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Original Research Article

Detection of polymorphism in booroola gene and growth differentiation factor 9 in Lori sheep breed

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Abstract

***Purpose:** This study was carried out for detection of possible polymorphisms in Booroola gene (FecB) and growth differentiation factor 9 [GDF9] in Lori sheep breed.*
***Methods:** Blood samples were taken from the jugular vein of the sheep and their DNA content extracted using modified salting-out method. The quantity and quality of extracted DNA was examined by absorption spectrophotometry and gel electrophoresis. Using two pairs of specific primers, two DNA fragments were amplified from FecB (190 bp) and exon 1 of GDF9 (462 bp) genes. The polymerase chain reaction (PCR) products were digested using AvaII and HhaI restriction enzymes for FecB and GDF9 genes, respectively.*
***Results:** In FecB locus, two genotypes, viz, wild type (+/+) and carrier-mutants (B/+), were detected with genotype frequencies of 0.948 and 0.052, respectively. Also, at GDF9 locus, two genotypes, namely, wild type (+/+) and carrier-mutants (+/-), were detected with genotype frequencies of 0.67 and 0.33, respectively. Heterozygous genotype frequencies for both loci showed higher than homozygous genotypes. None of the sheep carried homozygous genotype for both of the FecB and GDF9 variants in this breed.*
***Conclusion:** The results of the present study reveal slight polymorphism in FecB and GDF9 loci of Lori sheep breed.*

***Keywords:** Polymorphism, Lori sheep, Booroola gene (FecB), Growth differentiation factor 9 (GDF9), Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), Genotype, Mutants, Loci*

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INTRODUCTION

Improved animal genetic resources with versatile characteristics are necessary to confront growing human demand. Improving the reproductive efficiency in sheep could be one of the key factors in increasing farm profitability. The reproductive potential of the flock is determined by genetics, and modified by flock management - namely ewe nutrition and selection. Although the genetic quality of a sheep herd is important, heritabilities for most reproductive traits are less than those for many other traits (0.05 to 0.15), and opportunities for within-breed selection are limited. Therefore, trying to improve the reproductive efficiency of a sheep herd by genetic selection is slow and difficult. Various mutations influencing ovulation rate and litter size

in sheep provide additional opportunities to rapidly adjust genetic potentials, but require careful breeding management [1].

In recent years, a number of natural genetic mutations have been identifiedin the bone morphogenetic protein (BMPR1B or FecB) and the growth differentiation factor 9 (GDF9) genes [2-10]. These genes were selected again to detect the probably effects on sheep prolificacy. However, it was certain that these mutations had great association with the ovulation rate of different sheep breeds.

Booroola gene (FecB) was the first major gene for prolific breeding identified in sheep and it is a dominant autosomal gene that has been mapped to the sheep chromosome 6q23-31[11]. It has been shown that, FecB is caused by a point mutation in position 830 (A to G) leading to an Arginine>Glutamine transition in the bone morphogenetic protein 1B receptor (BMPR-1B) expressed in oocytes and granulose cells [12,13].

The growth differentiation factor 9 (GDF9) is an autosomal over-dominant inheritance pattern gene that has been located to the sheep chromosome 5. It has been shown to be essential for growth and differentiation of early ovarian follicles [14]. The mutation in this gene is caused by a point mutation (G to A) leading to an Arginine > Histidine transition, leads to increased ovulation rate and twin or triplet births in heterozygotes, but animals homozygous for GDF9 mutation are sterile due to arrested follicular development from the primary stage of growth in some prolific breeds of sheep [15].

The Lori sheep breed is one of the largest mutton breeds in Iran and the origin of the Lori sheep is the Lorestan province. This breed is kept mainly to produce meat. Identification of major genes for prolific breeding is appropriate for improved production performance and is useful for breeders of this breed. Therefore, the present investigation was conducted to evaluate the presence of FecB and GDF9 mutationsin Lori sheep breed.

EXPERIMENTAL

Animal experiment

Lori sheep examined in this study were fat-tailed sheep, with large size and meat type. These sheep have strong constitution, good traveling ability with suitable conformation as a mountain sheep. Blood samples were collected randomly from 150 Lori sheep from the jugular vein, using vacuum blood collection tubes containing EDTA and stored at 4 °C.

Genomic DNA extraction

Genomic DNA was extracted by salting out procedure with minor modifications [16]. DNA quantity and purity of each sample were assessed by spectrophotometer and agarose gel electrophoresis, which were suitable for a PCR protocol application.

PCR-RFLP analysis

Based on the methods described by Wilson *et al* [12] and Davis *et al* [17], forced PCR–RFLP DNA test was used to detect the mutations of FecB and GDF9 in Lori breed sheep. The primer sequences used for the FecB AvaII site and GDF9 HhaI site are presented in Table 1.

Polymerase chain reactions were performed in a 25 µL reaction mixture containing approximately 2.5 µL of 10 X PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, 50-100 ng of ovine genomic DNA, and 1 U of Taq DNA polymerase (Promega, Madison, WI).

The amplification conditions for primers of the FecB gene were as follows: denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 59 °C for 60 s, and extension at 72 °C for 60 s; with a final extension at 72 °C for 10 min. The amplification conditions for primers of the GDF9 gene were carried out using 35 cycles of denaturation at 94 °C for 5 min; followed by denaturation at 94 °C for 45 s, annealing at 60 °C for 40 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 10 min.

Table1: Primer sequences used for the FecB AvaII site and GDF9 HhaI site

Locus	Primer Sequence
FecB	Forward: 5′-CCAGAGGACAATAGCAAAGCAAA-3′
	Reverse: 5′-CAAGATGTTTTCATGCCTCATCAACACGGTC-3′
GDF9	Forward: 5' -GAAGACTGGTATGGGGAAATG-3'
	Reverse: 5' -CCAATCTGCTCCTACACACCT-3'

The PCR products were digested with AvaII restriction enzyme (Fermantas Life Sciences, Canada) at 37 °C for 16 h. After digestion, FecB mutation yield 160 and 30 bp fragments (B/B) or non-carrier products remain uncut at 190 bp (+/+). Heterozygotes should produce fragments of 190, 160 and 30 bp (B/+). Also, the 462 bp PCR products were digested at 37 °C for 9 h with HhaI restriction enzyme. After digestion with restriction enzymes, all products were separated using electrophoresis in 2 % agarose gel and visualized with ethidium bromide. Digestion of the 462 bp fragment in GDF9 gene with HhaI restriction enzyme can reveal three genotypes. Homozygous carriers should produce fragments of 410 and 52 bp (-/-), the homozygous non-carriers (wild type) should produce fragments of 254, 156 and 52 bp (+/+) and heterozygotes should produce fragments of 410, 254, 156 and 52 bp (+/-).

Ethical matters

The animals were reared in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals [18]. Furthermore, the blood sampling procedures and handling of animals complied with the ethical guidelines of the Boroujerd University's Ethical Committee, Islamic Azad University, Boroujerd branch, Iran (approval ref no. 2014-845) [19].

Statistical analysis

Genetic diversity parameters, genotype and allele frequencies of FecB and GDF9 genes were calculated using POPGEN software (version 1.32). Hardy-Weinberg equilibrium for the population was also analyzed using Chi-square (X^2) test [20].

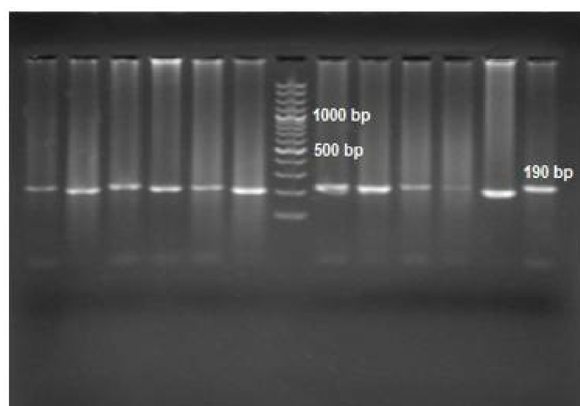
RESULTS

A DNA fragment with a size of 190 bp was amplified from exon 3 of FecB and 462 bp from exon 1 of GDF9 genes successfully. We analyzed the status of the FecB and GDF9 mutations in Lori breed and none of the individuals carried homozygous genotype for both FecB and GDF9 variants in this breed. Two genotypes, +/+ (190 bp), B/+ (190, 160 and 30 bp) for FecB and two genotypes +/+ (254, 156 and 52 bp), +/- (410, 254, 156, 52) for GDF9 were detected in Lori sheep (Figure 1 and 2). Also, the RFLP pattern of FecB and GDF9 genes are given in Figs 1 and 2, respectively.

Allelic and genotypic frequencies of the FecB gene and the GDF9 gene are presented in Table 2. For theBMPR-IB gene, frequencies of genotypes +/+ and B/+ were 0.948 and 0.052 respectively. For the GDF9 gene, frequencies of genotypes +/+ and +/- were 0.67 and 0.33 respectively. In both loci, wild types were dominant genotypes.

The result of chi-square analysis indicated that the population of Lori sheep for FecB locus was in Hardy-Weinberg equilibrium. Although due to the low samples genotype (B/B) was not identified. On the contrary GDF9 locus was not in agreement with Hardy-Weinberg equilibrium (p < 0.05).

Combined genotypic frequencies of the FecB and GDF9 mutations are presented in Table 3. Combined genotype +/+,+/+ (both wild type) had the highest frequency (0.661), whereas B/+,+/+ had the lowest frequency (0.017). These findings indicate that about 2/3 of the study population for both loci is wild-type.



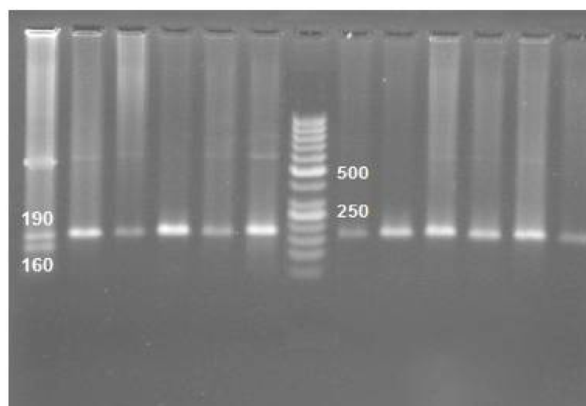


Figure 1: RFLP patterns of PCR amplification of FecB. Left: PCR product of FecB gene; right: Different genotypes of FecB gene, (+/+): 190 bp, and (B/+): 190, 160 and 30 bp.

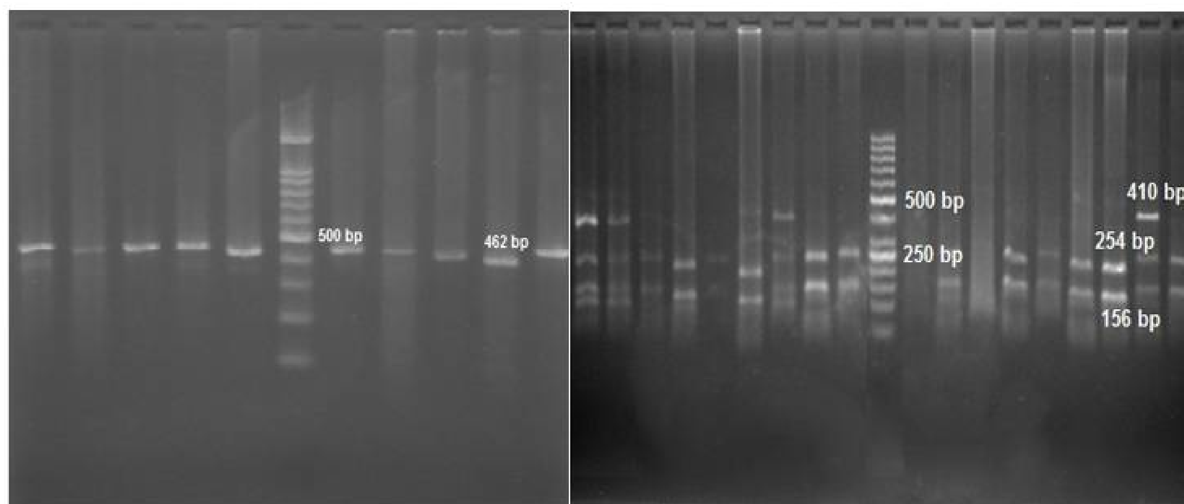


Figure 2: RFLP patterns of PCR amplification of GDF9. Left: PCR product of GDF9 gene (462 bp band); right: different genotypes of GDF9 gene; (+/+): 254, 156, and 52 bp, and (+/-): 410, 254, 156, and 52 bp.

Table 2: Allelic and genotypic frequencies of the FecB and GDF9 genesin Lori sheep breed

Gene	No. of animals	Allelic frequency		Genotypic frequency			*P*-value
FecB	150	+ 0.974	B 0.026	+/+ 0.948	B/+ 0.052	BB 0.00	0.791^{ns}
GDF9	150	+ 0.835	- 0.165	+/+ 0.670	+/- 0.330	-/- 0.00	0.039^{*}

B = FecB mutation; - = GDF9 mutation; + = wild-type; ns = non-significant; *$p < 0.05$

Table 3: Combined genotypic frequencies of FecB and GDF9 genes in Lori sheep

Parameter	Combined genotype			
Genotypic frequency	+/+,+/+ 0.661	B/+,+/+ 0.017	B/+,+/- 0.035	+/+,+/- 0.287

B = FecB mutation; - = GDF9 mutation; + = wild-type

Table 4: Genetic diversity parameters for FecB and GDF9 genes in Lori sheep breed

Gene	Effective number of alleles	Observed heterozygosity	Expected heterozygosity	Nei index	Average heterozygosity
FecB	1.05	0.052	0.051	0.051	0.051
GDF9	1.38	0.330	0.277	0.276	0.276

The observed and expected heterozygosity, effective number of alleles and Nei index for FecB and GDF9 genes in Lori sheep breed are shown in Table 4. Average heterozygosity as well as Nei index showed this breed did not have an appropriate genetic variation.

DISCUSSION

The results of the present experiment are consistent with those of Asadpour *et al* [21] which suggested that B/B genotype is not found in Zel sheep breed. Javanmard *et al* [5] and Bahrami *et al* [9] reported that none of the individuals carried homozygous genotype for the GDF9 variant in the breeds studied. In contrast to these reports, Moradband *et al* [3] observed that, all three possible genotypes for the GDF9 are present in Baluchi sheep population. Also, our findings are in conflict with Mahdavi *et al* [10] who reported that the BB, B+ and ++ genotypes have been identified in Kalehkoohi sheep breed 0.13, 0.446 and 0.424, respectively. In fact, this is the first report of homozygous carriers of the FecB gene in Iranian sheep breeds. Mahdavi *et al* [10] pointed out that the result of their study support the concept that BMPR-IB significantly affected litter size and thus it could be used for marker assisted selection programmers for the genetic improvement of reproductive characteristics in this breed.

The result of chi-square analysis indicated that population of Lori sheep for FecB locus was in

Hardy-Weinberg equilibrium, although due to the low samples used for this study, genotype (B/B) was not identified. In turn GDF9 locus was not in agreement with Hardy-Weinberg equilibrium ($p < 0.05$). According to our findings, Shi *et al* [8] reported that Hardy-Weinberg equilibrium for the FecB locus in Cele black sheep. In contrast to this report, Bahrami *et al* [9] and Kasiriyan *et al* [6] observed that populations of Hisari and Sangsari sheep were in the Hardy-Weinberg equilibrium at the GDF9 locus. Also, another study showed that the population of Kalehkoohi sheep breed at the FecB locus was not found to follow the Hardy–Weinberg equilibrium [10].

As shown in Table 4, lower heterozygous level was observed in both genes especially the FecB locus in the studied population. According to the present data, this appear to be due to the closed herd and the limited number of males used for reproduction. Therefore, reduce the level of heterozygosity was observed.

The observed number of alleles at the two investigated loci was similar to that previously reported by Hanrahan *et al* [15]. The remarkable thing is that in the present study, the effective number of alleles at the FecB Locus was near 1 (1.05), this means that only one allele of the two alleles (wild allele) in the population plays an important role. This is due to the vast difference in the frequencies of alleles in the population. This is true for loci GDF9 with less intensity (1.38). Notter *et al* [1] pointed out that there may be some possible exceptions, but we should not ignore that, generally there is ample evidence that increasing lambing rate leads to more profit and so is desirable.

Although meat production deserves the first priority in all regions and all of the Iranian sheep breeds are used as meat producers but some of them are known for other products, Lori sheep is the well-known meat type sheep but Kalehkoohi is the breed with relatively fine fiber, which is known as carpet wool producer. In Lori sheep, twinning rate is 5.4 twins per hundred lambs born. Lambing rate is 109 lambs per hundred ewes lambing. It seems that is not significant. According to the researchers [10], the finding of the FecB mutation in Kalehkoohi sheep breed, was an interesting result, as it was claimed that was the first case in which FecB mutation has been detected in fattailed sheep breeds in the Middle East.

Lori and Kalehkoohi sheep breeds are located in Lorestan and Markazi provinces, respectively. The two provinces are in the same neighborhood and breeding areas of these two breeds are too near together, and so it can be expected that the confluence happened between the two breeds. Furthermore, the detection of the FecB mutation in Lori sheep breed could be more exciting than Kalehkoohi sheep breed because they are much bigger than the Kalehkoohi, and therefore likely to prove more acceptable to sheep breeders as a source of FecB. According to the results, mutant allelic frequency for FecB locus was 0.026 (Table 2). However, Booroola gene has been introduced in many sheep populations around the world. Nevertheless, introgression of the FecB gene can improve the fecundity of non-prolific sheep flocks and this has already been widely demonstrated in Iran.

Beside, at the GDF9 locus, because the heterozygous genotype (+/-) was 1/3 of the population, mutant allelic frequency was 0.165 (Table 2). Moradband *et al* [3] pointed out that the mutation in GDF9 gene was associated with litter size in Baluchi sheep. They reported that FecB mutations are not present among this selected population of Iranian Baluchi sheep breed. Also, they explained that preliminary polymorphism analysis performed on mutation in GDF9 locus suggested a major gene inheritance of prolificacy in Baluchi population. Similarly, it seems that there is a homologous situation in Lori sheep breed.

Generally, there is no doubt that genes FecB and GDF9 play an important role in reproductive function, but, these findings indicate that selection for these genes in the studied population does not exist.

In addition, other major genes related to high prolific breeding may also be present. There have been several recent research findings in relation to inheritance patterns and DNA testing of major genes for prolific breeding that have the potential to significantly increase the reproductive performance of sheep flocks throughout the world. These findings will also enhance knowledge of the control of reproductive regulatory mechanisms, for example studies on the inhibin α gene [22], the melatonin receptor 1A gene [23] and the prolactin gene [24]. Therefore, the prolific breeding of the ewes could not be totally accurately predicted by the FecB and GDF9 genotypes alone. More extensive screening is required to fully reveal the mechanisms underlying the prolific breeding of Iranian sheep.

CONCLUSION

The results obtained indicate that the native sheep breed studied shows light polymorphism

at FecB and GDF9 loci. The identified single nucleotide polymorphism (SNP) in the genes might be useful as a molecular marker in association studies.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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