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Short Communication

Identification of the *GDF9* mutation in two sheep breeds by using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) technique

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A genetic mutation with major effects on the litter size in sheep was recently identified in the growth differentiation factor (GDF9) gene of the TGF-B super family (transforming growth factor). GDF9 gene has been localized to chromosome 5 in sheep. In order to evaluate the GDF9 gene polymorphism, blood samples were collected randomly from 42 Kordi sheep and 44 Arabic sheep from Kordestan and Khozestan provenance. Exon 1 from GDF9 gene was amplified to produce a 462 bp and exon 2 from GDF9 gene was amplified to produce a 139 bp fragment. The amplified fragment of Exon 1 was digested with Hin6I restriction enzyme and the amplified fragment of exon 2 was digested with DdeI restriction enzyme. Exon 1 revealed two alleles, (denoted A and B) and exon 2 revealed a single allele. In these populations for exon 1, AA and AB genotypes were observed. With attention to the role of a GDF9 in increasing ovulation rate, it seems that this gene can be used as a marker for increasing the twin rate.

Key words: Polymorphism, PCR- RFLP, GDF9 gene.

INTRODUCTION

Multiple ovulations in mammals are a complex trait influenced by genetic and environmental factors. Primates and many ruminants typically release a single oocyte at each cycle whereas species such as mice and pigs are capable of rearing many offspring, have consistently high ovulation rates. Current models of follicular selection indicate that multiple ovulation is controlled both by concentrations of follicle stimulating hormone (FSH) near the time of follicular selection and by intraovarian factors (Montgomery et al., 2001). Ovulation rate in mammals is determined by a complex exchange of hormonal signals between the pituitary gland and the ovary and by a localized exchange of hormones within ovarian follicles between the oocyte and its adjacent somatic cells (Davis, 2005; Davis, 2004).

Genetic mutation with major effects on the ovulation rate in sheep was recently identified in gene growth differentiation factor (GDF9) of TGF-B super family (transforming growth factor-B) (Knight and Glister, 2003; Knight and Glister, 2006). The TGF-B super family contains over 35 members, many of which have been shown to be important for regulating fertility. GDF9 is oocytespecific growth factor that appears to play key roles in granulosa cell development and fertility in most mammalian species (Su et al., 2004). Animals homozygous for the GDF9 mutation are anovulatory whereas animals heterozygous for GDF9 have higher than normal ovulation rate (Chu et al., 2005). This shows that GDF9 is essential for normal folliculogenesis in sheep. GDF9 has been located to chromosome 5 in sheep (Hanrahan et al., 2004). The aim of the present investigation is to study polymorphism in GDF9 in two Iranian sheep breeds.

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Abbreviations: GDF9, Growth differentiation factor; **TGF-B,** transforming growth factor; **FSH,** follicle stimulating hormone; **PCR-RFLP,** polymerase chain reaction- restriction fragment length polymorphism.

MATERIALS AND METHODS

A total of 42 Kordi sheep and 44 Arabic sheep by a high litter size were examined in this study from Kordestan and Khozestan provenance. Genomic DNA was isolated by using corrected phenol-chloroform extraction method (Mohammadi and Saberivand, 2006). A 462 bp fragment of exon 1 and 139 bp fragment of exon 2 of

Table 1. Primers sequences used to amplify fragment of the GDF9 gene.

Name	Sequence (5'-3')	PCR product size(bp)	RFLP site sequences
Hin6I-RFLP	F:GACTGGTATGGGGAAATG R:CCAATCTGCTCCTACACACCT-	462	G/CGC
DdeI-RFLP	F:CTTTAGTCAGCTGAAGTGGGACAAC R:ATGGATGATGTTCTGCACCATGGTGTGAACCTG	139	C/TGA

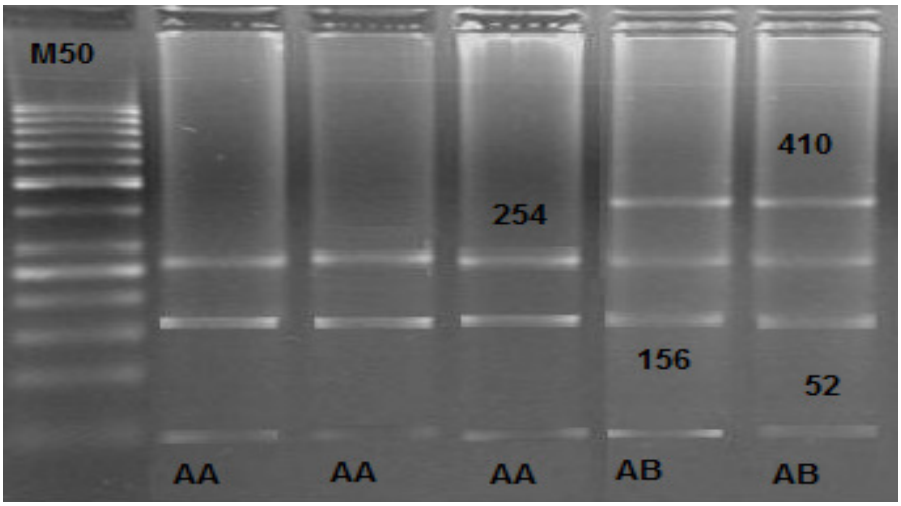

Figure 1. GDF9 genotyping by PCR-RFLP method using Hin6I.

GDF9 gene was amplified using primers (Table1). Primers sequences were established by Hanrahan et al. (2004). Polymerase chain reaction (PCR) was carried out for both fragments in a final volume of 25 µl containing approximately 33.3 ng of genomic DNA, 1 unit of Taq DNA polymerase, 2.5 µl of 1 x PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTP and 0.4 pM of each primer. Amplification conditions for 462 bp included an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 45 s, 58°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The amplification for 139 bp fragment consisted of initial denaturation at 94°C for 5 min, followed by 33 cycles at 94°C for 30 s, 62°C for 40 s, and 72°C for 30 s, followed by a final extension at 72°C for 4 min. The PCR products were separated by 1.2% (w/v) agarose gel electrophoresis. The amplified fragment of exon 1 was digested with 10 units of Hin 6I restriction enzyme and 10 µl of PCR product at 37°C for 8 h in a water bath and 15 µl of 139 bp PCR product was digested with 10 units of DdeI restriction enzyme at 37°C for 10 h. The digested PCR products were subjected to 2.5% (w/v) agarose gel electrophoreses and stained with ethidium bromide, the banding being visualized and documented. POPGENE software was used to estimate the gene and genotype frequencies, the heterozygosity and effective number of alleles (Yeh et al., 1999).

RESULTS AND DISCUSSION

All samples were evaluated with PCR- restriction fragment length polymorphism (RFLP) technique using Hin6I and DdeI restriction enzymes. The use of PCR-RFLP to detect new mutation is only feasible if such mutation creates or destroys a restriction target for the enzyme used. For the 462 bp PCR GDF9 fragment, three Hin6I restriction sites were found in the AA as 52, 156 and 254 bp fragment (wild-type). In the AB genotype, four restriction sites as 52, 156,254 and 410 bp (heterozygous type) were found (Figure 1).

For the 139 bp PCR, GDF9 fragment were observed with two DdeI restriction site in the AA genotype as 31 and 108 bp (wild type). The results indicated that there was no polymorphism in this exon (Figure 2).

Among 42 Kordi sheep examined with Hin6I, two genotypes were observed; frequencies were 0.81 and 0.19% for AA and AB, respectively. This gives frequencies of 0.91 and 0.09 for A and B alleles, respectively (Table 2). Chi square test was used and indicated that the population followed Hardy-Weinberg equilibrium (X^2 = 0.024), so low genetic diversity in this population was observed. The allele's frequencies of Hid6I in Arabic sheep were 0.92 and 0.08 for A and B alleles, respectively, and two genotypes were observed; genotype frequencies were 0.84 and 0.16 for AA and AB, respectively (Table 2). Chi square test was 0.001 which means genetic diversity was low in this breed too.

The GDF9 allele frequencies estimated for Hin6I in the present study were approximately similar to the Hanrahan et al. (2004). In addition to the allele frequencies of DdeI, it is also similar to Chu et al. (2005) previously reported. GDF9 is one of the main genes in granulose cell develop-

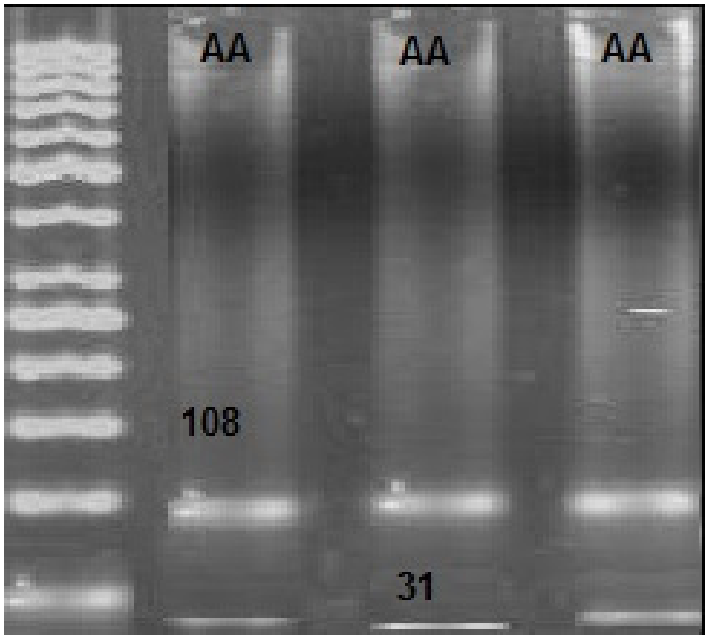

Figure 2. GDF9 genotyping by PCR-RFLP method using DdeI.

Table 2. Genotype and allele frequencies of Hid6I.

Breed	Genotype frequencies		Allele frequencies	
	AA	AB	A	B
Kordi	0.81	0.19	0.91	0.09
Arabi	0.84	0.16	0.92	0.08

ment and fertility in most mammalian species. The findings presented in this study indicate that two breeds are polymorphic by using Hin6I, and using DdeI is monomorphic in two breeds. Further investigations could be carried out taking into consideration all possible genotype at different loci and using other restriction enzymes for recognizing the variants.

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