white breed and Holstein heifer crossbred revealed the frequency of AA genotype as 0.79 and indicated its relation with increased milk yield (Gilmanov *et al.,* 2021). The genetic variability of the prolactin gene and its impact on milk composition was assessed in 225 animals consisting of Sahiwal, Rathi and Kankrej breeds; in which they reported the frequency of AA genotype as 0.27, and association of AA genotype with milk protein (Aggrawal *et al.,*2020). Another study, they reported the frequency of the AA genotype as 0.27, and the association of AA genotype with milk protein (Agrawal *et al.,*2020). PRL polymorphism study in Lithuanian cattle revealed the frequency of AA genotype as 0.87 and indicated its relation with milk fat percent (Miceikiene *et al.,* 2006). The AA genotype found in the current Gaolao cattle population is relatively more related to increased milk yield and protein per cent which can be a promising candidate for selection of cattle for high milk yield.

The association of a marker with a trait is population as well as family dependent. The LD that exist in our population may or may not exist in the reviewed populations. Due to this reason, the associations may differ for same gene between diverse set of populations.

However, they reported an association of the B allele with milk yield (Semerci *et al.,* 2022). PRL association in 125 Russian Red Pied Cattle revealed A allele frequency as 0.8 and an

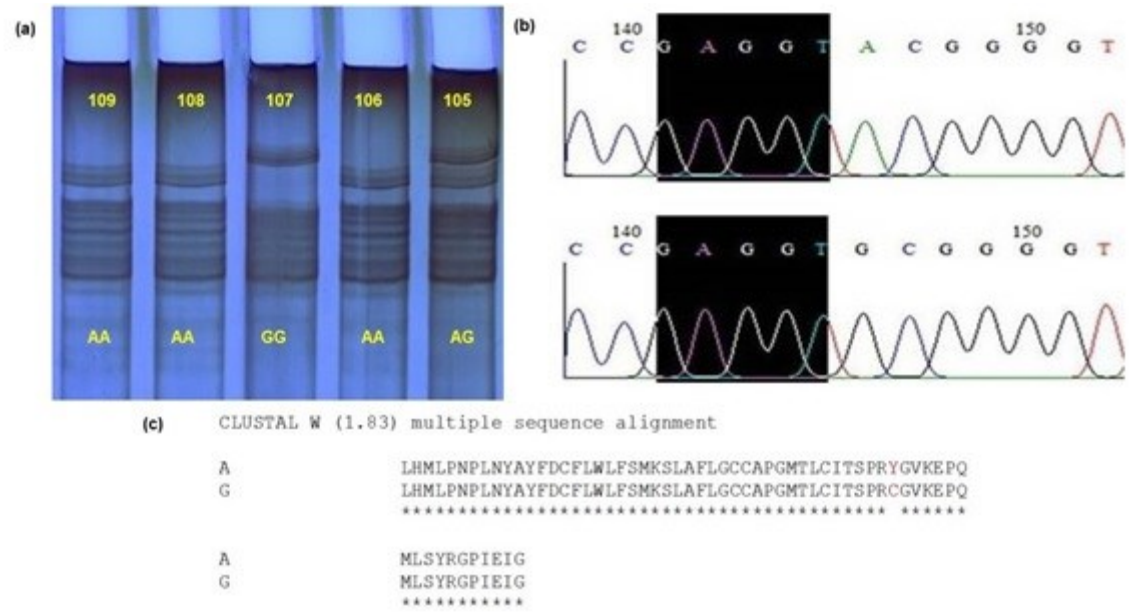

Figure 2. PRL G2-SSCP sequences results for intron-3,4 and exon-4 region of 249 bp genr fragment of prolactin gene (PRL) (a) polymorphic PRL G2-SSCP gel exhibiting three SSCO genotype patterns (b) chromatogram depicting SNP-A>G at 146th position in 201 bp PRL sequence (c) PRL G2-SSCP sequence variant aligment results indicating change in amino acid tyrosine Y (TYR) to cystine C (CYS) due to SNP A>G at 146th positin in 201 bp PRL sequence.

association of B allele with milk yield (Alipanah *et al.,* 2007). Based on the current study results and related studies, the exon – 3 region of the bovine PRL gene is an informative locus. The identified PRL-*RsaI* AA genotype can be a potent variant for the gene-assisted selection of dairy cattle.

PCR amplification and PRLG2- single-strand conformation polymorphism (SSCP)

The PRLG2-SSCP analysis of 249 bp amplicon encompassing intron 3, exon 4, and intron 4, regions of th e PRL gene was carried out in 56 samples at 10 % non-denaturing PAGE, 16^0C at 10-20 Volts/cm for 6:40 hrs duration (Figure 2). The silver staining of PRLG2 gene fragments of the PRL gene revealed polymorphism exhibiting three SSCP patterns with frequency as A = 0.23, B = 0.61 and C = 0.16 at PRLG2-SSCP locus (Figure2).

The sequences for PRLG2-SSCP patterns A, B and C were analyzed using Bio-Edit and Clustal alignment tools, revealing transition SNP A>G at 146th position in 201 bp PRLG2 sequence, which influenced the change in tyrosine to cysteine amino acid (Figure2). The association analysis of genotypes at PRLG2-SNPA>G-146 locus using logistic regression analysis did not reveal the significant association of the genotypes with milk production traits within the Gaolao cattle population (Table 5).

Prolactin gene fragment of 249 bp (PRLG2) encompassing intron-3, exon -4, and intron-4 region exhibited three SSCP patterns confirmed by direct sequencing. It revealed transition SNP A>G at 146th position influencing tyrosine to cysteine amino acid change. The association analysis did not reveal the significance of the genotypes with milk traits. Similarly, screening of intron 3, exon 4, and intron 4, region fragment of PRL gene using PCR-SSCP in 586 Chinese Holstein cattle revealed eight SNPs (Dong *et al.,* 2013). Out of them, the SNP at 7545 loci in exon-4 had revealed higher milk yield for A allele in the experimental population. Exon -4 region of 294 bp of prolactin gene was genotyped in 200 Sahiwal and Achai cattle samples and revealed the frequency of A allele as 0.19 and 0.44, of G allele as 0.81 and 0.56 in Sahiwal and Achai cattle breed populations, respectively (Ishaq *et al.,* 2013). The PRL polymorphism at exon -4 gene region in 186 animals of Holstein cattle

Table 5. Least Squares Means and Standard Errors (SE) for Milk Traits at Polymorphic SSCP Loci Genotypes of Prolactin Gene in Gaolao Cattle

1. PRLG2-SNP-A>G-146th	n	Fat% ± SE	SNF% ±SE	Lactose% ± SE	Protein % ± SE	Milk Yield ± SE	LN± SE
AA	34	4.36± 0.99	8.41± 0.19	4.62± 0.09	3.10± 0.05	5.12± 0.20	2.68± 0.20
AG	12	4.34± 0.09	8.41± 0.12	4.55± 0.07	3.09± 0.08	5.54± 0.36	2.50± 0.49
GG	10	4.1± 0.35	8.53± 0.16	4.70± 0.12	3.20± 0.10	5.30± 0.38	3.40± 0.59
Total	56	4.31± 0.09	8.43± 0.12	4.62± 0.05	3.11± 0.04	5.28± 0.15	2.77± 0.19
p-value	-	0.54	0.93	0.73	0.61	0.67	0.28
2. PRLG4-SNP-A>T-105th	n	Fat% ± SE	SNF% ±SE	Lactose% ± SE	Protein % ± SE	Milk Yield ± SE	LN± SE
AA	25	4.00± 0.14	8.71± 0.09	4.33± 0.06	3.14± 0.05	3.86± 0.35	2.80± 0.37
AT	5	4.12± 0.19	8.32± 0.22	4.35± 0.12	3.36± 0.13	5.80± 0.58	2.00± 0.44
TT	26	4.15± 0.08	8.64± 0.07	4.36± 0.06	3.16± 0.04	4.05± 0.33	3.73± 0.21
Total	56	4.08± 0.07	8.64± 0.05	4.34± 0.04	3.16± 0.03	4.12± 0.23	2.69± 0.20
p-value	-	0.65	0.19	0.95	0.18	0.07	0.55
3. PRLG5-SSCP	n	Fat% ± SE	SNF% ±SE	Lactose% ± SE	Protein % ± SE	Milk Yield ± SE	LN± SE
A	36	4.20± 0.09*	8.65± 0.07	4.35± 0.06	3.12± 0.04	4.50± 0.03	2.92± 0.28
B	17	3.96± 0.13	8.71± 0.11	4.38± 0.08	3.10± 0.05	3.73± 0.51	2.18± 0.23
C	3	3.37± 0.22	8.61± 0.25	4.31± 0.05	3.20± 0.10	3.17± 0.17	2.67± 0.33
Total	56	4.08± 0.08	8.67± 0.06	4.36± 0.04	3.16± 0.03	4.20± 0.24	2.68± 0.20
p-value	-	**0.02**	0.85	0.92	0.35	0.23	0.25

* Significant ($p<0.05$)

reported AA genotype frequency as 0.26 and AB genotype as 0.52, and for BB genotypes 0.22. They did not find an association of genotype with milk traits. The exon-4 region of the PRL gene was analyzed using PCR-SSCP in 392 Chinese Holstein cattle and reported 0.894 for allele A and 0.106 for allele B. Their association study revealed that genotype BB was responsible for higher milk yield (Hu *et al.,* 2009). Effects of milk traits were analyzed PRL SNP polymorphism for exon-4 in 315 Holstein cows and reported non-significant association results for the polymorphism (Rincon *et al.,*2013). The genetic variability of PRL was studied using PCR-RFLP in 262 animals of Bali cattle and reported gene frequency of allele A as 0.0467 and G allele as 0.9533 (Paramitasari *et al.,*2015). The effect of the prolactin (PRL) gene was investigated at the locus in 268 Iranian Holstein Bulls and reported the frequency of A allele as 0.069 and G allele as 0.931. They reported that the G allele was unfavorable for milk and protein yield. studied PRL polymorphism using SSCP and sequencing within exon-4 in 186 black and white cows and 138 Jersey cows and reported six SNPs by SSCP and direct sequencing. The association study revealed AG genotypes in black and white cows exhibited the highest milk yield (Brym *et al.,* 2005). PRL polymorphism was studied in 125 Holstein dairy

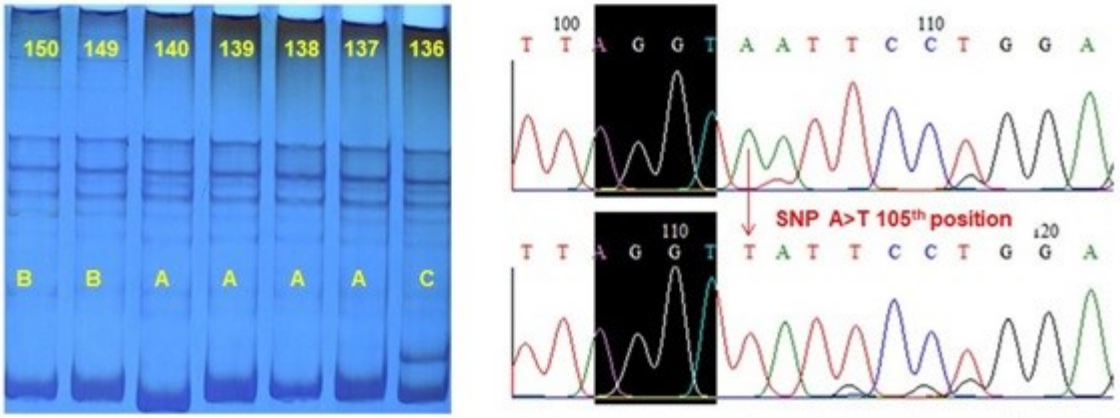

Figure 3. PRLG4-SSCP sequencing results of exon-r region of 207 bp gene fragment of prolactin gene (PRL G4) (a) PRLG4 PCR-SSCP gel profile of three pstterns, A, B, and C (b)chromatogram pattern of SNP A-T at 105th position in exon-5 sequence of prolactin gene in Gaolao cattle.

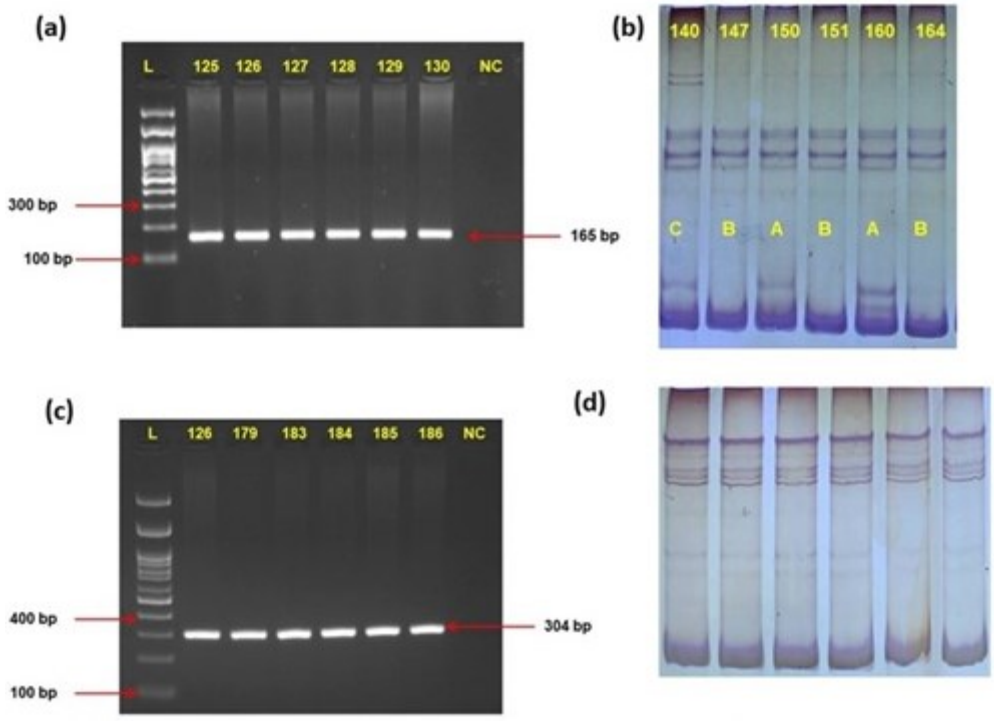

Figure 4. PCR amplication and SSCP results (a) PRL G5-PCR amplication of 5'-flanking and exon-1 region-615 bp (b) polymorphic PRL G5-SSCP resolved in 12% non-denaturing PAGE stained by silver staining (c) PRL G6-PCR amplication of CDS region-304 bp (d) monomorphic PRLG6SSCP patterns resolved in 10% denaturing PAGE stained by silver staining.

cows using RFLP and recorded the P allele as 0.824 and the C allele as 0.176. They reported that genotype PP exhibited more milk yield than other genotypes (Thuy *et al.,* 2018).

PCR amplification and PRLG4-SSCP Analysis

The PRLG4-SSCP analysis of 207 bp amplified exon-5 region of PRL gene (Figure 3)

was carried out in 56 samples in 10% non-denaturing PAGE, at 16^0C at 10-20 volts/cm, for 7 hrs duration using Vertical Gel Unit. The silver staining of PRLG4 gene fragments revealed polymorphism exhibiting three SSCP patterns with the frequency of Pattern A=0.446, B=0.464 and C = 0.089 at the locus (Figure 3). The sequences for PRLG4-SSCP patterns A, B and C were analyzed using Bio-Edit and Clustal alignment tools, which revealed transversion synonymous SNP A>T at 105^{th} position in 175 bp PRLG4 sequence (Figure 3). The association analysis of genotypes at the PRLG4-SSCP locus did not reveal a significant association of the genotypes with milk traits within the Gaolao population (Table 5).

In the present study, 207 bp fragment exon-5 region of PRL gene (PRLG4) was screened using SSCP and sequencing, which revealed three SSCPs after sequencing revealed transversion synonymous SNP A>T at 105th position. It did not reveal any significant association of the genotypes with milk traits within the Gaolao cattle population (Table 5). PRL gene polymorphism was analyzed using direct sequencing in 100 Pakistani cattle and reported 16 SNPs in all breeds indicating polymorphism. They found SNP 8447 was shared by all the studied breeds (Uddin *et al.,* 2013).

PRLG5-SSCP and PRLG6-SSCP Analysis

The PRLG5-SSCP analysis of 165 bp amplified 5' flanking and exon 1 region of PRL gene (Figure 4) was carried out in 56 samples in 12% non-denaturing PAGE, at 17^0C at 10 to 20 volts/cm for 7:10 h duration. Silver staining of PRLG5 gene fragments revealed polymorphism exhibiting three SSCP patterns (Patterns A, B and pattern C) at the PRLG5-SSCP locus (Figure 4). The frequency of these three PRLG2-SSCP patterns was found (A = 0.65, B = 0.30 and C = 0.05) in 56 Gaolao cattle. The sequences for PRLG5-SSCP patterns A, B and C were analyzed using Bio-Edit and Clustal alignment tools, revealing transition indel SNP N>T at the fifth position in the 112 bp PRLG5 sequence (Figure 4).

The association analysis of SSCP genotypes at PRLG5- SSCP locus revealed a significant association of the genotypes with milk fat percent within the Gaolao cattle population (Table 5). The PRLG6-SSCP analysis of 304 bp amplified CDS region of PRL gene (Figure 4) was carried out in 28 samples in 8% non-denaturing PAGE, 5:00 h duration, at 20°C at 10 to 20 volts/cm using Vertical Gel Unit. Silver staining of PRLG6 gene fragments of the PRL gene revealed monomorphism exhibiting single SSCP patterns (Pattern A) at the PRLG6-SSCP locus (Figure 4). The Prolactin gene of 165 bp fragment consisting of 5' flanking region and exon-1 region (PRLG5) in Gaolao cattle revealed three SSCP patterns PRLG5- SSCP A pattern revealed a significant association with fat percent in Gaolao cattle population. PRLG6 had SSCP monomorphism and was hence not processed for sequencing.

CONCLUSION

The genetic variation was found at exon-3, intron-3, exon-4, intron-4, exon-5, 5' flanking and exon-1 regions of the Prolactin gene in Gaolao cattle. The 'AA' genotype at PRLG1-*Rsa*I locus at exon-3 region of the Prolactin gene was associated with milk yield. The 5'flanking exon-1 region revealed an association of the SSCP-A genotypes with the milk Fat% trait. Prolactin gene fragments PRLG2-SSCP and PRLG4-SSCP revealed SNP A>G at the 146th position and SNP A>T at the 105^{th} position respectively. The identified genetic variation and association at PRLG1-*Rsa*I locus with milk yield will aid in the selection and breeding plans for the genetic improvement of Gaolao cattle.

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