Animal Biotechnology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/labt20

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To cite this article: Aftab Ullah, Rajwali Khan, Syed Muhammad Suhail, Ijaz Ahmad, Farhan Anwar Khan, Muhammad Subhan Qureshi, Nazir Ahmad Khan, Amel Ayari-Akkari & Dalia Abd El Moneim Ahmed (2022): Bioinformatics analysis and the association of polymorphisms within the caprine *GDF9* gene promoter with economically useful traits in Damani goats, Animal Biotechnology, DOI: 10.1080/10495398.2022.2154676

To link to this article: https://doi.org/10.1080/10495398.2022.2154676

ANIMAL BIOTECHNOLOGY
https://doi.org/10.1080/10495398.2022.2154676

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Bioinformatics analysis and the association of polymorphisms within the caprine *GDF9* gene promoter with economically useful traits in Damani goats

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ABSTRACT

The blood sample from 60 Damani does were collected and genomic DNA was extracted, and DNA integrity were investigated. A 447 bp promoter fragment of the *GDF9* gene was amplified and Sanger sequenced for the identification of *GDF9* gene polymorphism. Three novel SNPs were identified at positions g. 97(T > A), g. 142 (G > G) and g. 313(C > T) in the promoter region of the caprine *GDF9* gene which significantly ($P < 0.05$) influenced litter size, body measurement, and milk production traits in Damani goats. The genotype CT of SNP1 significantly ($P < 0.05$) improved litter size, genotype GG of SNP2 significantly ($P < 0.05$) enhanced milk production, while the genotypes CC of SNP3 significant ($P < 0.05$) increased body measurement traits in Damani goats. Moreover, in SNP1 loss of 3 transcription factors (TF) binding sites occurred, SNP2 caused loss of two TFs binding sites, and SNP3 caused loss of a single TF binding site. Similarly, SNP1 and SNP2 caused the gain of three new potential TF binding sites, and SNP3 caused gain of two new TF binding sites. It is concluded that caprine *GDF9* gene could be used as a candidate gene for litter size, milk production and body measurement traits in Damani goats through marker-assisted selection for future breeding program.

KEYWORDS

Damani goat; caprine GDF9; SNPs; litter size; body measurement traits

Introduction

Damani goats are found in southern part of Khyber Pakhtunkhwa province specifically in Dera Ismail khan and Bann districts.[1] They are medium to small sized with average weight of male 30 kg and female 35 kg.[2,3] Damani goat provides significant economic value in terms of meat, milk and skin. However, the twining percentage is very low in Damani goat as compared with other goat breeds found in the country.

Breeding is a complex process, and reproduction traits like ovulation rate and litter size are genetically regulated by several genes known as fecundity (Fec) genes.[4] Several important genes, including three related oocyte-derived components were identified to influence fecundity in sheep such as, *Bone morphogenetic protein Receptor* (*IB*), the *Bone morphogenetic protein receptor15* (*FecX FecB*), and *Growth differentiation factor 9* (*GDF9*) also known as *FecGs*.[5,6] The *GDF9* gene belongs to the transforming growth factor-b superfamily. It is an important growth and differentiation factor in female reproduction during the early stages of folliculogensis.[7] The *GDF9* gene controls several primary enzymes emitted by granulosa cells, which regulates ovulation, implantation and female reproduction. It also plays a synergistic role with *bone morphogenetic protein 15* in cumulus proliferation, hyaluronic acid synthesis initiation, oocyte microenvironment restoration.[8] The *GDF9* mRNA and protein were found at all phases of development in caprine ovarian follicles, as well as in luteal tissue.

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/10495398.2022.2154676

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Moreover, during long-term in vitro culture of goat prenatal follicles, *GDF9* and FSH can maintain follicular integrity and increase primordial follicle activation and integrity. It also regulate goat fecundity by high expression in the ovary and connection with other fecundity-related genes in different tissues.[9,10] Previously, significant associations were found between SNPs (Single nucleotide polymorphism) found in different location of *GDF9* gene with traits of economic importance in goat. A variant of *GDF9* gene in exon I (g.234C > A) exhibited significant association with fertility traits in Markhoz goat in Iran.[11] Moreover, the polymorphism in the coding region sequence of *GDF9* gene showed significant association with reproductive traits in different breeds of goats[12] such as in Assam hill goat,[13] black Bengal goat,[14] Haimen goat, and Xuhuai goat.[15] Thus, *GDF9* is a known marker gene for female reproduction traits. The candidate gene is one of the latest tool used in breed improvement strategy against traits of economic importance under marker-assisted selection.

Marker-assisted selection increases the rate of genetic improvement and reduces the cost of pedigree and progeny testing to preselect animals that inherited chromosomal segments of greater genetic worth,[16,17] such as SNPs which is easy, inexpensive method to select the genome with high density, and the results can be computed as soon as DNA obtained, and hence selection in both sexes can be done early in life.[18,19] The identification of causal variants increase the accuracy of genomic selection and is considered efficient tool for the analysis of association between genetic polymorphism and traits of economic importance,[20] especially reproduction traits in small ruminants.[21] SNPs in the promoter region is very important as it contains transcription factor binding sites which regulates the function of respective marker genes.[22] Previously, we explored the association of SNPs in the promoter region of *STAT3*,[23] *SIRT3*,[24] *KLF3*,[25] *SIX1*,[22] *SIX4*[26] and *TORC2*[27] genes with carcass quality and body measurement traits in Qinchuan beef cattle. Similarly, the polymorphism in the promoter of *KISS1* and *BMPR1B* genes exhibited significant association with reproductive traits in goats[28] and sheep,[29] respectively. These SNPs can cause loss or gain of important transcription factor binding sites and can lead to activation or suppression of the target genes. Therefore, we hypothesized that genetic variants of *GDF9* gene promoter may influence body measurement and reprodu000ctive traits in Damani goat. Hence, the present study was designed to explore the *GDF9* gene promoter region and investigate their associations with body measurement and litter size tratis. Therefore, this study was executed to characterize *GDF9* gene polymorphism, mapped its genotypes association with reproduction traits through marker assisted selection in Damani goat breed.

Materials and methods

A total of 60 representatives does of Damani goat breed were randomly selected. Damani goat are found in southern part of Khyber Pakhtunkhwa province of Pakistan, specifically in Dera Ismail khan and Bann districts. They are medium to small sized with average weight of male 30 kg and female 35 kg. The experimental animals were handled as per standard operating procedures laid down by Ethical committee of the Faculty of Animal Husbandry and veterinary sciences the University of Agricultural Peshawar. The blood samples were collected from selected animals using standard protocol in sterile environment. The animals were properly and humanly restrained before samples collection. The jugular vein was evaluated and 70% ethanol was used as disinfectant, and total 3 cc blood was collected aseptically in the 5 cc sterile syringes and then blood was transferred into EDTA tubes. The samples were transported at 4 °C temperature into the Histo-pathology laboratory in Faculty of Animal Husbandry and veterinary sciences, The University of Agricultural Peshawar. Phenotypic data was also collected from these goats about litter size and body measurement traits as described by.[30] Qualitative (discrete) traits including horn, shape, ear length, tail length head length and quantitative parameters such as body weight, body length, chest girth, animal height at withers, rump and neck length . All the measurements were done with the help of an inching tape. The animals were brought to stand on a plan surface during its body measurements. Body weight was recorded through digital weighing balance.

Estimates of conservation and biological evolution

The *GDF9* gene is located on chromosome *7* of the *caprine* genome, with the total length of 4720 bp and 3 exons (*NC_030814.1)*. The ORF length is 1362 bp and the putative protein contains 453 amino acids (Figure 1). The protein sequences of GDF9 proteins were extracted for seven different species as XP 006050617.1 *Bubalus bubalis*, NP 777106.1 *Bos taurus*, XP 019820461.1 *Bos indicus*, XP 005890493.1 *Bos mutus*, NP 001272637.1 *Capra hircus*, NP 001136360.2

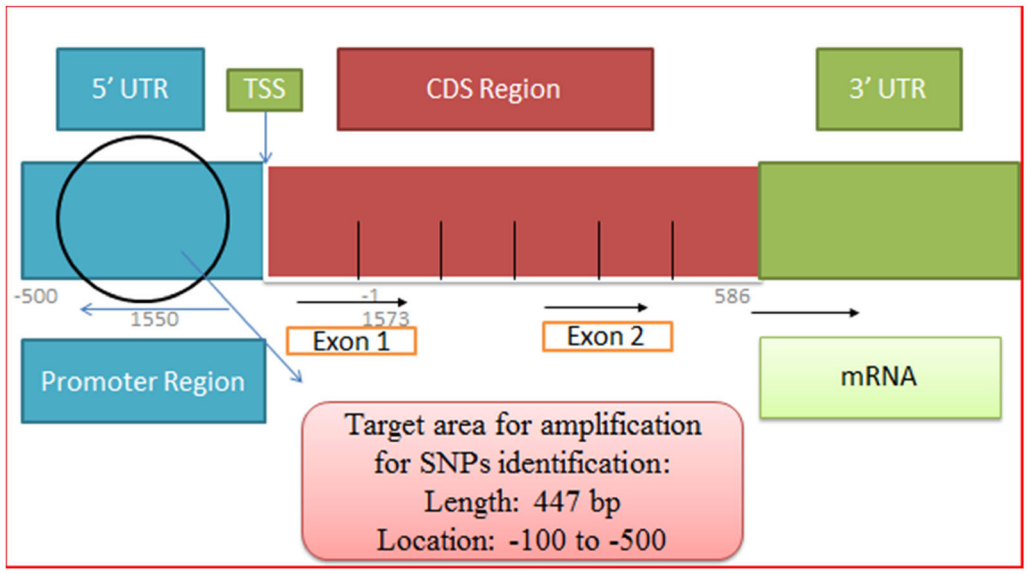

Figure 1. Structure of *GDF9* gene (www.ncbi.nlm.nih.gov).

Ovis aries, and NP 996871.3 *Gallus gallus* were extracted from NCBI database in FASTA format.[31]

DNA extraction

The non-enzymatic salting out procedure was applied to extract DNA from the blood.[32] In the first stage, 300 ul of heparinized blood was extracted and placed in 1.5 Eppendorf tubes using a micropipette. 900 ul of TKM1 and 50 ul of 1x triton-X were added. Firstly, the RBCs were lysed through heating, WBCs were pelleted through centrifugation and protein were precipitated through vortex. The Eppendorf tubes were centrifuged the proteins were precipitated and settled down, the supernatant was safely transferred to new Eppendorf tubes contained 300 ul isopropanol, and the DNA were precipitated through gentle rotation. Finally, the Eppendorf tubes were centrifuged for five min at 8000 rpm to pellets the DNA. The supernatant was removed, and then the DNA pellets were air-dried. After drying 50 ul of TE buffer was added to the Eppendorf tubes. The isolated DNA quality and quantity was evaluated through Nano drop (Calibri Micro volume Spectrometer, Titertek Berthhold® Germany) and the DNA samples with the 260/280 ratio of ~1.7 to ~2.0 was used as a template for PCR reaction.

Genotyping through PCR reaction

The 447 bp promoter region was amplified using specific primers for identification of single nucleotide polymorphism in *GDF9* gene (NC_030814.1). The PCR reaction was prepared in 100 µl PCR tube containing 5 µl master mix (Dream Taq Green, Thermo Scientific), 1.5 µl of each forward and reverse primer, 5 µl of genomic DNA as a template and 12 ul PCR grade deionized water was added to make the volume 25 µl. A pair of primers used in the present study are forward 5′ AAGCGTGTTTATATGCAG AGTG.3′ and reverse 5′GGTCCCGTGGTGTAGTGG 3′, with the PCR reaction conditions given in Table 1.

Table 1. PCR reaction conditions.

PCR Steps	Temp °C	Time	Number of Cycles
Initial denaturation	94 °c	5 min	1
Denaturation	94 °c	30 sec	34 x
Annealing	57 °c	30 sec	
Extension	72 °c	1 min	
Last extension	72 °c	10 min	1

Gel electrophoresis and DNA sequencing

The PCR amplified products were gel electrophoresed in 100 mL, 2% agarose gel and run in 1X TE buffer. The gel was observed and pictures were captured through gel documentation system (FastGene, FAS-Digi, Europe). The confirmed PCR amplicons were sequenced through BGI® Shanghai, China (Figure 2(A–B)).

Data analysis

The sequence were analyzed with bioinformatics tools such DNA star laser gene software (https://www.dnastar.com/software/lasergene/) for SNPs identification. The online bioinformatics tool (Genomatix software) was used to find transcription factor binding sites. The

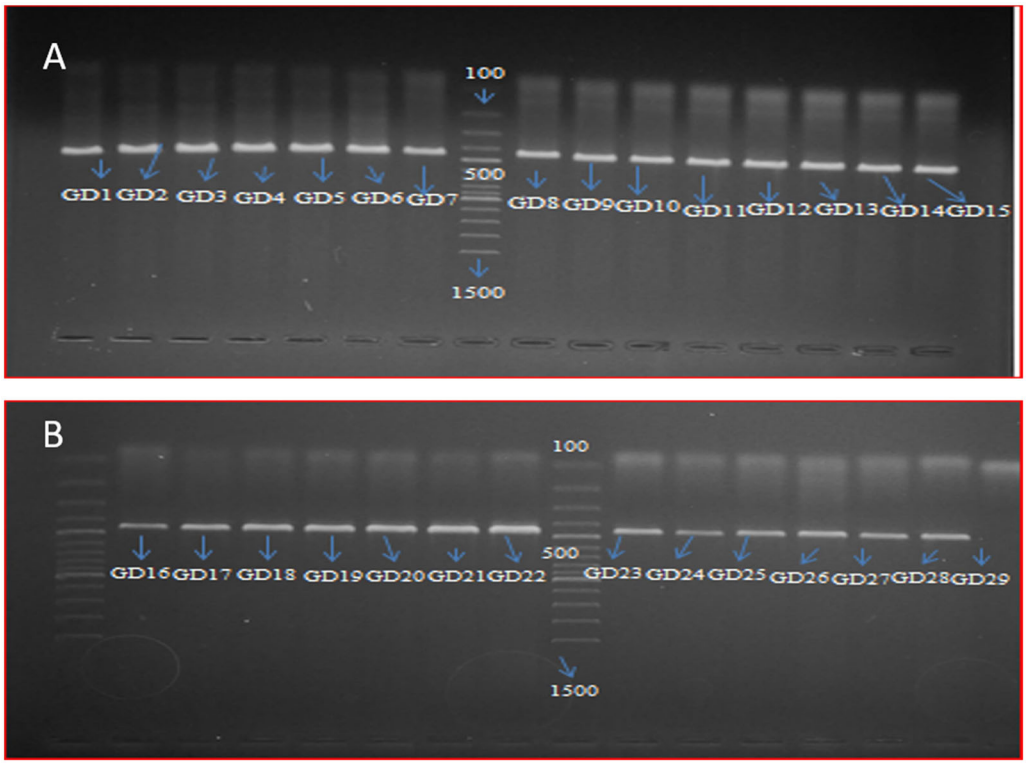

Figure 2. PCR products of the caprine *GDF9* gene in Damani goats.

statistical analysis through general linear model (GLM) using SPSS 20.0 software (SPSS, Inc., Chicago, USA) was applied for the association analysis among SNPs and target traits (body measurement and litter size).

The statistical linear model was

$$Yijk = \mu + \alpha i + \beta j + \Sigma ijk$$

Where Yijk each response variable to be measured in each animals

μ= is the overall mean of the population

i = ith effect of genotype A (1, 2)

j = jth effect of genotype B (1, 2)

Σ= random residual errors

The data was recorded, means between the group was compared through Duncan multiply range test.

Results

In-Silico *analysis of the GDF9 protein*

The protein sequences of GDF9 proteins were extracted for seven different species as XP 006050617.1 *Bubalus bubalis,* NP 777106.1 *Bos taurus,* XP 019820461.1 *Bos indicus,* XP 005890493.1 *Bos mutus,* NP 001272637.1 *Capra hircus,* NP 001136360.2 *Ovis aries,* and NP 996871.3 *Gallus gallus* were extracted from NCBI database in FASTA format (Table S1). As shown in Figure 3, multiple sequence alignment (MSA) of the GDF9 protein exhibited highest level (100%) of conservation among three species (*Bos taurus*, *Bos indicus*, and *Bos mutus*), two species *Capra hircus* and *Ovis aries* shows similarity and exhibited 20% variation from other livestock species in sequence of GDF9 protein. However, the tertiary (60%) conservation levels was found in the GDF9 protein sequence of *Gallus gallus* species as shown by grey and white colors, respectively.

The Evolutionary relationships of taxa was inferred through Neighbor-Joining method represented through phylogenetic tree (Figure 4).[33] The optimal tree with the sum of branch length = 1.24571241 is shown (next to the branches). The evolutionary distances were computed using the Poisson correction method[34] and are in the units of the number of amino acid substitutions per site. The analysis involved 8 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 212 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.[35,36] This analysis predicted that FGF10 protein sequence is conserved among all six livestock species. The *Bos taurus* and *Bos indicus* are very closely related, followed by cross of *Bos mutus* and *Bubalus bubalis*. The *Ovis aries* and *Capra hircus* are located on the same branch. However, *Gallus gallus* are located far away from the common livestock species on phylogenetic tree.

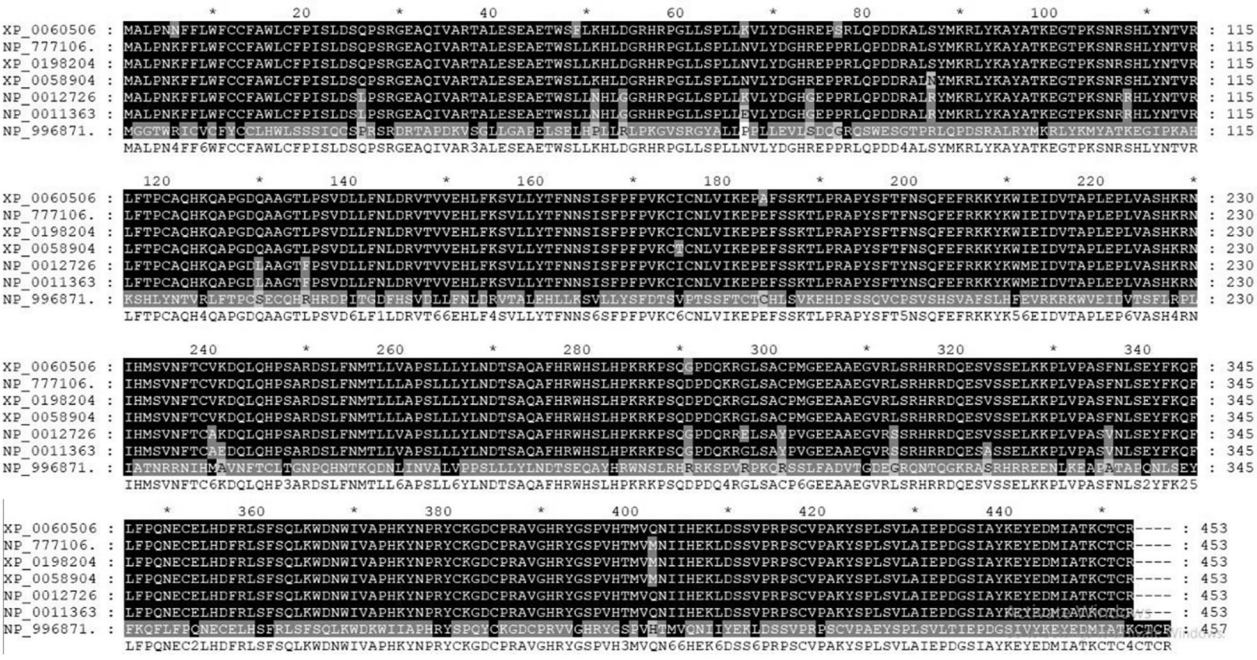

Figure 3. The GDF9 Protein Sequences (Multiple sequence alignment) of seven species. The conservation levels (similarity levels) are shown through background shading of sequence text; the black shades represents primary level of conservation which reflect 100% similarity; the grey shades represent secondary level of conservation that reflects 80% similarity; and white shade shows tertiary level of conservation i.e. 60% similarity.

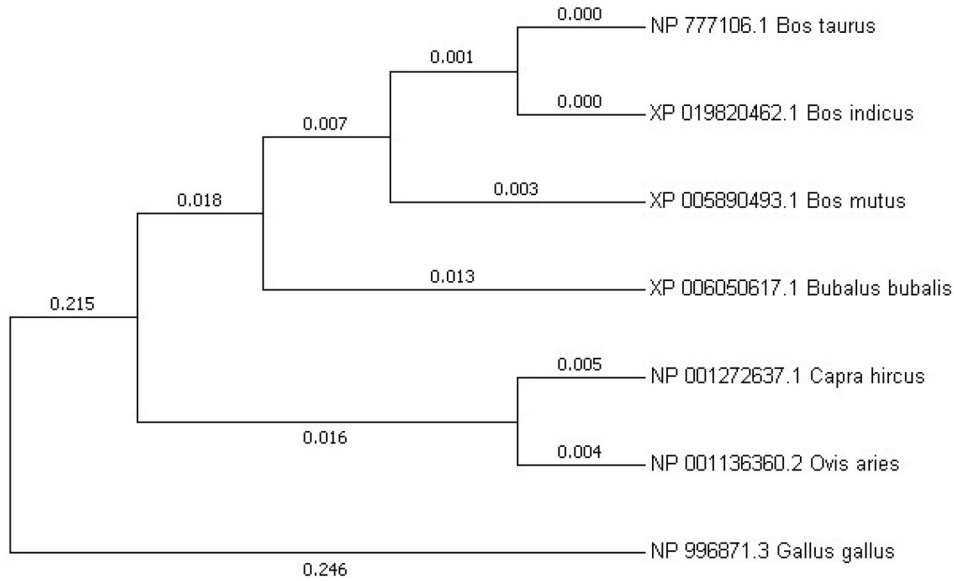

Figure 4. The evolutionary history of species represented through Neighbor-Joining Phylogenetic tree (A). Motif Locations in the FGF10 protein sequence of eight species. The p-value shows the significance of the motif site. The length of the color block shows the position, strength and significance of a particular motif site. The motif sites length is proportional to the negative logarithm of the p-value of the motif site. These colors are given through motif analysis performed through MEME suit system.

The conserved protein hits of FGF10 protein sequence in eight species was searched through NCBI CDD-batch search and hidata was downloaded in concise form, explored through TBtools software against eight species for FGF10 protein sequences. We found 21 conserved domain hits of Fibroblast Growth Factor (FGF) and 1 FGF superfamily in FGF10 protein sequences of all common livestock species (*Bos taurus*, *Bos indicus*, *Bos indicus* x *Bos taurus*, *Bubalus bubalis*, *Bos mutus*, *Capra*

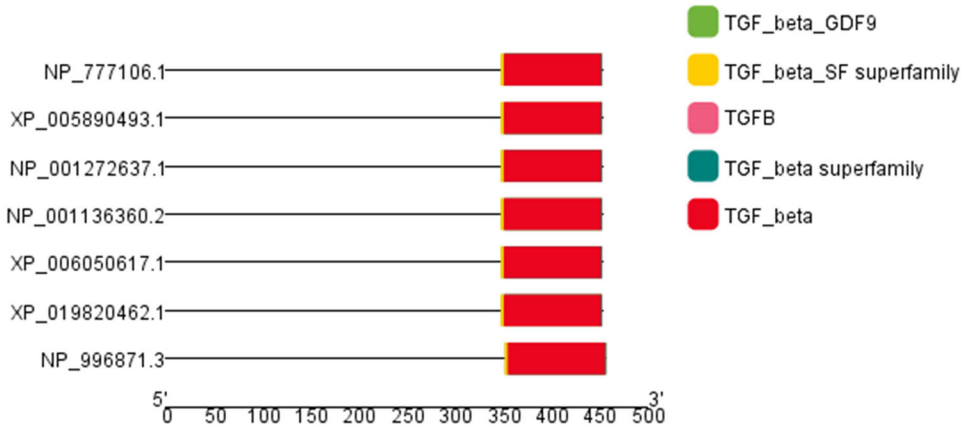

Figure 5. Location of conserved domain hits within FGF10 protein sequences of *Bubalus bubalis*, *Bos taurus*, *Bos indicus*, *Bos mutus*, *Capra hircus*, *Ovis aries*, and *Gallus gallus* species. Conserved protein domains within FGF10 protein sequences in seven different species represented by different color blocks, reflects specific hits of different domain family in different species.

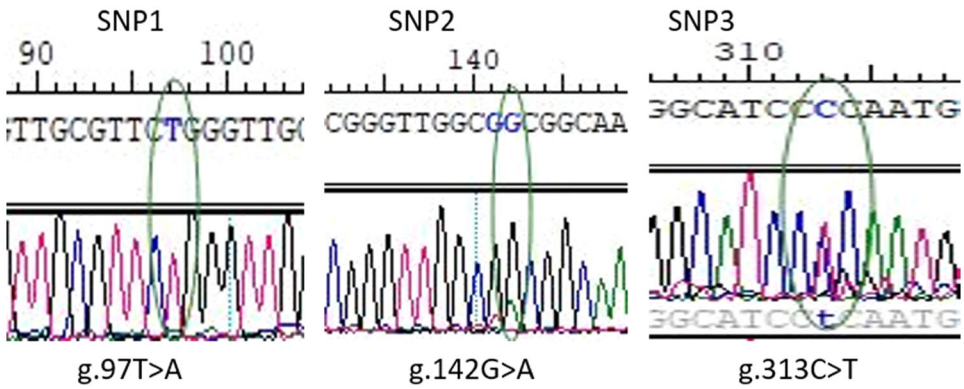

Figure 6. Position of single nucleotides polymorphism in the promoter region of *GDF9* gene.

Table 2. Identification of Single nucleotide polymorphism and genotypes.

SNPs	GENOTYPES 1	GENOTYPES 2
SNP1	TA	TT
SNP2	GG	GA
SNP3	CT	CC

Table 3. Haplotype identification of the caprine *GDF9* gene in Damani goats.

Haplotype	g.97C > A	g.142G > A	g.313C > T
hap1	T	G	C
hap2	A	G	T
hap3	T	G	C
hap4	T	A	C

hircus, and *Ovis aries)*, however only 3 FGF domain hits and a single FGF superfamily conserved domain hits was found in *Mus musculus* species (Figure 5).

SNP identification

Three SNPs were identified at loci g.97 T > A g.142 G > A g.313 C > T in the promoter region of the Caprine *GDF9* gene (Figure 6). In SNP1 two genotypes produce TA and TT, while SNP2 yielded two genotypes including GG and GA. The genotypes generated due to SNP3 were CT and CC as shown in Table 2.

Haplotypes identification

There are four haplotypes found of the caprine *GDF9* gene in Damani goats (Table 3).

Association of different genotypes with litter size and body measurement traits

Three Single Nucleotide polymorphisms located in the promoter region of the caprine *GDF9* gene exhibited associations with body measurement and litter size traits in Damani goats (Table 4). In SNP1, the goats with genotype TA revealed significantly ($P < 0.05$) larger litter size and larger neck length than genotype TT, while no significant variation were found in body weight, body length, heart girth, wither height, head and ear length, tail length, milk production. In SNP2, the goats along with genotype GG exhibited significantly ($P < 0.05$) larger milk production than genotype GA, while no significant variations were found in

Table 4. Association of different genotypes with litter size and body measurement traits (in inches).

Locus	Geno-	Litter	BW	BL	HG	WH	RH	HDL	NL	EL	TL	MP
SNP1	TA	1.33 ± 0.48	46.06 ± 5.96	29.6 ± 1.35	32.2 ± 1.53	30.80 ± 1.47	30.77 ± 1.09	6.60 ± 0.32	9.94 ± 0.18	4.42 ± 0.25	6.20 ± 0.86	0.73 ± 0.25
	TT	2.00 ± 0.00	43.00 ± 10.39	29.3 ± 2.30	31.3 ± 2.31	31.00 ± 2.30	31.00 ± 1.73	6.33 ± 0.57	9.60 ± 0.34	4.13 ± 0.40	5.83 ± 0.28	0.66 ± 0.28
	P-value	$p < 0.05$	0.47	0.78	0.38	0.60	0.72	0.26	$P < 0.05$	0.11	0.48	0.69
SNP2	GG	1.42 ± 0.51	46.71 ± 6.62	29.7 ± 1.54	32.4 ± 1.65	31.00 ± 1.75	30.85 ± 1.29	6.60 ± 341	9.94 ± 0.19	4.41 ± 0.25	6.03 ± 0.84	0.78 ± 0.25
	GA	1.50 ± 0.577	41.50 ± 0.52	29.0 ± 1.15	31.0 ± 1.15	30.50 ± 0.57	30.50 ± 0.57	6.40 ± 0.46	9.70 ± 0.34	4.43 ± 0.25	6.50 ± 0.57	0.50 ± 00
	P-value	0.81	0.16	0.40	0.12	0.58	0.60	0.35	0.07	0.33	0.32	$p < 0.05$
SNP3	CT	1.57 ± 0.53	43.00 ± 6.65	29.1 ± 1.61	31.4 ± 1.88	30.71 ± 1.88	30.71 ± 1.38	6.41 ± 0.38	9.74 ± 0.25	4.25 ± 0.32	5.92 ± 0.83	0.71 ± 0.26
	CC	1.36 ± 0.504	47.18 ± 6.28	32.54 ± 1.57	32.5 ± 1.57	31.00 ± 1.41	30.81 ± 1.07	6.64 ± 0.34	9.98 ± 9.19	4.45 ± 0.25	6.27 ± 0.78	0.72 ± 0.26
	P-value	0.41	0.19	0.35	0.16	0.71	0.86	$p < 0.05$	$p < 0.05$	0.16	0.39	0.92

Geno- (Genotype), BW(Body weight), BL(Body length), HG(Heart girth), WH(Wither height), RH(Rump height), HDL(Head length)NL(Neck Length)EL(Ear length).

litter size, body weight, body length, heart girth, wither height, head and ear length, tail length in both genotypes of SNP2. In SNP3 the goats along with genotype CC revealed significantly ($P < 0.05$) larger head length and larger neck length than genotype CT, however no significant variation were found in litter size, body weight, body length, heart girth, wither height, rump height, ear length, tail length and milk production in both genotypes of SNP3.

Transcription factor binding site predictions

In Genomatix software analysis showed that mutations due to SNP1 caused gain and loss of transcription factor binding sites shown in (Table 5 and Table 6 and Figure 7). (https://www.genomatix.de/solutions/genomatix-genome-analyzer.html). The variant C > T in SNP1 caused the loss of three important transcriptional factor binding sites, (AHRANT.02, MYC.02, EPAS1). In SNP2, the G > A also caused loss of two important transcriptions factor binding sites (NFKAPPAB50.01, ESRRA.01). In SNP3, variant C > T also caused the loss of one important transcriptional factor binding site (ZFP57.01) as shown in Table 5. Similarly, in SNP1, due to variant C > T three transcriptional factor binding sites were gained (ZF5.03, HELT.01, HSF1.01), while in SNP2, the G > G create gain of three transcription factor (GCM1F, HIVEP.02, SF-1.01) and SNP3 C > C also caused gain of two transcription factors binding sites (P53.05, P53.03) as shown in Table 6.

Discussion

The *GDF9* belong to transforming growth factor (TGF-) superfamily, is an oocyte-derived growth factor and is secreted by oocytes in growing ovarian follicles. It is essential for growth and differentiation of early ovarian follicles.[37] The polymorphisms of the *GDF9* gene play an important role in the reproductive process of both sheep and goats.[12] The expression levels of *GDF9* in the ovaries were higher in high prolific small Tail Han sheep than that in low prolific specimens, which indicated that *GDF9* may play a positive regulator role in the lambing performance.[38] In the present study, in-silico analysis was performed to characterize GDF9 protein in different mammalian species, different bioinformatics tools exhibited conservation and sequence homology in the protein sequences of GDF9 protein in *Bos taurus*, *Bos indicus*, *Bos indicus* x *Bos taurus*, *Bubalus bubalis*, *Bos mutus*, *Capra hircus*, *Ovis aries*, and *Mus muculus* species.

Table 5. Loss of transcription factor binding sites due to changes in promoter sequence through SNP1 (C to T), SNP2 (G to G), and SNP3 (C to T).

SNP	loci	Name of TF	TF Binding site sequence	strand	Score*	Ci- value
SNP1	g-97 C > A	MYC.02 Myelocytomatosis oncogene (c-myc proto-oncogene)	cgcgccgCGTGagtgcc	_	92.5	>60
		AHRANT.02 Aryl hydrocarbon / Arnt heterodimers, fixed core	gcgtcgcgccGCGTgagtgccctgg	_	92	>60
		EPAS1.01Endothelial PAS domain protein 1 (HIF2A)	tcgggCGTGcatccttcgcgtcgcg	_	109	>60
SNP2	g-142G > A	NFKAPPAB50.01NF-kappaB (p50)	ttgGGGAtgcccttg	+	94	>60
		ESRRA.01Estrogen-related receptor alpha	cactacttcagcAAGGgcatccc	–	98.9	>60
SNP3	g-313C > T	ZFP57.01Krueppel-associated box-containing zinc-finger protein 57 (KRAB-ZFP 57)	actTGCCgccgcc	+	96.5	>60

The "*" shows "Core Similarity score".

Based on bioinformatics analysis, we can predict functional similarity of *GDF9* gene in different livestock species. Therefore, based on the bioinformatics analysis, the functional and regulatory significance of the caprine *GDF9* gene could be extended to other livestock species.[39,40] To exploit and mapped the genetic variants of *GDF9* gene, three single nucleotide polymorphisms were identified in the promoter region of the caprine *GDF9* gene in Damani goats and explored their associations with body measurement and litter size traits. The genotype CT significantly ($P < 0.05$) improved litter size traits. In SNP2, the genotype GG exhibited significantly ($P < 0.05$) larger milk production. In SNP3 the goats with genotype CC significantly ($P < 0.05$) affected body measurement traits such as larger head length and larger neck length.

Previously, genetic association was performed with phenotypic traits such as age, body weight, body length, parity and litter size Black Bengal goats.[41,42] Although a few genes were recognized for effecting litter size and body weight, body length, hearth girth, weather height neck length.[43] However, the desirable body measurement at certain age and parity may be required for optimum metabolic activity which in turn affect hypothalamus—pituitary axis for increased ovulation that ultimately contribute to the number of successful fertilization and subsequently litter size. In the present study, the Genotype CT of SNP1 significantly improved litter size traits of Damani goat. The bioinformatics analysis of the promoter sequence coupled with molecular investigation exploited polymorphism in the caprine *GDF9* gene. The variation due to SNP1 caused gain and loss of transcription factor binding sites. The genotype C > T in SNP1 caused the loss of three important transcriptional factor binding sites, (AHRANT.02, MYC.02, EPAS1). Similarly, the SNP1 genotype C > T caused gain of three important transcriptional factor binding sites, (ZF5.03, HELT.01, HSF1.01). The loss of three important transcriptional factor binding sites due to SNP1 are (MYC.02 Myelocytomatosis oncogene (c-myc proto-oncogene), HELT.01Hey-like bHLH-transcriptional repressor, and HSF1.01Heat shock factor 1. The MYC.02, Myelocytomatosis oncogene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. The gain of a new TF binding site at the same loci is AHRANT.02 Aryl hydrocarbon/Arnt heterodimers, fixed core. The AHRANT.02 is a receptor complex heterodimer transcription factor, comprising the basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) domain aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) proteins, mediates the toxic effects of TCDD (2, 3, 7, 8 tetrachlorodibenzo-*p*-dioxin). Another most important TF binding site gained in the same location due to SNP1 are ZF5.03. The Zinc fingers are small protein domains in which zinc plays a structural role contributing to the stability of the domain. Zinc fingers are architecturally varied proteins that are found in a wide range of biological processes, including replication and repair, transcription and translation, metabolism and regulation, cell proliferation, and death. Zinc fingers bind to a wide range of substances, including nucleic acids, proteins, and tiny molecules, and serve as interaction modules.[44] Therefore, the phenotypic effect of the genotype CT at loci g.97 could be due to the gain and loss of these TF binding sites in the promoter region of *GDF9* gene in Damani goats and hence this genotype could be used as molecular marker for litter size traits.

In SNP2, the genotype G > G also caused the loss of two important Transcriptions factor binding sites (NFKAPPAB50.01, ESRRA.01). While in SNP2, the genotype G > G caused a gain of three important transcription factor binding site (GCM1F, HIVEP.02, SF-1.01). Important role, the loss of two important transcriptional factor binding sites, in SNP2 G > G genotypes of promoter region of the *GDF9* gene. The ESRRA.01 (estrogen-related receptor alpha) is an

Table 6. Gain of new transcription factors binding sites due to change in promoter sequence through SNP1 (T to C), SNP2 (G to G) and SNP3 (C to C).

SNP	loci	Name of TF	TF Binding site sequence	strand	Score[a]	Ci-value#
SNP1	g-97 C > A	ZF5.03ZF5 POZ domain zinc finger, zinc finger protein 161 (secondary DNA binding preference)	cgcgtCGCGccgcgtga	−	100	>60
		HELT.01Hey-like bHLH-transcriptional repressor	cactCACGcggcgcg	+	96	>60
		HSF1.01Heat shock factor 1	cgcggcgcgacgCGAAggatgcacg	+	98.9	>60
SNP2	g-142G > A	GCM1F.03Glial cells missing homolog 1 (secondary DNA binding preference)	ggcatCCCCaatgtg	−	99.5	>60
		HIVEP.02Human immunodeficiency virus type I enhancer binding protein 1	caagggcaTCCCCaa	−	100	>60
		SF1.01SF1 steroid genic factor 1	tcagCAAGggcatcc	−	97.5	>60
SNP3	g-313C > T	P53.05Tumor suppressor p53	ggctCAAGcccgggacttgccgccg	+	100	>60
		P53.03Tumor suppressor p53 (3′ half site)	tcgggttggcggcggCAAGtcccgg	−	97.5	>60

[a]Core Similarity score; red colored alphabets reflect the core sequence nucleotides within the TF binding site; encircled nucleotide represents mutated nucleotide. #The Ci-vector (consensus index vector) for the matrix represents the degree of conservation of each position within the matrix. The maximum Ci-value of 100 is reached by a position with total conservation of one nucleotide, whereas the minimum value of 0 only occurs at a position with equal distribution of all four nucleotides and gaps.
shows Ci-value.

orphan nuclear receptor (NR) that significantly influences cellular metabolism. The ESRRA is predominantly expressed in metabolically-active tissues and regulates the transcription of metabolic genes. In SNP2 G > G genotypes of promoter region of the *GDF9* gene. The GCM1F (Glial Cells Missing Transcription Factor 1) is a Protein Coding gene. It plays a vital role in human early embryo development. Gene Ontology (GO) annotations related to this gene include DNA-binding transcription factor activity and RNA polymerase II proximal promoter sequence-specific DNA binding. Transcription factor involved in the control of expression of placental growth factor (PGF) and other placenta-specific genes. Therefore, we can conclude that, the genotypes GG of SNP2 may perform its role due to these TFs located in the promoter region of GDF9 gene in Damani goats.

In SNP3 the genotype CT also caused the loss of one important transcriptional factor binding sites (ZFP57.01). However, the genotype CC caused gain of two new transcription factor binding site (P53.05 and P53.03), and also caused the loss (KRAB-ZFP 57) binding sites in promoter region of *GDF9* gene. The Krüppel-associated box (KRAB) domain is a transcription repression module from the largest family of transcriptional regulators encoded by higher vertebrates. The KRAB can mediate both irreversible and reversible regulation of endogenous genes, depending on embryonic developmental stage. During the early embryonic stage, KRAB produced persistent DNA methylation inside the KRAB binding site, resulting in irreversible gene repression. Important role, the gain of two important transcriptional factor binding sites, in SNP3 CC genotypes of promoter region of the *GDF9* gene. The tumors suppressor protein p53 promotes or represses the expression of a range of target genes involved in cell cycle control, senescence, and death. It fulfills its role as genome protector by interacting with a complex network of functional domains that are independently folded but fundamentally disordered. This sheds light on p53's structural complexity, the molecular underpinnings of its inactivation in cell growth cancer, and therapeutic options for restoring p53 activity in malignancies and diverse biological functions. It promotes the transcription of genes that negatively inhibit cell cycle progression, contributing to genome stability. We can conclude from the present study, that TFs located in the promoter region are gained or lost due to SNPs at loci g.97C > A, g.142G > A and g.313C > T may regulate body measurement, milk production and litter size traits in Damani goats. Therefore, genotype CT, GG and CC

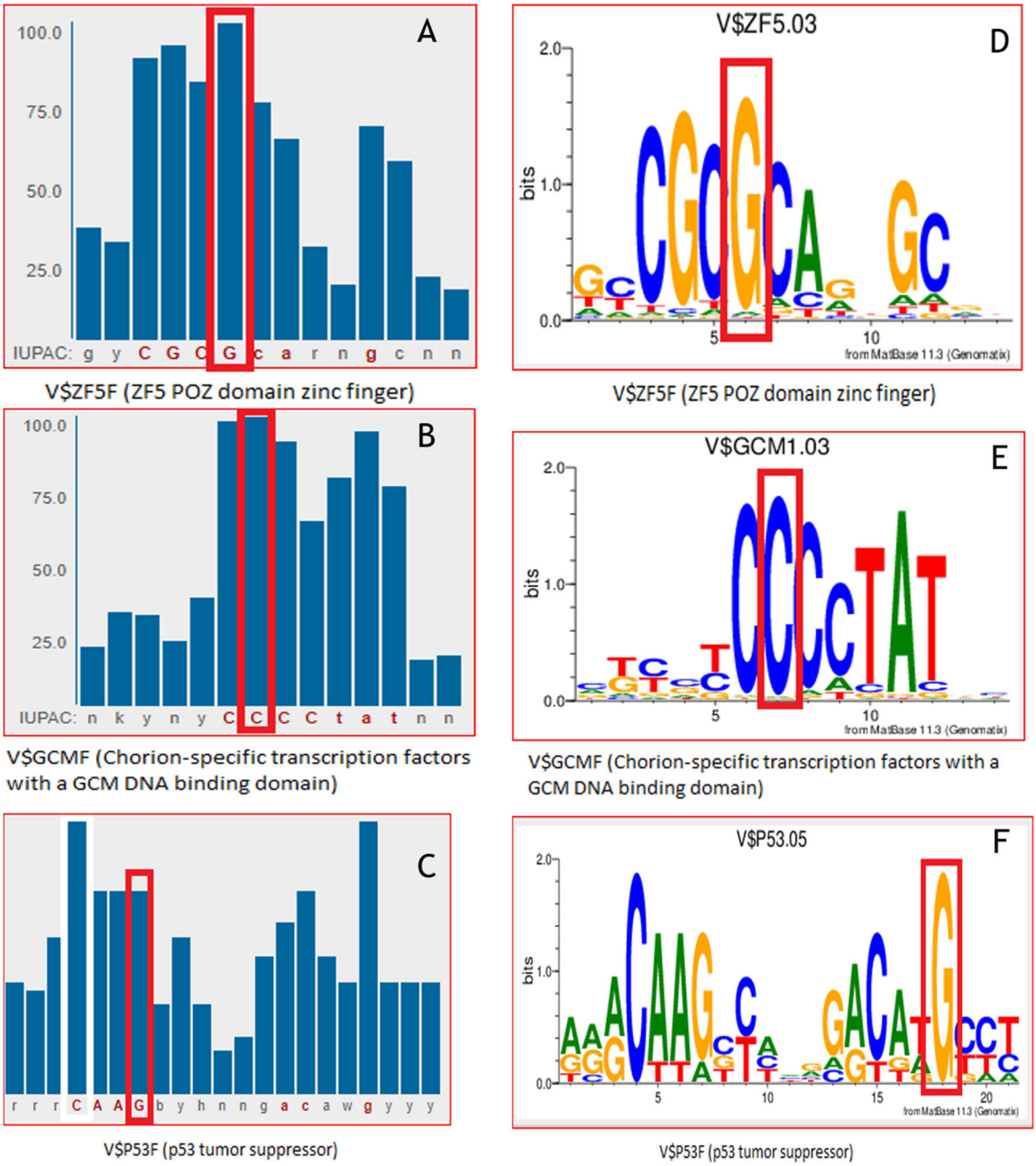

Figure 7. Variation in the core sequences of TFs binding sites due to SNPs in the promoter region of caprine GDF9. The sequence logos and profiles of transcription factor binding sites changed due to SNPs in the promoter of GDF9 gene. (A–F) represents the sequence log and profile of transcription factor binding sites in promoter region of GDF9 gene.

of GDF9 gene could be used as genetic variants for litter size, milk production and body measurement traits in marker-assisted selection.

Conclusions

The results of the current study concluded that genotypes TA at loci g 97, GG at loci g.142, and CC at loci g.313 of *GDF9* gene promoter significantly ($P < 0.05$) increased litter size, milk production, and body measurement traits respectively in Damani goats. Therefore, genetic variants of *GDF9* gene promoter (TA, GG, CC) at loci g 97, g.142, and g.313 respectively, could be used as candidate genes for economic traits in Damnai goat through marker-assisted selection for future breeding program.

Authors contribution

R.K and A.K conceived the idea, performed experiment, and wrote the manuscript. S.M.S, I.A MSQ, F.A.K, NAK, AAA, and DMA assisted in analysis, editing and revision

the draft manuscript. R.K supervised overall research and analyzed the data.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The authors extend their appreciation to the Deanship of Scientific Research at King Khaled University for supporting this work through small Groups Project under grant number [RGP.1/381/1443].

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