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ASSOCIATION BETWEEN THE PROLIFICACY OF ROMANOV SHEEP BREED AND FECUNDITY GENE, GROWTH DIFFERENTIATION FACTOR 9 GENE AND PROLACTIN GENE GENOTYPES

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ABSTRACT

Prolificacy data of Romanov sheep breed were retrieved from Sekhra Station (Jordan) through five parities. The data were used for studying the effect of Fecundity (FecB), Growth Differentiation Factor 9 (GDF9) and Prolactin genes on the prolificacy of Romanov ewes. GDF9, FecB and Prolactin genes genotypes were investigated in Romanov ewes by using the PCR-RFLP technique. The Mixed Model of SAS software was used for analyzing the data. The different gene genotypes and the parity were inserted in the Model as fixed effects while the dams were inserted as Random. Fecundity gene was observed to be monomorphic, the wild type genotype of Fecundity gene was found in the Romanov ewes. Prolactin and GDF9 genes were observed to be polymorphic in Romanov. The results revealed non-significant differences in the prolificacy of the ewes that carry AA and BB of Prolactin gene genotypes. The GDF9 gene genotypes showed significant ($P \leq 0.0001$) differences in prolificacy. The homozygous MM genotype ewes produced 0.792 more lambs born per lambing than the heterozygous NM genotype. Selection based on GDF9 mutation may help in improving the prolificacy of Romanov sheep.

Key words: Romanov Sheep, fecundity, gene association

INTRODUCTION

The Romanov sheep (*Ovis aries*) breed is well known for its high prolificacy; however, maximizing litter sizes by selection using ovulation rates may not be acceptable for the sheep producer because of its complicated procedure. Extreme litter sizes with five lambs or more has occurred in the Romanov and the Finnsheep breeds. High prolificacy rate may not acceptable because of its effect on ewe health especially when it is associated with low milk production (SanCris-Tobal-Gaudy *et al.*, 2001). In addition to this, the limitations of the number of teats (two) in sheep may restrict the ability of the ewes to suckle more than two lambs, but this can be avoided by introducing the artificial rearing system (milk replacers by artificial teats). The desired level of fecundity is fluctuated according to the production system. For instance, in pastoral systems, twins are more desired than triplets while triplets or quadratics or even more are acceptable in the intensive systems (Simm, 1998; Notter, 2008).

The prolificacy of the sheep is controlled by one or many genes such as: fecundity gene (FecB) (McNatty & Henderson, 1987; Montgomery *et al.*, 1992; Baird & Campbell, 1998 & Yan *et al.*, 2005), growth differentiation factor (GDF9 gene) (Hanrahan *et al.*, 2004; Moradband *et al.*, 2011 & Liandrisa *et al.*, 2012) and prolactin gene (PRL) (Oul *et al.*, 2007; Chu *et al.*, 2008 & Jawasreh *et al.*, 2014). The FecB gene was reported by many researchers (Davis *et al.*, 2002; Yan *et al.*, 2005; Guan *et al.*, 2006; Ghaffari *et al.*, 2009; Asadpour *et al.*, 2012 & Jawasreh *et al.*, 2013) to be a major gene controlling prolificacy.

The ovulation rate and litter size were affected by the mutations that found in the fecundity genes, the additive genetic effect was detected in some of those genes such as fecundity gene that has been

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found to be a major gene in Booroola Merino sheep (Wilson *et al*., 2001).

The GDF9 gene polymorphisms observed in the Norwegian white sheep (Vage *et al*., 2013), Cambridge and Belclare sheep (Hanrahan *et al*., 2004) and Awassi sheep (Jawasreh *et al*., 2014) were not associated with prolificacy. The lack of association between prolificacy and the GDF9 gene in some breeds and its major effect in others could be attributed to the other fixed alleles or multiple mutations interaction. Davis *et al.* (2006) indicated that fertility may be affected by high number of genes that are expressed in suitable way and much of them need to be activated by the effect of other fixed or genetic interactions. Fecundity genes can be expected to be found in prolific breeds with high variations in litter size and ovulation rate. Inconsistent inheritance patterns and high repeatability can also be as a sign for indicating the presence of fecundity genes in sheep. This may include Swedish Fine wool, Finnsheep, East Friesian dairy, Romanov; Blue face Leicester and Barbados Black belly breeds (Davis *et al*., 2006). The low heritability estimate of the reproductive traits can lead to an ineffective selection program, so selection using known genes with major effect on fertility traits is an effective approach to reduce the generation intervals and speed up the genetic gain in ovulation rate and litter size. The objective of the present study was to investigate the polymorphisms of Prolactin, Fecundity and GDF9 genes in Romanov sheep and to investigate the relationship between the genes polymorphisms and Romanov sheep breed prolificacy.

MATERIALS AND METHODS

The experiment was conducted at Sekhra Center for Research and Extension, Sekhra- Jordan (Latitude: 32.33 and Longitude: 35.75, 900 m above sea level). Five ml of blood samples were collected from the jugular vein of 35 Romanov ewes and predicted for 70 ewes using segregation analysis of GENPROB software (Ker & Kinghorn, 1996) by combining the obtained genotypes and the available pedigree information. In addition to Romanov population two Afec Awassi ewes DNA (carriers for one copy of fecundity gene (FecB)) were used as a positive control for FecB gene only. Another DNA sample from Awassi sheep known to be a carrier for the heterozygous genotype was also used as positive control for the heterozygous AB genotype. Blood samples were placed in ice box and then stored at 4°C until DNA extraction and genotyping.

Genomic DNA extraction

DNA was extracted from the collected blood using E. Z. N. A blood DNA kit (OMGA- bio-tek, Inc.). DNA quality was tested using spectrophotometer and confirmed using 1.5% of Agarose Gel electrophoresis.

PCR Amplification and Restriction Fragment Length Polymorphism (RFLP) Analysis (PCR-RFLP) method was carried out for detecting the polymorphisms that may found in the growth differentiation factor gene (GDG9; FecG1), Prolactin gene (PRG) and Fecundity gene (FecB). Primer sequences, annealing temperatures, digestion enzymes and PCR product length for each gene are presented in Table 1. In PCR-RFLP assay, PCR reactions were set up in a final volume of 20 μL using 10 μL nuclease free water, 2 μL (50 ng) of genomic DNA, 2 μL Primer forward (10 pmol/ μL), 2 μL (10 pmol/ μL) Primer reverse and 4 μL of HOT FIREPol® DNA Polymerase, then specific modified PCR protocol for each primer was applied depending on the annealing temperature as describe in Table 1. The PCR products of each of PRL, FecB and GDF9 genes were digested by 10 U of *Hae III, AvaII* and *HhaI*, enzymes, respectively for 3 hours at 37ºC then the agarose gel electrophoresis was used for obtaining the exact genotype of each individual.

Table 1. Primers sequences, annealing temperatures, and PCR product length of the targeted genes

(Primers 5'→3')	Annealing	Product length
PRL-F: ACCTCTCCTCG-GAAATGTTCA PRL-R: GGGACACTGAAGGACCAGAA	56°C	1,209 bp
FecB-F: CCAGAGGACAA-TAGCAAAGCAAA FecB-R: CAA- GATGTTTTCATGCCTCATCAACACGGTC	60°C	190 bp
GDF9-F: GAAGACTGG-TATGGGGAAATG GDF-R: CCAATCTGCTCCTACACACCT	58°C	462

Genotype imputation

The relationship among all individuals from the pedigree with known genotypes was used for predicting the genotype of the non-genotype relatives using GENEPROB software of Ker and Kinghorn (1996) which was achieved by the segregation analysis. The accuracy of the predicted genotypes was estimated by obtaining the genotype probability index (GPI) of Kinghorn (1997).

Genotype probability index for animals with a GPI of 0% were excluded from the data set and those of high GPI along with the genotyped animals were used for studying the association between the prolificacy and the genes that used in this study.

To guarantee the quality of the analyses, data were edited and abnormal records or out of biological limit were deleted. Gene and genotype frequencies were calculated according to Falconer and Mackay (1996) procedure.

Analysis of variance was performed using a mixed model to evaluate the effect of parity, PRP and GDF9 as fixed effects and dam nested in sire and residual as random effects. Statistical analyses were carried out with the version 9.1 of the Statistical Analysis System (SAS, 2004).

RESULTS AND DISCUSSION

The studied population included 70 ewes and two Afec Awassi ewes (carriers for one copy of fecundity gene (FecB) used as a positive control, for the FecB gene). The PCR product (190 bp) of the FecB gene was successfully amplified by Thermal Circulation Machine at 60ºC annealing temperature (Figure 1), while Figure 2 displayed the PCR-RFLP results of FecB gene by *AvaII* digestion enzyme. The FecB gene was investigated by many authors (Souza *et al*., 2001; Davis *et al.,* 2002; Yan *et al*., 2005; Guan *et al*., 2006; Ghaffari *et al.*, 2009 & Asadpour *et al*., 2012) to be a major gene for prolificacy.

The digestion products resulted in a wild type (++) ,190 bp band, in all Romanov sheep studied population, while the Afec Awassi showed the heterozygous genotype (+ -), (190 bp and 160 bp) (Figure 4). These results were in agreement with Kumar (2006) in Mulpura sheep; Davis (2006) in Blueface, Leicester, D'Man, East Friesian, Finn, Galician, Romanov, Teeswater, Chios, Mountain Sheep, Barbados Blackbelly, German White headed mutton, Lleyn and Loa breeds; Michailidis *et al.* (2008) in Chios and Florina Greek breeds; Ghaffari *et al.* (2009) in Shal Sheep; EL-Hanafy and El-Saadani (2009) in Egyptian breeds, Rahmani, Ossimi, Awassi, Barki and Awassi x Barki; Moradband *et al.* (2011) in Baluchi Sheep; Abulyazid *et al.* (2011) in crossbred Egyptian sheep; Abouheif *et al.* (2011) in Najdi and Naeimi breeds in Saudi Arabia. The Afec Awassi genotype that holds the heterozygous (+-) FecB gene was reported to be prolific as reported by Gootwine *et al.* (1993), (2001), (2003) and Jawasreh *et al.* (2013).

According to our results in FecB gene polymorphism and referring to the monomorphic (absence of heterozygosity (not polymorphic) genotypes it can be concluded that the genetics factor controlling twinning in Romanov sheep may not related to the mutation in FecB gene and it is possible that some other genes rather than FecB gene may control twinning in Romanov sheep.

The point mutation of the FecG1 at GDF9 locus was investigated in the Romanov sheep breed according to Moradband *et al.* (2011). The PCR product was obtained giving a band of size 462 bp (Figure 3). This product was also digested by the *HhaI* restriction enzyme to give us as stated by

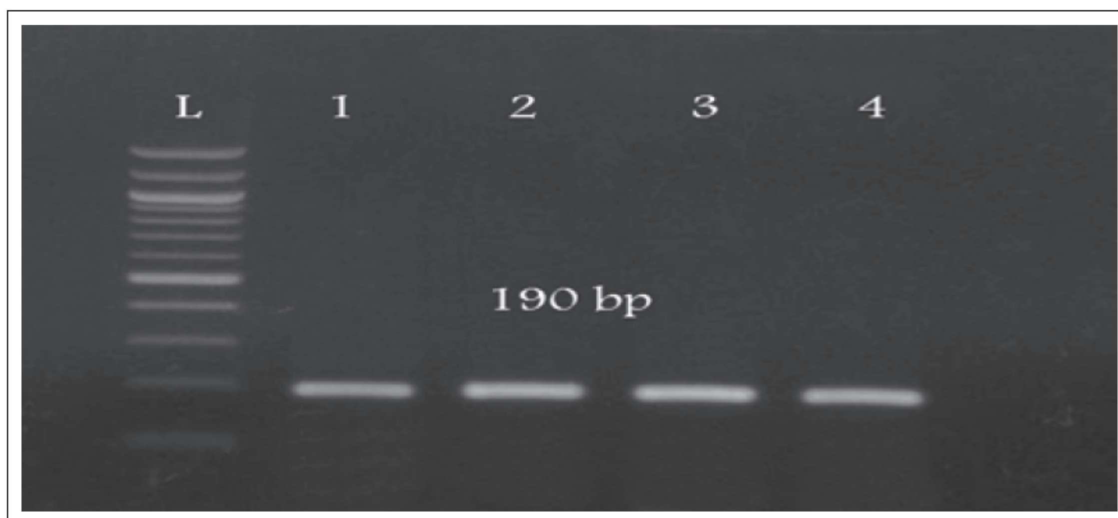

Fig. 1. PCR product of FecB gene (190bp) analyzed by electrophoresis. L = ladder (100bp) and 1-4 = PCR product of FecB gene.

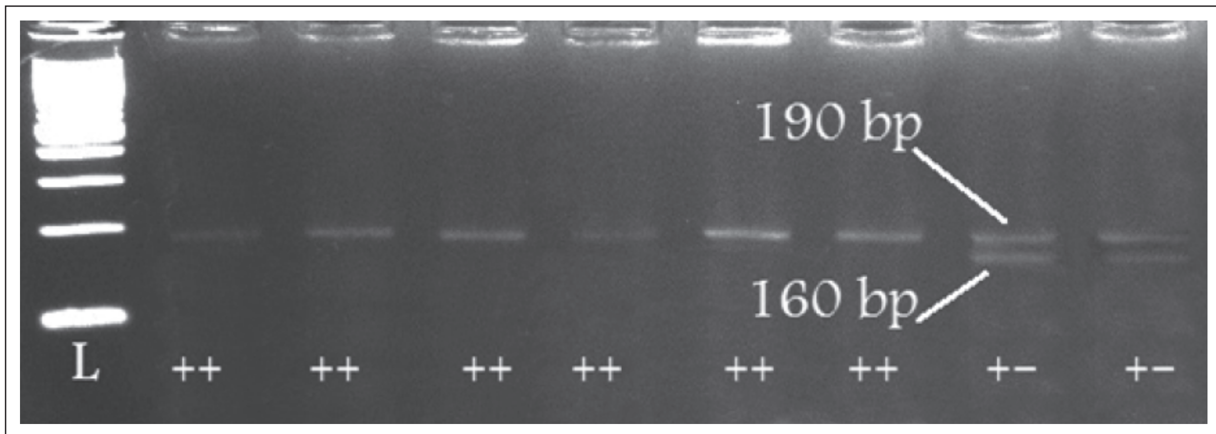

Fig. 2. PCR-RFLP results for FecB gene by *AvaII* restriction enzyme on 3% agarose gel. L = ladder 100 bp, (++) genotype = Romanov sheep with only 190 bp band, (+-) genotype = Afec Awassi animals with 190 bp and 160 bp bands (positive control).

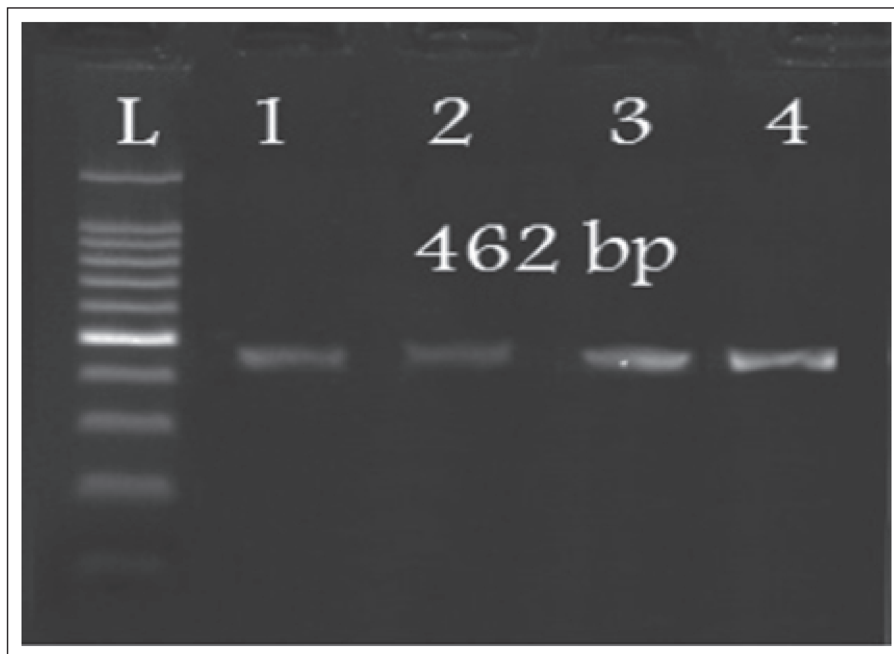

Fig. 3. PCR product of GDF9 gene (462bp) analyzed by Agarose Gel electrophoresis = ladder 100bp and 1-4 = PCR product of GDF9 gene.

Moradband *et al.* (2011), three bands of 52, 156 and 254 bp in case of homozygous animals for the M allele (MM), four bands of sizes of 52, 156, 254 and 410 bp in case of heterozygous genotype (NM) (Figure 4), while the homozygous genotype (NN) for the B allele (NN) 52 and 410 bp that reported by Moradband *et al.* (2011) in Baluchi breed; Liandrisa *et al.* (2012) in Chios breed; Hanrahan *et al.* (2004) in Belclare Cambridge breeds did not appeared in the studied Romanov sheep population. The same two genotypes were also detected by Liandrisa *et al.* (2012) findings in Jawasreh *et al.* (2014) in Awassi sheep who indicated the absent of NN individuals in their studied populations.

The allele frequencies of GDF9 gene were calculated to be 0.79 and 0.207 for A and B alleles, respectively, while the genotype frequencies were 0.585 and 0.415 for AA and AB genotypes, respectively. Allelic frequencies in Awassi sheep population were 0.951 for A allele (mutant allele) and 0.049 for B allele while the genotypic frequencies were 0.889 for AA genotype and 0.123 for AB genotype (Jawasreh *et al*., 2014).

Highly significant ($P<0.0001$) differences were obtained between the prolificacy obtained for AA and AB GDF9 genotypes (Table 2). The overall mean of the prolificacy obtained for AA-GDF9 genotype was 2.732 ± 0.17 LB/L while it was 1.94

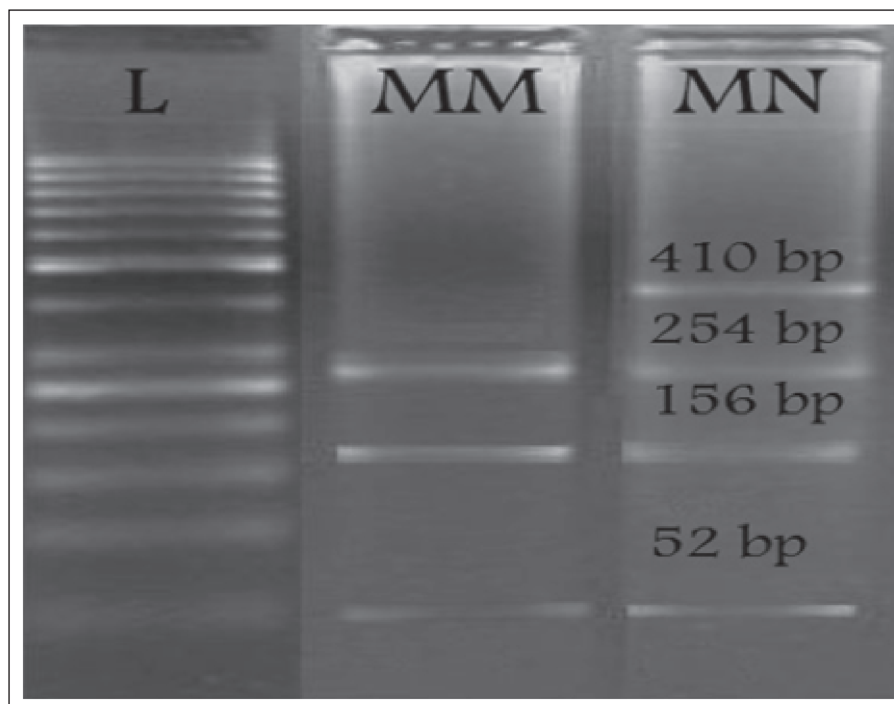

Fig. 4. PCR-RFLP results for GDF9 gene by *HhaI* restriction enzyme on 3% agarose gel. L = ladder 50 bp, MM = genotype with 52, 156 and 254 bp bands, MN = genotype with 52, 156, 254 and 410 bp bands.

Table 2. Least squares means ± S.E of the Romanov sheep breed prolificacy in the different genotypes obtained for GDF9 and Prolactin genes

Genotype	Number	Mean (LB/L)	S.E	P-value*
GDF9				
AA	41	2.732	0.17	0.0001
AB	29	1.94	0.18	
Prolactin				
AA	64	2.234	0.1	0.4814
BB	6	2.436	0.28	

*adjusted for the effect of Parity, GDF9: Growth differentiation Factor 9 gene, S.E. Standard error. LB/L: lamb Born per Lambing.

± 0.18 LB/L for the heterozygous genotype. Growth differentiation factor-9 (GDF-9) has been reported to play crucial roles in determining folliculogenesis, ovulation rate and litter size in sheep (Moore *et al*., 2004).

Prolactin gene (1200 bp) (Figure 5) was amplified by PCR at 56ºC, the A variants appeared with 540, 370, 147, and 152 bp amplified fragments while B variant displayed 517, 370, 147 and 152 bp (Figure 6). Only two genotypes were detected but the heterozygous individuals were not found in the studied population. These results were consistent with the results obtained by Orford *et al.* (2010).

The A allele of the prolactin gene was calculated to be 0.914 and it was 0.086 for the B allele. The AA genotype frequency of the Prolactin gene was calculated to be 0.91 (Table 2). The AB genotype was not appeared in the Romanov breed at the contrary the three genotypes were found in Awassi sheep with allelic frequencies were 0.757 for A allele and 0.243 for B allele and the genotypic frequencies were 0.646 for AA, 0.221 for AB and 0.131 for BB (Jawasreh *et al*., 2014). Ramos *et al.* (2009) found that a value of 0.639 was recorded to the A allele and 0.361 value to the B allele in Serra da Estrela breed, 0.57 to A allele and 0.43 for B

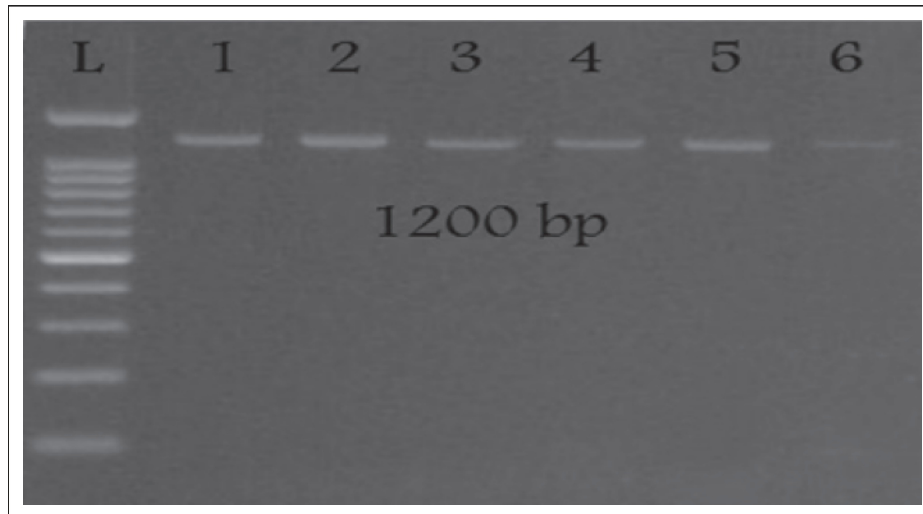

Fig. 5. PCR product of prolactin gene (1200bp) analyzed by electrophoresis. L = ladder 100bp and 1-6 = PCR product of Prolactin gene.

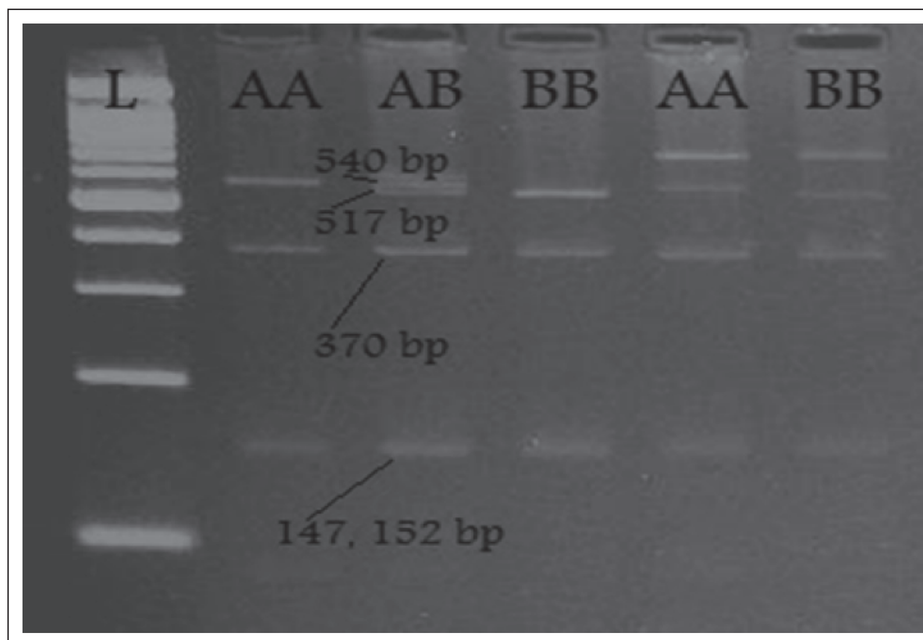

Fig. 6. PCR-RFLP results for Prolactin gene by *HaeIII* restriction enzyme on 3% agarose gel. L = ladder 100 bp, AA = genotype with 540, 370, 147, and 152 bp bands, BB = genotype with 517, 370, 147, and 152 bp bands and AB = genotypes with 540, 517, 370, 147, and 152 bp bands set as Positive control not included in the analysis.

allele in white Merino breed and 0.722 to A allele and 0.278 for B allele in Black Merino.

No association was detected between the two variants found in PRL gene and prolificacy of Romanov. The prolactin gene was associated with the prolificacy of Awassi sheep as reported by Jawasreh *et al.* (2014) but in the Romanov sheep breed no association was observed. The population size and the low number of the BB-PRL genotype may lead to this result and/or the mutations in PRL gene are not responsible for the high prolificacy in Romanov sheep.

CONCLUSION

The Romanov sheep breed prolificacy is affected by the GDF9 gene mutation. The AA genotype produces more prolificacy than the AB genotype; it

may be used as a selection criterion for improving the prolificacy of Romanov sheep.

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