

Novel Single Nucleotide Polymorphisms (SNPs) in Two Oogenesis Specific Genes (*BMP15*, *GDF9*) and Their Association with Litter Size in Markhoz Goat (Iranian Angora)

Research Article

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ABSTRACT

BMP15 and *GDF9* are two oogenesis specific genes play a pivotal role in female fertility in mammals and potential for improvement of prolificacy in marker-assisted selection. The aim of present research was to investigate the variation and association between *BMP15* and *GDF9* polymorphism and litter size in Markhoz goats. The sequence variability of the different amplified fragments utilized for genotyping was further investigated by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and sequencing. Sequencing technology allowed the detection of two nucleotide variations: A both non-functional mutation (G/A) transition at nt 1026 known as P3) and a synonymous mutation at 1049 position which for the first time was found at the *BMP15* gene. Also, *GDF9* gene exhibited a polymorphism on *BcnI* site (1159 bp) within exon 2 of amplified PCR products (glutamine/proline). Our analysis using general linear model (GLM) statistical methods showed litter size was significantly influenced by genotypes of *GDF9* ($P < 0.01$). Heterozygous genotype showed higher litter size than homozygous genotypes ($P < 0.01$). The conformation of this finding would need large sample size and test to screen in other indigenous goats.

KEY WORDS *BMP15*, fecundity, *GDF9*, polymorphism.

INTRODUCTION

Iran is believed to be one of the main gateways for domestication of sheep and goat in the world. Despite of this fact that more than 25 million individual goats from 20 breeds of goats have been recognized in the country, only eight of these breeds classified as typical breeds (Tavakolian, 2000; Arefnejad, 2007; Rashidi, 2000). Markhoz goats are an interesting genetic material due to their considerable twinning percentage (25 percent) and a rare set of triplet type of birth phenomena in the herds (Rashidi, 2000). Historically, Markhoz goat was scattered in the provinces of Western

Azarbaijan, Kurdistan, and Kermanshah; however, it is currently distributed only in a small part of Kurdistan and a few villages of Western Azarbaijan provinces (Rashidi, 2000; Farshad *et al.* 2008). To stop the declining trend of the Markhoz breed, the benefit of breeding for increased fertility/litter size as a fundamental step to conserve indigenous breeds is paramount (Javanmard *et al.* 2011; Tavakolian, 2000). But, how precisely reproductive traits such as litter size is controlled remains a critical and important question in reproductive biology and is the major goal of research at this moment (Abdel-Rahman *et al.* 2013; Farshad *et al.* 2008). In mammals, the ovulation rate and

the litter size is a result of well regulated interactions of endocrine and paracrine mediators (Silva, 2005). The number of mature oocytes released during one reproductive cycle and estrous is determined by complex exchange of endocrine signals between the pituitary gland and the ovary as well as the paracrine and autocrine signals within ovarian follicles involving the oocytes and its adjacent somatic cells (Knight and Glister, 2003).

The *BMP15* is a growth factor and a member of the TGF β super-family that is specifically expressed in oocytes (Abdel-Rahman *et al.* 2013). This gene regulates granulosa cell proliferation and differentiation by promoting granulosa cell mitosis and suppressing follicle stimulating hormone receptor expression, all of which play a pivotal role in female fertility in mammals (Hanrahan *et al.* 2004). The *BMP15* gene has been mapped to sheep chromosome X and contains 2 exons. Investigation on mutations for this gene in sheep showed that the same phenotype: homozygous carrier ewes are sterile but heterozygous carrier ewes had elaborated ovulation rate (Ahlawat *et al.* 2013). Ovine *GDF9* spans approximately 2.5 kb and contains two exons and one intron. Exon 1 spans 397 bp and encodes for amino acids 1-134, while exon 2 spans 968 bp and encodes for amino acids 135-456. The single intron spans 1.126 bp. *In vivo* changing of *GDF9* concentration led to incremental changes in ovulation rate in sheep (Hanrahan *et al.* 2004; Chu *et al.* 2011). The *BMP15* and *GDF9* fecundity alleles show an X-linked and an autosomal over-dominant inheritance pattern with infertility in homozygous females (Ahlawat *et al.* 2013). Despite of this, there is little information concerning the *BMP15* and *GDF9* genes in Iranian indigenous goats. Thus, the genetic basis of caprine prolificacy remains to be explored.

Based on the above considerations, the present study pertains to molecular characterization and screening of novel polymorphisms within prolificacy associated *BMP15* and *GDF9* genes and their association with litter size in Markhoz goat breed.

MATERIALS AND METHODS

Experimental animals and litter size evaluation

To explore the genetic variations within investigated candidate genes, a total of 120 mature Markhoz does with known pedigree information and litter size records at first kidding from two groups of Animal Husbandry Research Station (n=82) and around rural zones (n=38) were randomly selected based on phenotypical variation of prolificacy, geographical distribution and age of does. The does were managed for once a year kidding and were routinely bred to bucks through natural mating following heat detection.

At the start of the breeding season, the does were randomly mated with bucks and were not under any hormone therapy or synchronization of estrus using controlled internal drug releasing (CIDR) or flushing methods. Natural pasture was the main source of feed.

The quantity and quality of the pasture vary considerably during the year. With the dry season and with decreasing in the quantity and quality of the pasture, supplemental feeding was provided.

DNA extraction

All procedures involving animals were approved by the animal care and use committee at the respective institutions where the experiment was conducted. Blood samples were collected from the jugular vein of 120 mature female goats supplemented with 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH=8). All samples were delivered back to the laboratory in an ice box and stored in the laboratory freezer at -20 °C. Genomic DNA was isolated from frozen whole blood cells by using commercial genomic DNA extraction Kit (QIAGEN, Australia). Quality and purity of the entire extracted DNA was assessed using agarose gel electrophoresis (0.8%) and calculating the OD₂₆₀/OD₂₈₀ nm ratio determined with the Nanodrop (ND1000).

PCR, SSCP and sequencing mutation detection

The molecular analysis consisted of amplification of *BMP15* and *GDF9* fragments by PCR, followed by detection of polymorphisms by single-strand conformation polymorphism (SSCP), verification of single nucleotide polymorphisms (SNPs) by sequencing, and finally statistical analysis of SNPs.

Polymerase chain reaction

New primer sets designed for both genes according to coding region and most variable region of investigated genes in previous reports and available information of NCBI. The sequences of the forward and reverse primers for the amplification of the *GDF9* and *BMP15* genes are given in Table 1. The PCR was performed in a 25 μ L reaction using the master mix kit (Fermentas, Russia) in a T-Personal thermocycler (Biometra co., Germany).

The PCR mixture contained: 50–100 ng of DNA, 2.5 μ L of 10 X PCR buffer (200 mM (NH₄)₂SO₄, 0.1 mM Tween 20%, 750 mM Tris-HCl (pH 8.8), 2.5 mM MgCl₂, 200 μ M dNTPs, 3 μ L mix of oligonucleotids (10 pmol of each primer), 1 U Taq DNA polymerase (Dream Taq polymerase, Fermentas company) and 11 μ L ddH₂O. A total of 35 cycles was adapted for denaturation at 94 °C/1 min, annealing were variable depend on primer sequence and polymerization at 72 °C/2 min.

Table 1 Characteristics of amplified loci of the *GDF9* and *BMP15* genes

Genes	Primers	PCR (bp)	Annealing (°C)	Sequence of primers
<i>BMP15</i>	BMP15-O.a	529	54	GGCACTTCATCATTGGACACTG TGCACCTTTGCCGTACCTG
	SSCP-BP ₁	353	58	CGCTTTGCTCTTGTTCCTCT AGCAGCCAAGAGGTAGTGAGG
	SSCP-BP ₂	273	57	GGGTTCTACGACTCCGCTTC ATGATGGGCCTGAAAAGTAACC
	SSCP-BP ₃	312	55	CATGATGGGCCTGAAAAGTAAC GGAGTATGAGGGTATGATTGCC
	FecX ^B	153	62	GCCTTCCTGTGTCCCTTATAAGTATGTTCCCTTA TTCTTGGGAAACCTGAGCTAGC
<i>GDF9</i>	<i>GDF9</i> -ex2	1169	59	ATCCACCTGACGTTTAAGGC CCTCCAAAGGCATAGACAGG
	SSCP-GP ₁	296	55	TGGCATTACTGTTGATTGTTTT CAAGAGGAGCCGTACATCA
	SSCP-GP ₂	206	55	GATTGATGTGACGGCTCTCT GGGAATGCCACCTGTGAAAAG
	SSCP-GP ₃	198	57	CTTTTCACAGGTGGCATTCCC ATCACTCAGATTGACTGAAGCTGG
	SSCP-GP ₄	221	54	TCTTTTCCCCAGAATGAATGT CACAGGATGGTCTTGGCACT

Single strand conformation polymorphism

The SSCP methodology, for amount of PCR product, denaturing solution, acrylamide concentration, presence or absence of glycerol and running temperature at 4 °C and 20 °C, was optimized for each PCR product. Each PCR reaction was diluted in denaturing buffer (95% of formamide, 10 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue and 10 mM of EDTA), denatured at 95 °C for 10 min, cooled snap on ice and loaded on polyacrylamide gel (Wang *et al.* 2011).

The PCR fragments were analyzed in polyacrylamide gels 37.5:1 acrylamide-bisacrylamide (6% for SSCP-P1, 10% for SSCP-P2 and 8% for SSCP-P3 PCR products) without glycerol and with migration at room temperature with 80-120 V and 6 h. Subsequently, silver staining performed to detect the bands (Zhang *et al.* 2008). The size of the alleles was determined based on a 100 bp DNA size standard (Fermentas company) using the computer software BIO 1 D⁺⁺.

Sequencing of amplified fragments

For those loci showed polymorphism in pattern of bands, each pattern was a candidate for sequencing in the next step.

For sequencing and mutation detection, the resulting PCR products were analyzed by agarose gel and then, purified by AccuPrep® PCR Purification Kit of BIONEER. Because of a small range of PCR products only forward sequencing was conducted for each PCR product.

The PCR products were sequenced by MacroGen company and significant results submitted to the Genbank (Accession number is: EU095935). The sequence alignment was performed using BLAST and MAFFT and CLUSTW online multiple alignment tools (www.ebi.ac.uk). Electropherogram of raw sequence data gives us information on the length of the read and the height of the peaks which determines whether or not the peaks are within acceptable heights and peak resolution, uniform peak spacing and has a weak background noise in comparison for evaluation of quality of raw data.

Single nucleotide polymorphism detection

PCR-RFLP *BMP15*

For confirmation of the single base substitutions at the nucleotide position 1100 by sequencing genomic DNA covering the whole mature peptide coding region, a PCR-RFLP DdeI restriction enzyme (CTNAG cleavage site) was used for FecX^B polymorphic loci. After amplification of the 153 bp DNA fragment, restriction digestion of 8 µL of PCR product with 1 U DdeI and 2 µL enzyme buffer in 20 µL final volume was performed for 6 h at 37 °C.

PCR-RFLP *GDF9*

With direct sequencing of *GDF9*-ex2 to find new mutations we have identified a non-conservative substitution at nucleotide position 959. Restriction digestion of 8 µL of PCR product with 1-U *BcnI* and 2 µL enzyme buffer in 20 µL final volume was performed for 6 h at 37 °C.

Statistical analysis

Allele frequencies, most frequent allele, Hardy-Weinberg equilibrium test, observed and expected heterozygosity and average heterozygosity were calculated using POPGENE software (Version 1.3) (Yeh *et al.* 1997). The allele binning procedure was used for minimizing of genotyping error and validity of raw data. The statistical model fitted to evaluate the association of the genotypes of each gene and litter size included the fixed effects of the herd, parity, and birth year, random effect of sire and dam of kids. The reason of choosing birth year as fix effect is due to variations in the environment, management methods, feeding programs, and body condition of the dams. The two-way interactions between the fixed effects, effects of age of the dam as covariate were also included in the model. The data were analyzed by generalized linear mixed model (GLM) procedure of SAS (SAS, 1999). Mean allele class differences for the litter size traits were compared and then evaluated with Bonferroni correction post-hoc tests.

RESULTS AND DISCUSSION

In this study, we investigated the pattern and variability of *GDF9* and *BMP15* gene and their association with fecundity of Iranian native Markhoz goat.

We hypothesized herein that the variation of the litter size in Markhoz goat mostly related to novel and unique specific SNP in this breed and therefore, we did not use the reported polymorphism in other exotic or indigenous sheep/goat breeds for evaluation of fecundity in this indigenous breed.

SNP detection for *BMP15* gene

The Figure 1 indicated quality of PCR products of *BMP15* exon 2 different gene loci. Column 1, 2 is BP1 (353 bp), Column 3, 4 is BP2 (273 bp) and 5, 6 Column is BP3 (312 bp).

The SSCP analysis identified three conformational differences in the BP3 fragment (312 bp). But, no conformational patterns were detected for BP1 and BP2.

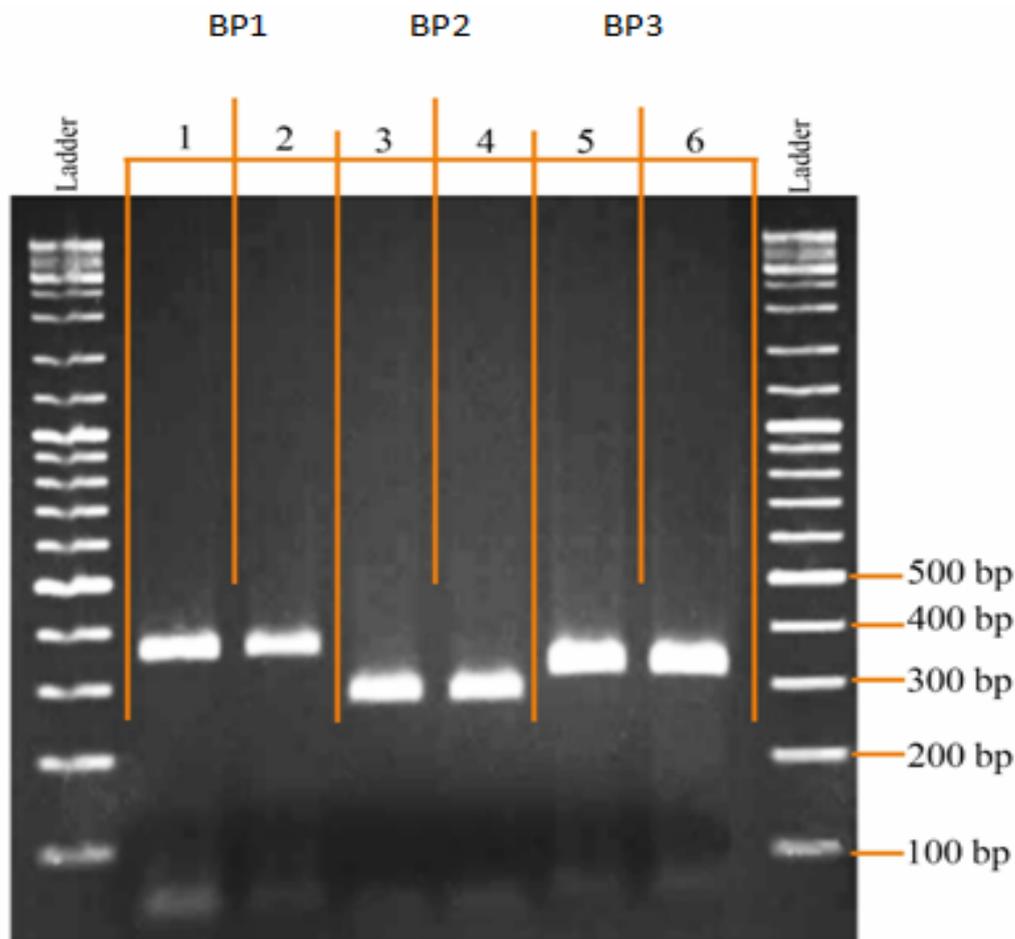


Figure 1 PCR products of *BMP15* exon 2 different gene loci
Column 1, 2 is BP1 (353bp), Column 3, 4 is BP2 (273 bp) and 5, 6 Column is BP3 (312 bp)

The sequence variability of the different amplified fragments utilized for genotyping was further investigated by PCR-RFLP, PCR-SSCP and sequencing.

Sequencing technology allowed the detection of two nucleotide variations: a both non-functional mutation (G/A) transition at nt 1026 known as BP3) and a synonymous mutation at 1049 position for the first time was found at the *BMP15* gene (Figures 3 and 4).

SNP detection for *GDF9* gene

PCR-SSCP analysis within the 312 bp fragment of *GDF9* identified three conformational differences in GP3 fragment but no conformational patterns were detected for GP1 and GP2.

Also, *GDF9* gene exhibited a polymorphism on *BclI* site (1159 bp) within exon 2 of amplified PCR products (glutamine/proline).

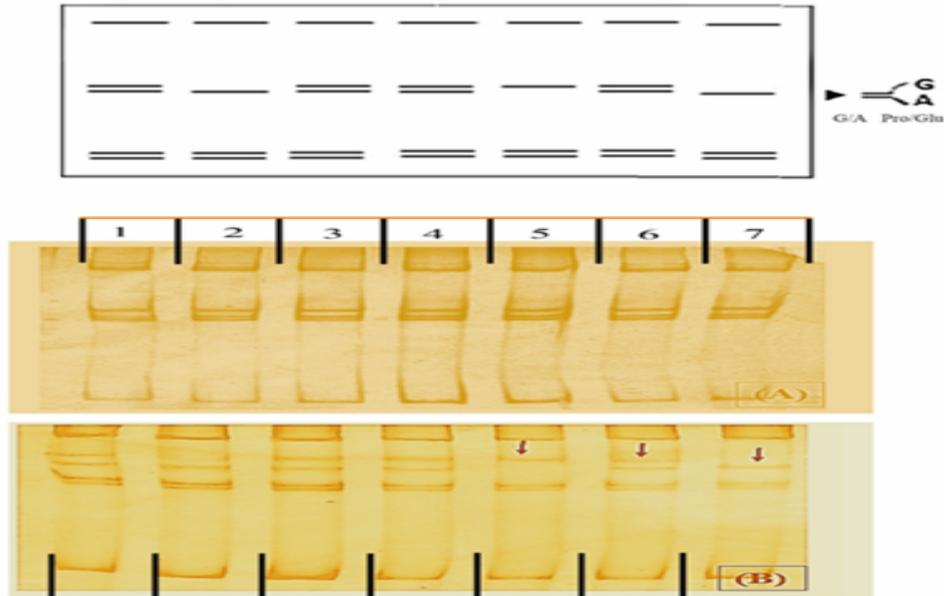


Figure 2 PCR-SSCP analysis of the 353 bp fragment using SSCP-GP1 primers within *GDF9* gene (A) SSCP analysis of the 312 bp fragment using SSCP-GP3 Primers (B) Analysis identified three conformational differences in GP3 fragment but no conformational patterns were detected for GP1 and GP2

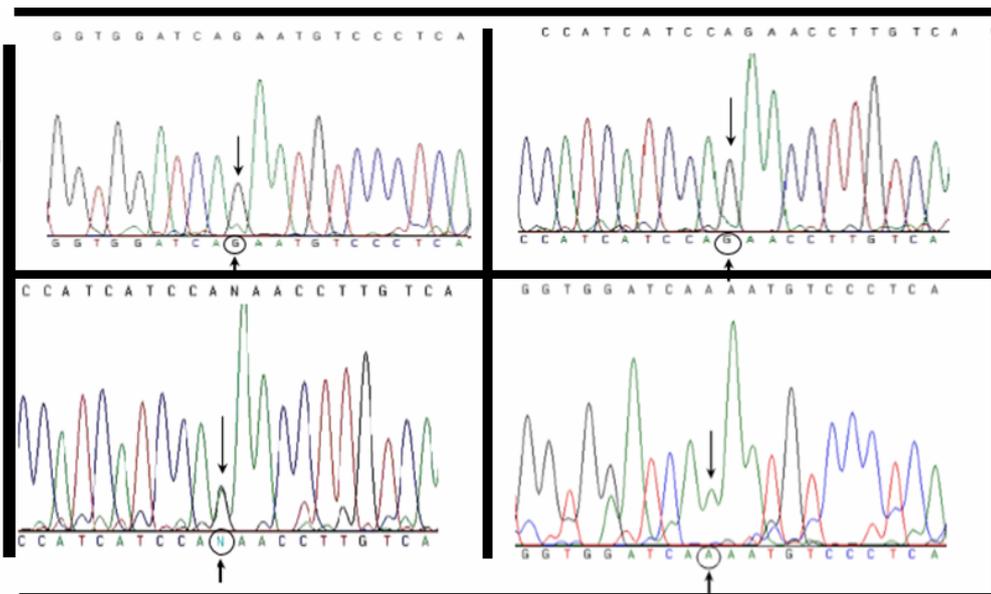


Figure 3 Sequencing results and evidence for existence of two novel SNP at nucleotide positions 1026 and 1053 positions of *GDF9* gene Upstream figures are wild alleles and downstream figures are mutant alleles

PCR-RFLP using *DdeI* within *FecXB* locus showed no polymorphic results for whole genotyped animals. *GDF9*-ex2 primers amplified 1169 bp of exon 2 and PCR product digested using *BcnI* which showed three genotypes. Analysis of PCR-RFLP for *GDF9* showed one polymorphic *BcnI* site in the 1169 bp PCR product within exon 2 of *GDF9* gene (Figure 4). The digested (-/-) PCR product exhibited one fragment of 1169 bp and for the (+/+) PCR product (Mutant type), the 1169 bp fragment was cleaved into two

fragments of 690 and 479 bp (wild type).

The frequency for the *BcnI* (-) alleles was 0.46 and 0.36 in the does from research station and rural zones, respectively. The allele frequencies between two groups was significantly different ($P < 0.05$) (Figure 5).

This mutation leads to substitution of a polar amino acid (glutamine) with a non-polar amino acid (proline). It seems that this mutation changes the structure and function of the *GDF9* protein.

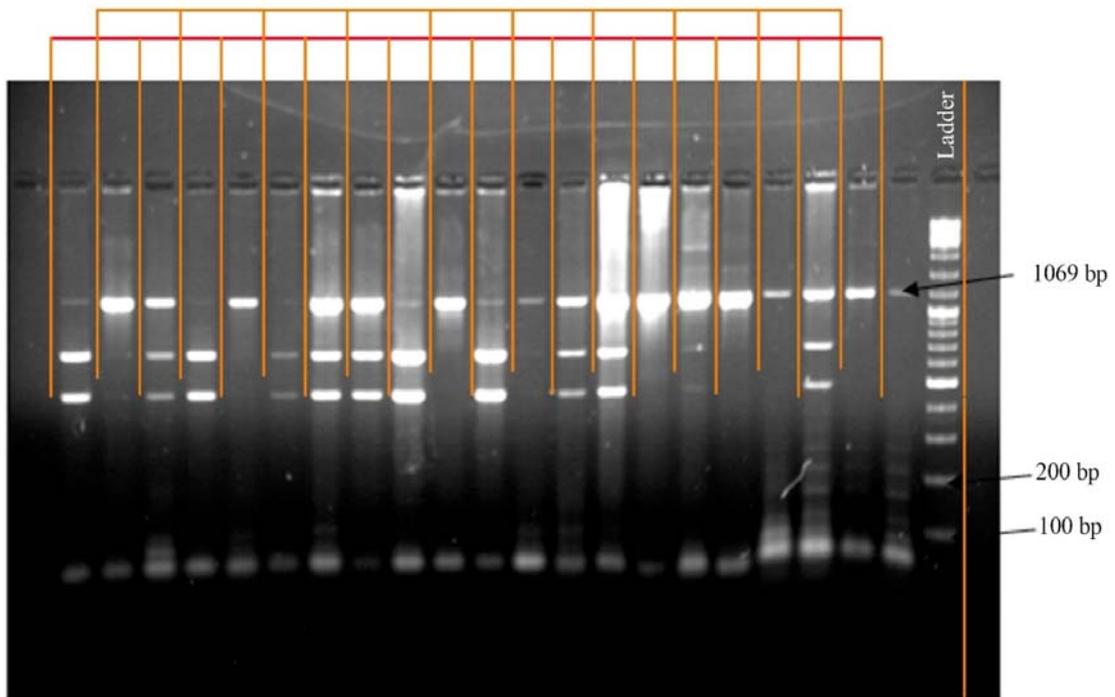


Figure 4 Analysis of PCR-RFLP for *GDF9 BcnI* site in the 1169 bp PCR product within exon 2 within *GDF9* gene

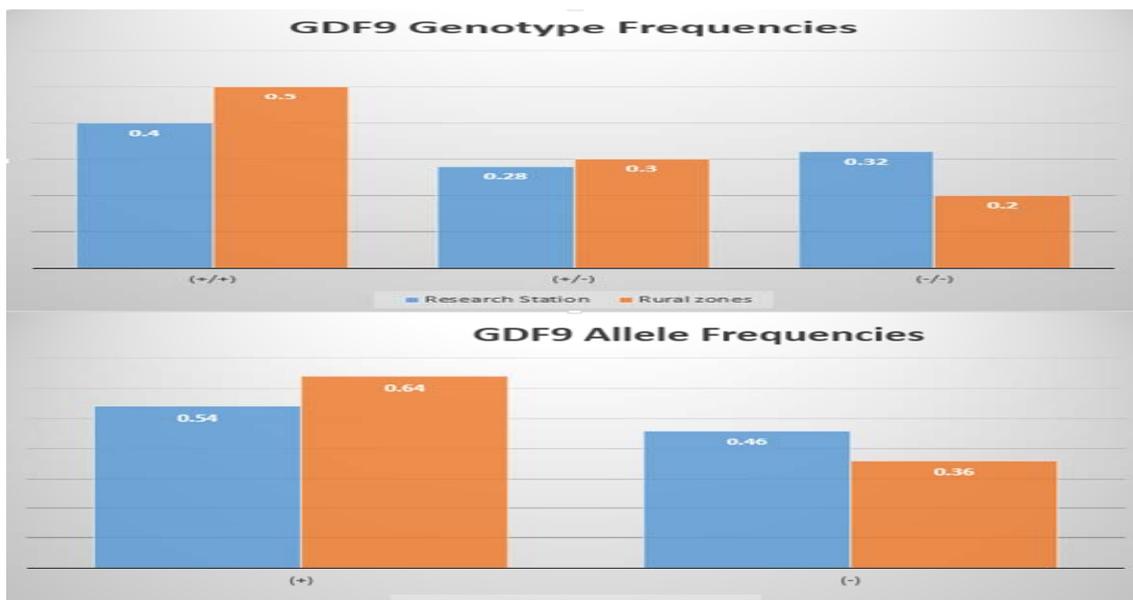


Figure 5 Genotype and allele frequencies of *GDF9* gene polymorphism between two groups of research and rural stations

Litter size was significantly influenced by genotype of the does for *GDF9* ($P < 0.01$). Heterozygous genotype showed higher (LSM±SE) litter size than homozygous genotypes ($P < 0.01$) in rural zones (2.66 ± 0.34 vs. 1.30 ± 0.18) and research station (2.41 ± 0.15) (Table 2).

Table 3 shows brief summary and comparison of our reports with similar study on fecundity candidate genes in goats. The reports of most of the similar works on monomorphic pattern of their indigenous goat breeds were in contrast of the present findings (Mehdizadeh *et al.* 2013; Shamik *et al.* 2009; Yuanqing *et al.* 2009; Deldar-Tajangookeh *et al.* 2009; Khatami Nejhada *et al.* 2010; Ganjali *et al.* 2012; Farag *et al.* 2013; Mohammadi and Alimahmoudi; 2011). Despite of this, our results are consistent with findings from earlier work on discovered polymorphism within the present candidate gene (Zhu *et al.* 2013; Xue-qin *et al.* 2009; Dutta *et al.* 2013; Ahlawat *et al.* 2013; Wang *et al.* 2013).

As logic, justification, the reason for a difference in the present result with other findings seems particularly due to different population and geographical and even more by technical staff, which may influence the analysis (Kasiriyana *et al.* 2011).

There are limitations in this study. To better understand how the ovary system organizes and coordinates fecundity during pregnancy, examination of single candidate gene polymorphism results alone may not be sufficient. This result is reasonable given that novel polymorphism, which can influence the primary function of the ovary during early pregnancy.

This study is groundwork for future investigations evaluating of fecundity rate in this specific indigenous goat breed. This present study demonstrated the designing specific primers based on caprine genome could preferable than using ovine primers and known SNPs for re-confirmation purpose in goat species.

Table 2 Association of *GDF9* gene polymorphism and litter size of Markhoz goat

Locus	N	<i>BcnI</i> (+/+)	<i>BcnI</i> (+/-)	<i>BcnI</i> (-/-)	P-value
Rural zones	38	(17) 1.30±0.18 ^b	(11) 2.66±0.34 ^a	(10) 2.0±0.26 ^{ab}	0.0001
Research station	82	(41) 1.57±0.16 ^b	(30) 2.41±0.15 ^a	(11) 1.80±0.10 ^b	0.0004

The means within the same column with at least one common letter, do not have significant difference ($P > 0.05$).

Table 3 Summary of similar study on fecundity candidate genes in goats

Breed	Country	N	Method	<i>BMP15</i>	<i>GDF9</i>	Association	Reference
Rayini	Iran	110	PCR-RFLP	M	M	No	Mehdizadeh <i>et al.</i> (2013)
Black Bengal	India	88	T-ARMS-PCR	M	M	No	Shamik <i>et al.</i> (2009)
Black		86					
Big foot	China	86	PCR-SSCP	-	p	Litter size	Zhu <i>et al.</i> (2013)
Jintang		86					
Haimen	China	170	PCR-SSCP	M	M	No	Yuanqing <i>et al.</i> (2009)
Guizhou	China	-	PCR-Sequencing	P	P	Not test	Xue-qin <i>et al.</i> (2009)
Assam hill	India	90	PCR-Sequencing	-	P	No	Dutta <i>et al.</i> (2013)
Iranian goat	Iran	-	PCR-RFLP	M	-	No	Deldar-Tajangookeh <i>et al.</i> (2009)
Black-Bengal							
Malabari	India		Direct sequencing	P	P	No	Ahlawat <i>et al.</i> (2013)
Osmanabadi							
Ganjam							
Alpain	Iran	50	PCR-RFLP	-	M	-	Khatami Nejhada <i>et al.</i> (2010)
Kamouri	Iran	40	PCR-RFLP	M	-	No	Ganjali <i>et al.</i> (2012)
Anglo-Nubian	Iran	25	PCR-SSCP	P	-	Litter size	Abdel-Rahman <i>et al.</i> (2013)
Zaraiby Damascus	Egypt	50	AS-PCR PCR-RFLP	M	-	No	Farag <i>et al.</i> (2013)
Najdi	Iran	50	PCR-RFLP	M	-	No	Mohammadi and Alimahmoudi (2011)

P: polymorphic pattern; M: monomorphic pattern and No: association study not done.

CONCLUSION

The sequence variability of the different amplified fragments utilized for genotyping was further investigated by PCR-RFLP, PCR-SSCP and sequencing in this study. Sequencing technology allowed the detection of two nucleotide variations, A both non-functional mutation (G/A) transition at nt 1026 known as P3) and a synonymous mutation at 1049 position, for the first time at the *BMP15* gene. Also, *GDF9* gene exhibited a polymorphism on *BcnI* site (1159 bp) within exon 2 of amplified PCR products (glutamine/proline). The litter size was significantly influenced by genotype of the does for *GDF9* ($P < 0.01$). Heterozygous genotype showed higher litter size than homozygous genotypes ($P < 0.01$). The *BcnI* polymorphism within Markhoz *GDF9* exon 2 gene could be further evaluated for use in marker assisted selection in litter size and fertility improvement of this breed. However, future investigations into the limitations research and any other research questions to improve our knowledge are necessary.

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