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Investigation of G1 (c.260G>A) polymorphism in exon 1 of GDF9 gene in Turkish sheep breed Karayaka

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Abstract: *GDF9* is an essential gene for normal follicular development and prolificacy in sheep. The aim of this study was to determine the genetic polymorphism and G1 mutation of *GDF9* gene exon 1 in 100 Karayaka ewes, belonging to four subpopulations in the Black Sea Region provinces of Turkey. Based on DNA sequence results, two alleles (A and G) and two genotypes (GG and GA) were determined with the restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR-RFLP) method. The frequencies of mutant (A) and wild-type (G) alleles were detected as 0.10 and 0.90. The frequencies of GG and GA genotypes were calculated as 0.79 and 0.21, respectively. Twenty-one percent of the studied animals were heterozygous GA genotype. The values for observed (H_o) and expected (H_e) heterozygosity for *GDF9*-exon 1 were estimated as 0.210 and 0.189, respectively. DNA sequence results revealed that Karayaka breed had the mutation G1 (c.260G>A). In conclusion, this mutation might be used as a potential marker to improve litter size rate in Karayaka sheep as evidenced in other sheep breeds.

Key words: Karayaka sheep, litter size, *GDF9* gene exon 1, PCR-RFLP analysis, *HhaI* restriction enzyme

1. Introduction

Sheep farming in Turkey plays an important role in terms of sociocultural structure, animal numbers, economic output, and animal production. Although Turkey has many sheep breeds, the production has not reached the desired level. In Turkey, sheep breeding has been mainly for meat production and less for milk and wool. The fact that meat yield is closely related to lamb yield has led to studies aiming to increase the number of lambs per ewe. Improving reproductive performance is an important issue to meet human demand for sheep products [1]. For this reason, the number of lambs per birth (litter size), in other words, fertility comes to the fore [2]. The used traditional breeding strategies on fertility were insufficient for increasing lamb production due to the low heritability of fertility [3,4]. Therefore, using genetic information of the genes linked to reproductive traits could affect the selection response in animal breeding [5].

Nowadays, the studies have been focused on mutations to develop advanced breeding strategies. With the development of molecular techniques, the mutations related to multiple births have helped genetic breeding programs that aim to improve litter size [6]. The studies of inheritance patterns and DNA testing of major genes for

fecundity have disclosed that major genes play a significant role in increasing the fertility performance in sheep flocks worldwide [7].

The effects of various candidate genes on multiple births in sheep have been reported. These genes are the bone morphometric protein receptor 1B (BMP1B), bone morphogenetic protein 15 (BMP15), and growth differentiation factor 9 (*GDF9*) genes. A member of the transforming growth factor-beta (TGF β) superfamily, the *GDF9* gene is located on ovine chromosome 5 [8]. The *GDF9* gene, which is expressed by oocytes, is an essential gene for normal follicular development in sheep. It consists of 2 exons and 1 intron separated by 1126 bp and codes a propeptide with 453 amino acid residues [9]. Eight different point mutations (G1–G8) have been identified in the *GDF9* gene in Belclare and Cambridge sheep [10]. Three mutations, namely G2, G3, and G5, cause nucleotide changes that do not result in an amino acid change, but the rest of those mutations, including G1, result in a change of amino acid (Arginine (R) \rightarrow Histamine (H)). A common feature of those mutations is that heterozygous ewes have been observed to have increased ovulation and birth rates in sheep [11].

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The mutation G1 has been reported in some Iranian sheep breeds, such as Baluchi, Sangsari, and Mehraban [11]. It was reported that the heterozygous ewes with this mutation were fertile and had a high litter size, while wild-type ewes had a smaller litter size [12]. In another study, it was found that ewes of AG genotype were the largest number of lambs (1.88) compared to AA genotypes (1.22) [3]. Paz et al. [13] reported that G1 mutation increased ovulation rate in heterozygous ewes in Chilota sheep [13]. Researchers indicated that this polymorphism could be used in the studies for improvement of fertility traits in Chilota sheep. Apart from its effects on ovulation rates, it was reported that lamb weight at birth in heterozygous ewes was higher than those in homozygous ewe as well [14]. Recently, the identification of *GDF9* gene has been of interest for some researchers [9,14]. In particular, the effects or presence of the mutations of this gene on fertility have been focused on small ruminants [9, 15–18].

In Turkish native sheep breeds, a mutation affecting fertility has not been reported on *BMP-15*, *BMPR-1B*, and *GDF9* genes [2,19–21]. Additionally, no study has included the G1 mutation in the *GDF9* gene. Numerous studies have confirmed a positive correlation between G1 mutation and litter size in sheep. Due to the important effects of *GDF9* on ovulation rates and since no studies have been performed on its polymorphism, there is clearly a need to study this gene. Therefore, we aimed to investigate the genetic polymorphism of *GDF9* exon 1 and G1 mutation in Karaya sheep.

2. Material and methods

In the present study, 100 blood samples that were previously collected from Karayaka sheep raised in the provinces of Samsun, Ordu, Giresun, and Tokat in the Black Sea region of Turkey were used. Blood samples were randomly taken from populations. Sample collecting was performed according to the Animal Care and Use Guidelines of Ondokuz Mayıs University of Local Ethical Committee (2013/64). Genomic DNA was extracted using a DNA isolation kit (IDPURE Spin Column, USA).

The samples were amplified with polymerase chain reaction (PCR) using primers (Forward;5'GAAGACTGGTATGGGGAAATG3', Reverse;5'CCAATCTGCTCCTACACACCT 3') [22]. PCR reactions were carried out in a final volume of 25 μ L, consisting of 12 μ L Tag DNA polymerase Master Mix red (2X) (1.5 mM MgCl₂ final concentration), 1 μ L (10 pmol/ μ L) of each forward and reverse primer, 1.5 μ L of DNA (30–50 ng), and 9.5 μ L ultrapure water. The PCR reaction was performed as follows: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 40 s, annealing at 64 °C for 40 s, extension at 72 °C for 5 min, and a final extension at 72 °C for 5 min. The PCR products of 462 bp

were run in 2% agarose gel electrophoresis and visualized with EtBr (500 μ L/mL in H₂O).

PCR products were digested with *HhaI* restriction enzyme in a final volume of 30 μ L, consisting of 1 μ L of fast digest enzyme, 10 μ L of PCR product, 2 μ L of green buffer, and 17 μ L ultrapure water. The reaction mix was first incubated at 37 °C for 20 min and then at 65 °C for 10 min. After digestion, the products were run in 3% agarose gel electrophoresis and visualized with EtBr (500 μ L/mL in H₂O).

To confirm the existence of the mutation, a total of 8 samples, belonging to homozygous and heterozygous individuals selected according to agarose gel visuals from each population, were subjected to sequence analyses in ABI 3100 capillary electrophoresis instrument. Obtained sequence results belonging to the mutant and wild-type alleles were aligned and compared with the *GDF9* sequences of the complete DNA of *Ovis aries* (GenBank no. AF078545.2) and Norwegian White sheep (GenBank no. HE866499.1) using ExPASy translate tool [23].

The frequencies of allele and genotype and observed and expected heterozygosity values were calculated using PopGene32 software program version 1.32 [24]. The Hardy–Weinberg equilibrium for the studied populations was analyzed using chi-square (X^2) test.

3. Results

After digestion of 462 bp PCR products of the *GDF9* exon 1 gene with *HhaI* restriction enzyme, a total of four DNA fragments were obtained indicating two genotypes as follows: wild homozygous ones, GG and heterozygous ones GA as shown in Figure 1.

The frequencies of allele and genotype were calculated for the investigated populations and summarized in Table.

Twenty-one percent of the studied animals were heterozygous genotype (GA), presented with four bands of 52, 156, 254, and, 410 bp. The frequency of homozygous or wild type genotype (GG) ewes was observed with the highest frequency of 79% and was denoted with three bands, 52, 156, and 254 bp.

A allele frequency mean was 0.10 through the studied populations and ranged from 0.08 in Samsun, Giresun, and Tokat to 0.16 in Ordu. As for allele G, its frequency mean was 0.90 and varied from 0.92 in Samsun, Giresun, and Tokat to 0.84 in Ordu. Consequently, the most frequent allele was G, and the lowest was A in the studied populations. Therefore, the most frequently found genotype for the Karayaka breed was homozygous or wild genotype (GG) with a frequency of 0.79. The heterozygous GA genotype was observed with a frequency of 0.21 and varied from 0.32 in Ordu to 0.16 in Samsun and Giresun. The homozygous AA genotype or mutant genotypes were not determined in the Karayaka sheep breed. Consequently,

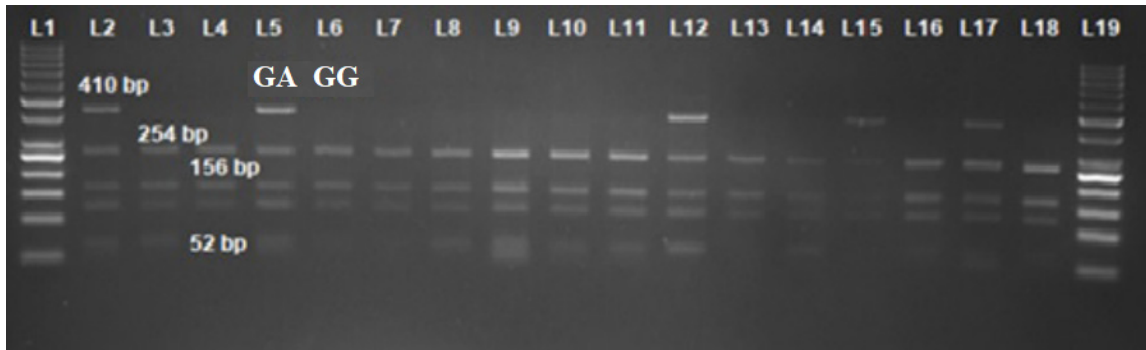


Figure 1. The determined genotypes at position c.260G>A for *GDF9*-G1 with the PCR-RFLP analysis. L1–L19 = 50 bp DNA ladder; L3, L4, L6–L11, L13, L14, L16, L18 = wild genotypes; L2, L5, L12, L15, L17 = heterozygous genotypes.

Table. Genotype and allele frequencies of *GDF9* exon 1.

Populations	N	Allele frequency		Genotype frequency		Heterozygosity			P-value
		G	A	GG	GA	Ho	He	X^2	
Samsun	25	0.92 (23)	0.08 (2)	0.84 (21)	0.16 (4)	0.160	0.150	0.139	0.709
Ordu	25	0.84 (21)	0.16 (4)	0.68 (17)	0.32 (7)	0.320	0.274	0.781	0.377
Giresun	25	0.92 (23)	0.08 (2)	0.84 (21)	0.16 (4)	0.160	0.150	0.139	0.709
Tokat	25	0.92 (23)	0.08 (2)	0.80 (20)	0.20 (4)	0.200	0.184	0.242	0.622
Total	100	0.90 (90)	0.10(10)	0.79 (79)	0.21 (21)	0.210	0.189	1.305	0.253

N, number of individuals; observed heterozygosity (Ho); expected heterozygosity (He); chi-square test for HWE (X^2), P-value (P).

Ordu had a higher prevalence of heterozygous genotypes and mutant alleles than other populations.

The observed (Ho) and expected (He) heterozygosity values were estimated for the studied populations and summarized in Table.

Considering all populations, Ho varied from 0.160 in Samsun and Giresun to 0.320 in Ordu with an average of 0.210, while He ranged from 0.150 in Samsun and Giresun to 0.274 in Ordu with an average of 0.189. Overall, the observed heterozygosity value was higher than expected. Consequently, the whole populations showed a low heterozygous level for the *GDF9* gene.

Concerning possible deviations from Hardy–Weinberg, the exact P-values for the studied populations, were calculated as shown in Table. The chi-square analysis results indicated that whole populations were in the Hardy–Weinberg equilibrium ($P > 0.05$).

Restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR-RFLP) PCR-RFLP products were confirmed by sequencing according to agarose gel visuals. Obtained sequence results belonging to the mutant and wild-type alleles were uploaded to National Center for Biotechnology Information database under accession numbers (MT295368–MT295371). DNA

sequence results proved the existence of a nucleotide change (G → A) at position 260 of the 462 bp DNA fragment of the *GDF9* gene exon 1. This identified nucleotide change or single nucleotide polymorphism (SNP) is called G1 mutation. To show the presence of nonidentified (a) and identified SNP (b) in the exon 1 of the *GDF9* gene, the chromatogram results of the DNA forward sequence are given, as seen in Figure 2. To show nucleotide change, sequencing results for both alleles were compared to the *GDF9* sequencing results for *Ovis aries* (GenBank no. AF078545.2) and Norwegian White sheep (GenBank no. He866499.1). The results proved that the Karayaka breed had A (mutant) and G (wild) alleles (Figure 3).

4. Discussion

Determination of nucleotide variations or major mutations on candidate genes affected by reproduction traits is an important subject for improving small ruminants' fertility traits. For this reason, researchers have tried to find the known or novel mutations in their native sheep breeds using different methods such as PCR-RFLP, PCR-SCCP, DNA sequencing, and GWAS analysis [11,25,26].

By using mostly the PCR-RFLP method, six different mutations (FecXI, FecXH, FecXG, FecXB, FecB, FecXG),

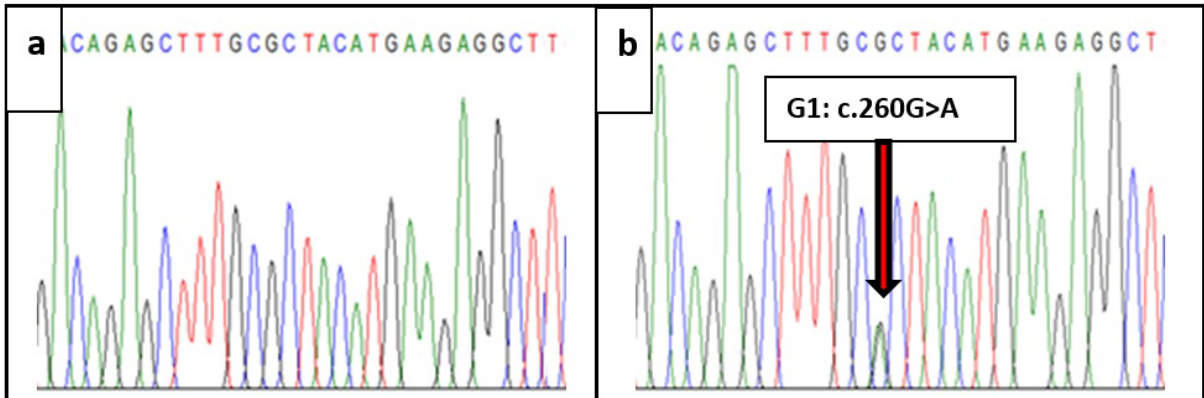


Figure 2. The nonidentified and identified SNP in the exon 1 of *GDF9* gene in Karayaka sheep breed (a: nonidentified region, b: the identified SNP).

<u>Ovis aries GDF9 seq.</u>	CATGGCGCTTCCCAACAAATTCTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTGTTTTCC	60
<u>Norwegian White seq.</u>	CATGGCGCTTCCCAACAAATTCTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTGTTTTCC	60
<u>G allele, Karayaka seq.</u>	CATGGCGCTTCCCAACAAATTCTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTGTTTTCC	60
<u>A allele, Karayaka seq.</u>	*****	
<u>Ovis aries GDF9 seq.</u>	TATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTGTAGCTAGGACTGCGTT	120
<u>Norwegian White seq.</u>	TATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTGTAGCTAGGACTGCGTT	120
<u>G allele, Karayaka seq.</u>	TATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTGTAGCTAGGACTGCGTT	120
<u>A allele, Karayaka seq.</u>	TATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTGTAGCTAGGACTGCGTT	120
<u>Ovis aries GDF9 seq.</u>	GCCAGATGACAGAGCTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGA	300
<u>Norwegian White seq.</u>	GCCAGATGACAGAGCTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGA	300
<u>G allele, Karayaka seq.</u>	GCCAGATGACAGAGCTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGA	300
<u>A allele, Karayaka seq.</u>	GCCAGATGACAGAGCTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGA	300

Figure 3. Comparison of the G and A alleles of the *GDF9* gene of Karayaka sheep with those of Norwegian White and *Ovis aries* complete

which are on *BMP15*, *BMPR1B*, and *GDF9*, were investigated in some Turkish native sheep breeds [19–21,27,28]. In most of those studies, the investigated genes had monomorphic structure. Until now, a major or novel mutation, which is related to litter size or ovulation rate, has not been determined in Turkish native sheep breeds. As some reasons for these results, researchers have focused on the importance of sample size, preferred method, and whether the breed is prolific or not [29].

For instance, the presence of *FecB* allele was not reported by Polat [30] who studied Chios sheep (406 samples), a prolific breed, while Davis et al. [31] found *FecB* allele in only twelve samples per breed in Hu and Han sheep. Similarly, Amini et al. [26] reported a novel T755C mutation associated with a litter size of Iranian Lori-Bakhtiari sheep (96 samples) by using the PCR-SCCP and DNA sequencing methods, while it was not reported in the same breed by Abdoli et al. [15] who conducted a study on ten ewes.

Consequently, it can be said that many factors will affect the succession in this kind of studies. Karslı and Balcıoğlu [19] pointed out that it is necessary to study with purebred sheep breeds and the maximum sample number. On the other hand, Dincel et al. [21] and Gursel et al. [20] emphasized that different mutations should be studied with an intensive screening.

In the present study, we tried to study Karayaka sheep bred as pure with an average sample number higher than those in some previous studies of native sheep breeds in Turkey. Moreover, it was considered studying with more populations because the genetic variations changed from one population to another, as evidenced by the previous studies on Karayaka sheep [32,33]. Maybe, this strategy provided determination of mutant allele in this study because the mutation was observed in only Tokat population.

There have been almost no studies of Turkish sheep breeds on exon 1 of the *GDF9* gene. A study carried out

by Çelikeloğlu et al. [34] investigated the *GDF9* exon 1 gene with eight samples, and the studied gene was monomorphic. This may be due to the used sample size and other factors as described above. In another study, Gursel et al. [20] found heterozygous genotypes for *FecX^G* and *FecG^H* mutations on the genes of *BMP15* and *GDF9* in Awassi, Chios, Imrose, and Kivircik sheep breeds while it was monomorphic for the mutations *FecX^I*, *FecX^H*, *FecX^B*, and *FecB* on *BMP15* gene in the mentioned breeds.

Up to now, G1 mutation, also called *FecG¹*, has not been determined on Turkish native sheep breeds. It shows that there has been a need to study this gene. Therefore, in the present study, a comparison of allele and genotype frequencies was not established for exon 1 of *GDF9* gene with Turkish sheep breeds.

In the study, the highest frequency genotype for the Karayaka breed was wild homozygous genotype GG (0.79) due to the low frequency of the A allele. This result was similar to the findings for Russian sheep breeds Salsk (0.88) and Volgograd (0.84) [3]. The frequency (0.21) of heterozygous genotypes (GA) in Karayaka sheep were similar to those of Colombian Creole hair sheep (0.24) and Volgograd sheep (0.16) [3,35]. Moreover, the frequencies of A (0.10) and G (0.90) alleles of *GDF9* exon 1 gene are consistent with those of the same studies (G: 0.89–0.94, A: 0.08–0.11). The allele and genotypic frequencies from this study are also in line with reports on Moghani sheep in which the allele frequencies were 0.78 for *FecG⁺* and 0.22 for *FecG⁻*, the genotype frequencies were 0.66 for *FecG⁺/FecG⁺* and 0.24 for *FecG⁺/FecG⁻* [36].

Mutant AA genotypes of the *GDF9* gene were not determined in the present study. This finding was similar to those reported by some researchers [12,28,29]. The absence of a mutant genotype can be explained by the low frequency (0.10) of A allele in the Karayaka breed, and mutant genotypes could suffer from embryonic death and reproductive defects [28]. This obtained result might also be due to differences in the biological effects of mutations, changing by species [37]. Similar results were observed in Chilean [12] and Iran fat-tailed sheep breeds [29].

There are conflicting results in the literature on associations of homozygous and heterozygous genotypes with litter size or ovulation rate. For example, a study conducted in Bangladesh sheep indicated the homozygous ewes (AA) had a higher lambing rate (2.00 ± 0.41) than heterozygous ones (GA) (1.59 ± 0.41). On the contrary, Russian Salsk and Volgograd breeds showed higher lambing rates (1.80 ± 0.12 to 1.88 ± 0.17) in heterozygous (AG) sheep compared to homozygous (GG) sheep (1.13 ± 0.09 to 1.22 ± 0.11) [3]. Jawasreh et al. [38] reported that the homozygous ewes (MM) of the *GDF9* gene produced 0.792 more lambs than that of heterozygous genotypes (NM). These results showed that prolificacy seemed to be

affected differently by genetic variants of *GDF9* gene exon 1.

Paz et al. [13] showed that mutation of *GDF9*-G1 or *FecG¹* led to the increased litter size in heterozygous ewes (1.56 ± 0.08) than that of the wild-type genotype (1.25 ± 0.09) in Chilota sheep breed. Similarly, Javanmard et al. [39] reported that heterozygous ewes for G1 had a litter size of 1.78, while the wild genotypes had an average litter size of 1.16 in Iran fat-tailed sheep breed. A similar result was also reported by Gorlov et al. [3]. However, in another study, it was reported that G1 mutation did not affect the reproduction of Mehraban sheep in Iran [12]. Similar effects were reported for Afshari, Ghezel, Lori-Bakhtyari, or Shal sheep breeds in Iran as well [29].

There was only one study on the polymorphism of *GDF9* exon 1 based on DNA sequencing carried out by Çelikeloğlu et al. [34]. The author did not report a mutation in Pırlak sheep. In the present study, DNA sequencing allowed the detection of nucleotide variation on *GDF9* gene. A SNP (c.260G>A known as G1 mutation) was found for the first time at the *GDF9* gene, in a Turkish sheep breed Karayaka.

In this breed, the existence of the G1 mutation might be due to more and large sampling from geographically distinct regions. Similar findings were observed in Belclare, Cambridge, and Mehraban ewes [10,30] but not in Rhmani × Barki, Barki, Ossimi Suffolk × Awassi, or Rhmani breeds (GenBank no; KT357481.1, KT357482.1, KT357484.1, KT357485.1, KT357486.1).

The G1 mutation causes an increase in ovulation rate or multiple births in heterozygous ewes. However, in homozygous ewes, it causes sterility due to the arrested follicular development [10]. On the other hand, some studies demonstrated that genotypes of *GDF9* exon 1 (AA, AG, and GG) have no significant effect on fertility traits [10,12,40]. Briefly, it can be said that ovulation rates in ewes heterozygous for the *GDF9* gene are mostly higher than those of homozygous animals [41,42]

A previous study demonstrated that Karayaka sheep flocks' twinning birth rate could be increased by approximately 3–6-fold using selection in favor of twin births [43]. Considering this and the findings obtained from the present study, it can be said that fertility traits might be improved in the Karayaka breed as evidenced in other sheep breeds. However, it is necessary to perform further studies in order to provide exact evidence the effects of G1 mutation on fertility traits in Karayaka and other Turkish sheep breeds.

In conclusion, we determined the exon 1 of *GDF9* gene was polymorphic in Karayaka sheep breed and found out G1 mutation (c.260G>A) on this gene. There were evidenced relationships between this mutation and litter size in other sheep breeds. For this reason, the similar

relationships should be investigated in native sheep breeds in Turkey.

Conflict of interest

The authors declare that they have no conflicts of interest.

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