



## Polymorphisms within Intron-4 and Exon-4 regions of *SPP1* gene and their association with milk traits in Gaolao cattle

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### ABSTRACT

*Secreted phosphoprotein 1 (SPP1)* is an acidic, phosphorylated glycoprotein found in fluids including milk and is involved in development of mammary gland and exhibits impact on milk composition and yield. The aim of study was to reveal *SPP1* gene polymorphisms and association with milk traits in Gaolao cattle. Blood was collected from 280 cows and test day milk was recorded. *SPP1G1-BsrI* locus in intron-IV was polymorphic and frequency of TT genotype was 0.99 and CT was 0.01, respectively. The association of *SPP1G1-BsrI* polymorphism with milk traits revealed significant difference in Milk yield and SNF% for CT genotype as compared to TT genotype. Sequencing of *SPP1G1-BsrI* locus-based TT and CT genotypes revealed G-A SNP at 32<sup>nd</sup> position. The *SPP1G5-SSCP* locus at exon-4 region was polymorphic with frequency for A pattern as 0.95 and for B patterns as 0.05. The association study, revealed significant differences for SNF% and Lactose% for *SPP1G5-SSCP* pattern A as compared to pattern B. Direct sequencing of SSCP pattern A and B of *SPP1G5-SSCP* revealed SNPs A-G at 71<sup>st</sup>, A-C at 109<sup>th</sup> and A-C at 208<sup>th</sup> position and 02 computational SNPs, viz. C-A at 109<sup>th</sup> position and C-A at 208<sup>th</sup> position. Identified significant association needs validation in large data sets with records for development of markers for bovine milk traits.

**Keywords:** Gaolao cattle, Polymorphism, Sequencing, *SPP1*

Currently, vast amount of animal genomic research work is being carried out to understand genetic mechanism behind milk production traits. Thus, exploring the genetic changes underlying preferred phenotypes is the target of today's animal producers. The possible genetic and economic benefits of gene assisted selection for improved productivity in dairy cattle have paved way for association of DNA markers with dairy traits (Korkuc *et al.* 2021).

*Secreted phosphoprotein 1 (SPP1)* is an acidic, phosphorylated glycoprotein found in many body fluids including milk and other tissues (Sodek *et al.* 2000). Previous studies relating to the polymorphism of the *SPP1* gene analysed its effects on milk yield and milk composition (Boleckova *et al.* 2012, Łuczak and Kulig 2013, Salehi *et al.* 2015, Manzoor *et al.* 2018). Genetic variants of *SPP1* had been proposed to be associated with milk fat (Khatib *et al.* 2007), milk yield (Mello *et al.* 2011), and protein content (Schnabel *et al.* 2005) in dairy cattle. It was also shown that the *SPP1* gene product has an essential role in the modulation of the expression of milk protein genes (Sheehy *et al.* 2009). Despite fulfilling so many functions, *SPP1* is treated by most authors as a positional candidate gene for milk performance traits harboring the

*QTL* region on BTA6 (Oztabak *et al.* 2008). SNPs play an important role in farm animal population structure, genetic differentiation, origin, and evolution research. Till date, many candidate genes or polymorphisms within these genes have been identified that have a positive correlation with milk production traits in dairy cattle.

The indigenous Gaolao cattle are fair milking, disease resistant, thermotolerant and the indigenous cattle breed adapted to harsh hot climate of Vidarbha region of Maharashtra. However, there are some unique animals in the breeding tract exhibiting high milk yield which need to be studied at gene level for understating the genetic mechanism behind milk production and its possible role in genetic improvement of the indigenous cattle resources. In view of above, the present study was planned with the objectives to detect *SPP1* gene polymorphisms and reveal association with milk traits in Gaolao cattle using PCR-RFLP and PCR-SSCP marker techniques.

### MATERIALS AND METHODS

*Experimental animals and DNA isolation:* Around 5 ml blood was collected from jugular vein in a centrifuge tube containing approximately 1.5 ml EDTA anticoagulant. The blood was collected from around 280 unrelated adult milking Gaolao cattle from the breeding tract, i.e. from farmer's herd located in villages of Wardha district and from Gaolao Cattle Breeding Farm, Hetikundi, Wardha. In the field conditions, the collected blood was mixed properly

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Table 1. Name of primer, region and product size for *SPP1* gene polymorphism study in Gaolao cattle

Name of Primer/region	Primer sequence	Product size (bp)/ Reference
<i>SPP1G1</i> / Intron-4	F:5' GCAAATCAGAAGTGTGATAGAC3' R:5' CCAAGCCAAACGTATGAGTT3'	290 bp/ Leonard <i>et al.</i> (2005)
<i>SPP1G5</i> / Exon-4	F:5' ACAGTAAGTGCTGTAAATGAATCT3' R:5' GCCTGCTTGGTGGCTATTTTT3'	362 bp/ Designed (AY878328.1)

F, Forward primer; R, Reverse primer.

and immediately kept in the ice box and transferred and later stored at 4°C temperature in refrigerator until extraction of quality DNA (Sambrook and Russell 2001). Around 15 ml test day milk was collected from the cows for estimation of milk components like Fat%, Protein%, SNF% and recorded.

**PCR amplification:** Reported and designed primers amplifying variable regions of bovine *SPP1* gene were selected for their use in current study (Table 1). The precise PCR amplification was optimised at 5 pM for *SPP1G1* and 10 pM for *SPP1G5* primers representing different regions. The primers were thoroughly optimised at different concentrations, quantity and different thermal cycling conditions.

**PCR-RFLP and PCR-SSCP analysis:** Restriction fragment length polymorphisms (RFLPs) were discovered by Botstein *et al.* (1980), which uses restriction enzymes that cut the DNA molecule at specific sites, called restriction sites, resulting in different fragments of variable lengths. The *BsrI* restriction enzyme was used for digestion of the amplified PCR products of *SPP1*. PCR-SSCP was used for detection of SNPs within amplified gene fragments based on the fragment mobility due to changes in secondary structure(s) in single-stranded DNA fragments in non-denaturing PAGE. The amplicons generated were visualised by electrophoresis and aliquot of 7 µl of each sample was mixed with 7 µl 2× SSCP gel loading dye. The denatured amplicons were resolved in 8% PAGE at low temperature (15-20°C) for duration of 05-06 h at 150V in the laboratory. The SSCP gel was stained using 0.01% silver nitrate and other procedure as per reported protocol with modifications in the laboratory (Orita *et al.* 1989).

**DNA sequencing, bioinformatics and statistical analysis:** Based on PCR RFLP and PCR-SSCP analysis results, PCR products amplified were selected for confirmation of mutation by sequencing. For the sequencing of *SPP1G1* and *SPP1G5* gene regions, quality PCR products and primers were sent. The obtained DNA sequences were edited and analyzed using bioinformatics tools. The relationship between the milk component and genotype was tested using one way ANOVA. The mathematical model used was:

$$Y_{ijklm} = \mu + F_i + S_j + L_k + P_l + GT_m + e_{ijklm}$$

Where,  $Y_{ijklm}$ , observation of milk yield;  $\mu$ , overall mean;  $F_i$ , fixed effect of Fat %,  $S_j$ , fixed effect of the SNF %;  $L_k$ , fixed effect of Lactose %,  $P_l$ , fixed effect of the Protein %;  $GT_m$ , random effect of genotype,  $e_{ijklm}$ , residual(error) effect of each observation. All the analysis was done by SPSS Version 20 (IBM, USA).

## RESULTS AND DISCUSSION

The *SPP1* is a highly phosphorylated glycoprotein in numbers of bovine tissues and milk. *SPP1* has been reported to be associated with milk production in cattle. It has shown that in the Holstein population, allele C of *SPP1* gene is associated with milk production traits (Khatib *et al.* 2007). In view of this, the study was planned to identify the genetic variation within *SPP1* gene and study association with milk traits in Gaolao cattle.

***SPP1G1-BsrI* PCR-RFLP analysis:** The PCR amplification of 290 bp fragment of intron-4 region of *SPP1* gene was carried out using *SPP1G1* primer (Fig. 1). For PCR amplification, the components were optimized at 10 pM concentration and thermal cycling conditions were optimized at 53°C annealing temperature. The 290 bp amplified products were digested with *BsrI* restriction enzyme as per the optimized PCR-RFLP protocol (Lali *et al.* 2020) with some minor modifications. Restriction digestion was carried out using 5 Units of *BsrI* restriction enzyme, 1× buffer and molecular biology grade water. The digested gene fragments were resolved in 3% agarose gel electrophoresis and later documented using gel documentation system.

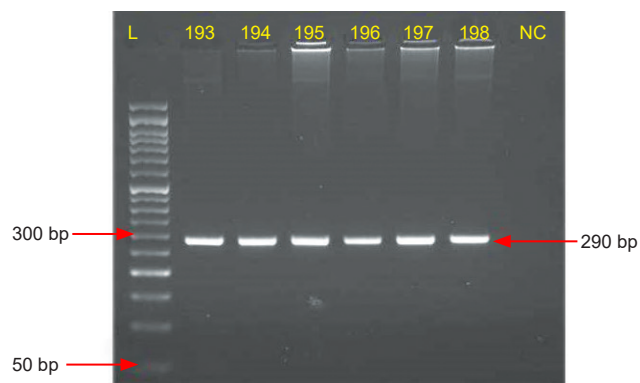


Fig. 1. PCR Amplification of 290 bp *SPP1G1* Intron-4 gene fragment (*SPP1G1* Primer) resolved in 2% agarose gel electrophoresis in Gaolao Cattle where, GB193-198=Gaolao cattle numbers, NC-Negative Control, L= 50 bp DNA Ladder (Hemedial).

*SPP1G1-BsrI* locus was found polymorphic (Fig. 2) with allele frequency for T alleles as 0.9946 and for C allele as 0.0054 (Table 2). An analysis of conformity with the Hardy-Weinberg principle showed that the population of Gaolao cattle has reached a genetic equilibrium with respect to *SPP1G1-BsrI* locus.

In present study, the frequency of TT genotype at *SPP1G1-BsrI* locus was high as compared to CC genotype.

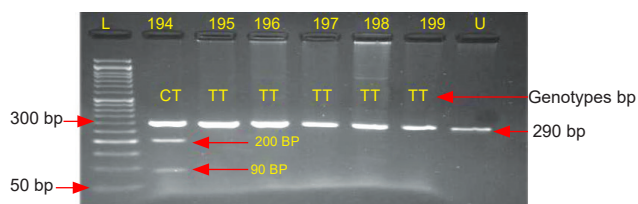


Fig. 2. Polymorphic PCR-RFLP (*SPP1G1-BsrI*) Intron-4 region of *SPP1G1* gene resolved in 3% agarose gel electrophoresis in Gaolao Cattle where, L, 50 bp DNA Ladder (Himedia, GB); 194-199, Gaolao Cattle Numbers; U, Uncut; TT, Genotypes (290 bp); CT, Genotype (290, 200 & 90 bp).

The other researchers also have reported similar kind of results. Sharma *et al.* (2019) conducted *SPP1* gene polymorphism study at intron-4 using *BsrI* enzyme and reported fixation of TT genotype (290 bp) in 147 animals of Sahiwal and Haryana cattle breeds. Rahmatalla and Brockmann (2015) investigated *SPP1* polymorphism and its effects for milk traits in 60 Sudanese dairy cattle breeds and reported fixation of T allele. Lali *et al.* (2020) studied effect of Osteopontin gene polymorphism on milk traits in 144 animals of Holstein Friesian crossbred cattle of Kerala and reported frequency of TT, CT and CC genotype as 0.60, 0.32, 0.08 respectively. In association study, they could not reveal any significant changes with different yield and composition traits of milk production. Oztabak *et al.* (2008) genotyped around 40 animals of South Anatolian Red (SAR) and East Anatolian Red (EAR) cattle using *SPP1-BsrI* PCR-RFLP and reported frequency of TT, CT and CC genotype as 0.55, 0.375, 0.075 respectively in South Anatolian Red (SAR). They reported frequency of TT, CT and CC genotype as 0.675, 0.325, and 0.00 respectively in East Anatolian Red (EAR). However, some of the other researchers had reported less frequency of T allele in contrast to the findings of present study.

***SPP1G1-BsrI* polymorphism and association analysis:** The association study of identified *SPP1G1-BsrI* polymorphism with milk traits using SPSS revealed that there was significant difference in Milk yield and SNF% traits for CT genotype as compared to TT genotype at *SPP1G1-BsrI* locus (Table 3) in 208 experimental Gaolao animals. It was found that the milk yield ( $7.33 \pm 0.67$ ) and

SNF% ( $9.03 \pm 0.08$ ) traits differed significantly at 5% level of significance for CT genotype as compared to TT genotype. However, other milk traits (Fat%, Lactose%, Protein% and Lactation Number) did not differ significantly at 5% level of significance for CT and TT genotypes at *SPP1G1-BsrI* locus.

Similar to the findings of the current study, Leonard *et al.* (2005) screened 214 Holstein cows using *SPP1-BsrI* PCR-RFLP and reported genotype frequency of TT, CT and CC genotype as 0.27, 0.48 and 0.25 respectively and also associated C allele with increase in milk protein % and fat % in the studied population. Khatib *et al.* (2007) carried out association of *SPP1-BsrI* polymorphism with milk production traits in 891 University of Wisconsin Holstein cattle populations and reported frequency of TT, CT and CC genotype as 0.26, 0.51 and 0.23 respectively and also associated C allele with increase in milk protein% and fat%. Mello *et al.* (2011) studied genetic diversity in 434 Girolando cattle using *SPP1-BsrI* PCR-RFLP in *osteopontin* gene and they revealed frequency of TT, CT and CC genotype as 0.52, 0.39 and 0.09 respectively. They observed that highest milk production in animals possessing at least one T allele indicating T allele might have role in milk production traits. Pasandideh *et al.* (2015) carried out association of bovine *SPP1* genes polymorphism with milk traits in 398 Holstein Friesian cattle and reported high frequency for C allele (0.47) as compared to the studies reported by other researchers. They reported association of T allele with milk fat percentage. Fontanesi *et al.* (2015) studied *SPP1* candidate gene polymorphism and association with milk traits in sires of Reggiana breed, they reported frequency of TT, CT and CC genotype as 0.82, 0.164 and 0.16 respectively and they reported association of C allele with increased milk yield and protein percentage. Boleckova *et al.* (2012) screened 505 Czech Fleckvieh cattle using *SPP1-BsrI* PCR-RFLP and reported frequency of TT, CT and CC genotype as 0.68, 0.28, 0.4 respectively. In association study, they also reported statistically significant trend of C allele association with protein percentage. Nasiri *et al.* (2014) studied *OPN* gene variations in 100 Iranian Holstein bulls and reported that

Table 2. Allelic and genotypic frequencies at *SPP1G1-BsrI* locus in Gaolao cattle population

Locus	N	Genotypes	No. of genotypes		Frequency		$\chi^2$
			Observed	Expected	Genotypic	Allelic	
<i>SPP1G1-BsrI</i>	280	TT	277	277.01	0.99	0.9946 (A)	0.005
		CT	03	2.99	0.01	0.0054 (B)	
		CC	00	0.01	0.00	-	

\*\*P<0.05.

Table 3. Average effect of milk production traits at polymorphic *SPP1G1-BsrI* locus in Gaolao cattle

Genotype	N	Fat % $\pm$ SE	SNF % $\pm$ SE	Lactose% $\pm$ SE	Protein% $\pm$ SE	MY $\pm$ SE	LN $\pm$ SE
TT	205	4.26 $\pm$ 0.21	8.64 $\pm$ 0.05	4.56 $\pm$ 0.03	3.15 $\pm$ 0.02	4.72 $\pm$ 0.11	1.41 $\pm$ 0.10
CT	03	4.73 $\pm$ 0.23	9.03 $\pm$ 0.08*	4.40 $\pm$ 0.25	2.90 $\pm$ 0.10	7.33 $\pm$ 0.67*	1.15 $\pm$ 0.67
Total	208	4.27 $\pm$ 0.21	8.65 $\pm$ 0.04	4.56 $\pm$ 0.03	3.15 $\pm$ 0.02	4.76 $\pm$ 0.11	2.82 $\pm$ 0.10

\*Indicates statistically significant at 0.05 (P<0.05) level.

Table 4. Average effect of milk traits at *SPP1G5-SSCP* in Gaolao cattle

SSCP Pattern	N	Fat% ± SE	SNF% ±SE	Lactose% ± SE	Protein % ± SE	Milk Yield ±SE	LN± SE
A	61	4.80± 0.67	8.66±0.05*	4.41±0.04*	3.15±0.03	4.16±0.25	2.56±0.18
B	03	4.13±0.24	7.96±0.43	4.07±0.09	2.95±0.19	5.00±0.57	3.67±0.88
Total	64	4.77±0.64	8.62±0.06	4.39±0.04	3.14±0.03	4.20±0.24	2.61±0.18

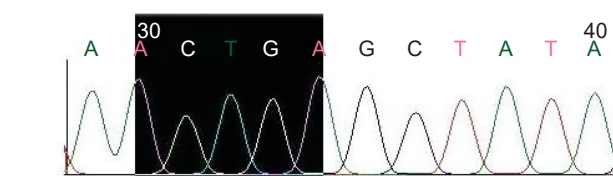
\*Indicates statistically significant at 0.05 (P<0.05) level.

this gene is essential for mammary gland growth as well as lactation. The amplicons of the PCR reaction were digested using the *BsrI* restriction enzyme. The PCR-RFLP results showed that the frequencies of CC, CT and TT genotypes of *OPN* gene were 0.17, 0.48 and 0.35 respectively, and the frequencies of C and T alleles were 0.41 and 0.59, respectively and they reported favourable effect of these two genes on milk production traits. Zakizadeh *et al.* (2015) screened population of 100 Brown Swiss cows using *SPP1-BsrI* PCR-RFLP in intron-4 region of *SPP1* gene and they reported genotype frequencies as TT, TC and CC genotypes as 0.32, 0.46 and 0.22, respectively and studied population was in accord with Hardy-Weinberg equilibrium. They didn't report significant association of genotypes with milk yield, Fat or Protein percent traits. Luczak and Kulig, (2013) studied genetic polymorphisms of *OPN* genes in 181 animals of Jersey cattle and reported frequency of TT, CT and CC genotype as 0.011, 0.411 and 0.578 respectively. They frequency of allele C (0.78) for allele T (0.22). They reported C allele was positively related with milk fat and protein content.

**DNA sequencing of polymorphic patterns at *SPP1G1-BsrI* locus:** In *SPP1G1-BsrI* Locus, the PCR products of the Intron-4 region of *SPP1* gene representing TT (GB195) and CT (GB194) genotypes were sequenced which revealed G-A SNP at 32<sup>nd</sup> position in 236 bp *SPP1* gene sequence (Fig. 3). BLAST based homology analysis of 236 bp *SPP1G1* sequence revealed sequence identity of 99.58 with European Cattle, 98.73 with Buffalo respectively.

***SPP1G5-SSCP analysis of 362 bp fragment of *SPP1* gene:*** One set of primer (*SPP1G5*) was designed using

#### SPP1G1-BsrI-TT



#### SPP1G1-BsrI-CT

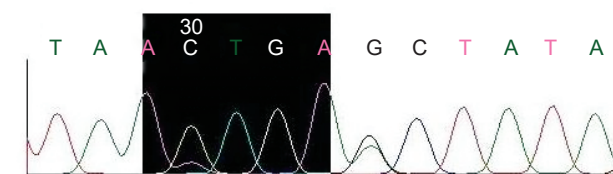


Fig. 3. SNPs at G-A at 32<sup>nd</sup> position in 236 bp sequence of *SPP1G1* amplicon in polymorphic *SPP1G1-BsrI* PCR-RFLP in Gaolao Cattle Population

GenBank sequence (AY878328.1) in such a way that 362 bp region of exon 4 of *SPP1* gene was amplified at 53°C annealing temperature (Supplementary Fig. 1). The PCR products were electrophoresed in 2% agarose gel at 100V for 45 minutes along with 50 bp DNA ladder as marker and visualized under Gel Doc System (Bio-Era).

The amplified PCR products of different fragments were analyzed by SSCP analysis. The different SSCP band patterns/variants were characterized by the number of bands and mobility shifts identified for the different fragments in *SPP1* gene. The silver staining followed by PCR-SSCP using 8% PAGE (49:1) for 6 hours of *SPP1* exon 4 gene fragments (*SPP1G5*) revealed polymorphism with two *SPP1G5-SSCP* patterns at *SPP1G5-SSCP* locus (Supplementary Fig. 2).

The frequency of these two *SPP1G5-SSCP* patterns was found (A = 0.95 and B = 0.05) in 64 Gaolao cattle. The comparison in SSCP Pattern A and B was tested using SPSS 16.0, which revealed significant differences for SNF % and Lactose % traits with SSCP patterns A exhibited as compared to patterns B (Table 4).

**DNA sequencing of polymorphic patterns at *SPP1G5-SSCP*:** The PCR amplicons (290 bp) of exon-4 region of *SPP1* gene exhibited A and B SSCP patterns indicating PCR-SSCP polymorphism. In view of sequence variation for the *SPP1G5-SSCP*, 02 PCR products were selected for SSCP pattern A and SSCP pattern B for sequencing. The edited sequence of 322 bp was analysed using bioinformatics software which revealed SNPs A-G at 71<sup>st</sup>, A-C at 109<sup>th</sup> and A-C at 208<sup>th</sup> position in 322 bp in *SPP1G5* amplified PCR Product in Gaolao Cattle (Supplementary Fig. 3).

The alignment of 322 bp *SPP1G5* amplified DNA sequence of *SPP1* gene of Gaolao cattle with *Bos taurus* reference sequence (AY878328.1) revealed 02 computational SNPs, viz. C-A at 109<sup>th</sup> position and C-A at 208<sup>th</sup> position. The sequence homology of *SPP1G5* amplified 322 bp sequence revealed sequence identity of 99.07 with European cattle, 98.80 with zebu cattle and 98.80 with sheep, 97.55 with buffalo, 96.34 with goat respectively. The identified polymorphism at exon-4 using SSCP at *SPP1G5-SSCP* Locus is not reported by other researchers in cattle, however Manzoor *et al.* (2018) analysed genetic variability at exon-IV region in *SPP1* gene and reported g.38329758 (T > C) SNP and its association with milk protein. In view of above, *SPP1G5-SSCP* locus may be useful in future marker development research for milk traits.

In conclusion, the findings of the current study will fill the information gap about gene sequence polymorphism at

Intron-4 and Exon-4 region of *SPP1* gene in Gaolao cattle. In studied region, *SPP1G1-BsrI* was found significant for milk yield and SNF% traits for CT genotype as compared to TT genotype. *SPP1G5-SSCP* polymorphism exhibited significant differences for SNF% and Lactose% traits with SSCP patterns A as compared to SSCP patterns B. The molecular genetic tools like PCR-RFLP, PCR-SSCP and SNP revealed genetic variation, thus proving them as effective tools for marker trait association study. The identified associations after validation in bigger database can be useful for selection and breeding decisions of indigenous dairy cattle.

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