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Molecular characterization of fecundity-related gene regions in some of Türkiye's native sheep breeds

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Abstract: In this study, the BMP15 and GDF9 gene regions were examined through DNA sequencing in some native sheep breeds of Türkiye (Kıvırcık, Karacabey Merino, Chios, Gökçeada, Çine Çaparı, Awassi, and Karakacan). In the BMP15 exon 2 region, two single nucleotide polymorphisms (SNPs) were identified: one synonymous SNP, P248P (proline/proline), and one nonsynonymous SNP, L251P (leucine/proline), resulting in a missense mutation. In the GDF9 exon 1 region, only a nonsynonymous SNP, R87H (histidine/arginine), was found. In the GDF9 exon 2 region, four SNPs were identified: two nonsynonymous SNPs, E241K (glutamate/lysine) and V332I (valine/isoleucine), and two synonymous SNPs, L159L (leucine/leucine) and E326E (glutamate/glutamate). The identified haplotypes in the GDF9 gene region showed a high D' (linkage disequilibrium) value, indicating a strong association. Furthermore, a complete correlation was observed between the E326E and V332I variants in the studied breeds. The Chios breed exhibited significantly higher frequencies of heterozygosity for the E326E and V332I variants compared to the Kıvırcık and Karacabey Merino breeds. The heterozygosity frequencies for the E326E and V332I variants in the Kıvırcık, Karacabey Merino, and Chios breeds were determined to be 0.08, 0.13, and 0.46, respectively. The higher frequency of heterozygosity observed in E326E and V332I variants within the Chios breed suggests their association with the trait of multiple births.

Key words: BMP15 gene, GDF9 gene, multiple births, sheep, SNP

1. Introduction

Litter size and ovulation rate, two vital factors influencing fertility, are influenced by specific known genes. Three significant gene regions, bone morphogenetic protein receptor 1B (BMPR1B), bone morphogenic protein 15 (BMP15), and growth differentiation factor 9 (GDF9) genes, have been identified as contributors to these traits [1]. Traditional breeding methods, characterized by extended timelines and reliance on phenotypic data, necessitate multiple generations to achieve genetic enhancements. Therefore, the integration of genetic studies and the adoption of marker-assisted selection (MAS) techniques into conventional breeding practices assume substantial importance for enhancing reproductive efficiency [2].

Bone morphogenetic protein 15 (BMP15), also known as the FecX gene, serves as the principal gene governing fertility, with predominant expression in oocytes. Notably, this gene is recognized as the most polymorphic site within the sheep genome [2]. Its functionality involves interactions with several other proteins, particularly through the SMAD pathway, regulating diverse cellular processes such as oocyte development and maturation [3]. The BMP15 gene is situated on the X chromosome and spans a length of 5.6 kilobases (kb). This gene comprises two exons and an intron region, encoding a protein of 393 amino acids [4]. Eight distinct mutations within the BMP15 gene region have been identified, each designated according to the associated sheep breeds. These mutations are denoted as follows: FecXI in Inverdale sheep, FecXG (Galway mutation) in Cambridge sheep, FecXH in Hanna sheep, FecXB in Belclare sheep, FecXL in Lacaune sheep, FecXR in Rasa Aragonesa, FecXGr in Grivette sheep, and FecXO in Olkuska sheep [2].

The GDF9 gene comprises one intron and two exons, with a total size of 2.5 kb, encoding a protein of 453 amino acids. Situated on the 5th chromosome [4], GDF9 has been established as a pivotal factor influencing ovulation and birth rates in sheep. Similar to BMP15, the autosomal GDF9 gene plays a crucial role in regulating follicular growth by modulating granulosa cell function [3,5].

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In this study, the molecular characterizations of the BMP15 and GDF9 gene regions of Türkiye's native sheep breeds (Kıvırcık, Karacabey Merino, Chios, Gökçeada, Çine Çaparı, Awassi, and Karakacan breeds) were examined.

2. Material and methods

In this study, a total of 311 individuals across seven breeds (Kıvırcık, Karacabey Merino, Chios, Gökçeada, Çine Çaparı, Awassi, and Karakacan breeds) were examined for the BMP15 gene region, and a total of 260 individuals representing three breeds (Kıvırcık, Karacabey, Merino, and Chios breeds) underwent examination for a part of the GDF9 gene region via DNA sequencing. All breeds, except for Merinos, were being raised within the scope of the "Conservation of Domestic Animal Genetic Resources" project carried out by the General Directorate of Agricultural Research and Policies (TAGEM). Moreover, two breeds, Çine Çaparı and Karakaçan, were under the threat of extinction. Sampling was conducted from the last remaining three herds protected by TAGEM. Sampling was conducted from three herds for the Chios breed (İzmir-Çeşme), two herds for the Kıvırcık breed (Kırklareli-Üsküp and Balıkesir-Bandırma), one herd for Gökçeada (Balıkesir-Bandırma), three herds for Awassi (Şanlıurfa), and one herd for the Karacabey Merino breed (Balıkesir-Bandırma). There were no relationships between herds of the same breeds, but a minimum level of inbreeding could be expected within each herd.

Blood samples, ranging from 6 to 8 mL, were collected from the jugular vein of each animal and transferred into EDTA blood tubes. All DNA extractions were performed using a commercial DNA extraction kit (GeneAll Exgene Blood SV) from blood. To amplify the targeted gene regions, 20–30 ng of DNA was utilized from each sample. The PCR mixture consisted of 10 mm Tris-HCl pH 8.3, 1 pmol of both forward and reverse primers, 0.5 µM dNTP (10 mM), 1.80 mM $MgCl_2$, and 0.25 U of Taq DNA polymerase. During the primer design, the NCBI online primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used with consideration given to primer length, annealing temperature, and primer base composition to achieve a GC content of 45%–50%. The primer sequences for the examined regions, the lengths of the amplified fragments (bp), and the positions of the amplified regions are provided in Table 1. These details are recorded in the NCBI Genbank database.

PCR products were sequenced using Applied Biosystems 3500 Genetic Analyzer and the BigDye Terminator v3.1 Cycle Sequencing Kit with the standard protocol. Chromatograms were visualized with the FincTV and aligned in the MEGA7 [6]. Observed heterozygosity, expected heterozygosity, p values from the Hardy-Weinberg (HW) equation, and minor allele frequencies (MAF) of the SNPs detected in the studied gene regions were calculated using the Plink 1.07 [7] program. Haplotype predictions and linkage disequilibrium (LD) analyses were conducted using the Haploview program (https://www.broadinstitute.org/) with embedded Phase v2.1 algorithm [8]. The results were then visualized using the same program.

Table 1. Primer sequences and fragment lengths for the BMP15 and GDF9 gene regions.

Gene regions	Primer sequences	Base pair (bp)	GenBank accession numbers
BMP15-EX1Y-F	5'-AAGCGTTATCCTTTGGGCTTTT-3'	565	JN655672 87-652
BMP15-EX1Y-R	5'-GGAGCCCTAAAGGGAAGCAAA-3'		
BMP15_EX2_F	5'-GGTCCAGAAAAGCCCAACCA-3'	744	JN655672 5910-6654
BMP15_EX2_R	5'-CTGAGCTAGCTGCACCTTTG-3'		
GDF9-EX1-F	5'-TAAGCACCTGGAAGTGGGAG-3'	994	AF078545 1267-2261
GDF9-EX1-R	5'-AGCAGGGCCAACTCCTTTATG-3'		
GDF9-EX2-F	5'-TCAGGAACCTTTCCATCAGTGG-3'	909	HE866499 551-1459
GDF9-EX2-R	5'-ATCAGGCTCGATGGCCAAAA-3'		

3. Results

No mutations were identified within the exon 1 region of the BMP15 gene. However, a T to C transition at position 248 within the exon 2 region led to a silent mutation, resulting in a synonymous single nucleotide polymorphism (SNP) denoted as P248P. The P248P SNP was observed in both Karacabey Merino and Kıvırcık sheep breeds. Another T→C transition occurred at leucine→proline (L251P), resulting from a missense mutation in the exon 2 region. The L251P mutation was detected in sheep breeds, including Karakacan, Merino, Çine Çaparı, and Chios. The chromatograms of the BMP15 gene are provided in Figures 1 and 2. Based on the computed values within the respective breeds, it was determined that the L251P variant was not found in the Hardy-Weinberg equilibrium in the Awassi and Chios breeds ($p < 0.05$). In contrast, the Hardy-Weinberg equilibrium was maintained in the remaining breeds for both variants (Table 2). Furthermore, it was observed that the two identified variants in the BMP15 gene did not combine to form haplotypes.

In the GDF9 gene, five SNPs were identified: one (R87H) inducing a missense mutation and amino acid substitutions within the exon 1 region, two (L159L, E326E) exhibiting silent mutations, and two (E241K and V332I) leading missense mutations in the exon 2 region. It was observed that only the R87H variant showed significant departures from the Hardy-Weinberg equilibrium in the Chios breed ($p < 0.05$), whereas the remaining variants maintained equilibrium in the studied breeds. Details of the SNPs detected in exon 1 and exon 2 regions are given in Table 3. The G→A transition at the 87th protein position in the GDF9 exon 1 region caused a missense mutation, resulting in an amino acid change from arginine to histidine. The heterozygosity frequencies of this mutation, detected in Kıvırcık, Karacabey Merino, and Chios breeds, were found to be 0.40, 0.19, and 0.11, respectively.

A part of the GDF9 gene exon 2 region was amplified. In this region a silent mutation, L159L SNP (leucine/leucine), was detected in Kıvırcık, Karacabey Merino, and Chios breeds with very high heterozygosity frequencies determined as 0.42, 0.45, and 0.49, respectively. SNP No. 241 (E241K), which was detected in Kıvırcık, Karacabey Merino, and Chios breeds, occurred as a result of A→G transition, converting glutamate (E) to the lysine (K). The heterozygosity frequencies of E241K were found to be 0.29, 0.15, and 0.22 in the Kıvırcık, Karacabey Merino, and Chios breeds, respectively. E326E and V332I SNPs were identified as the most significant findings in this study. The

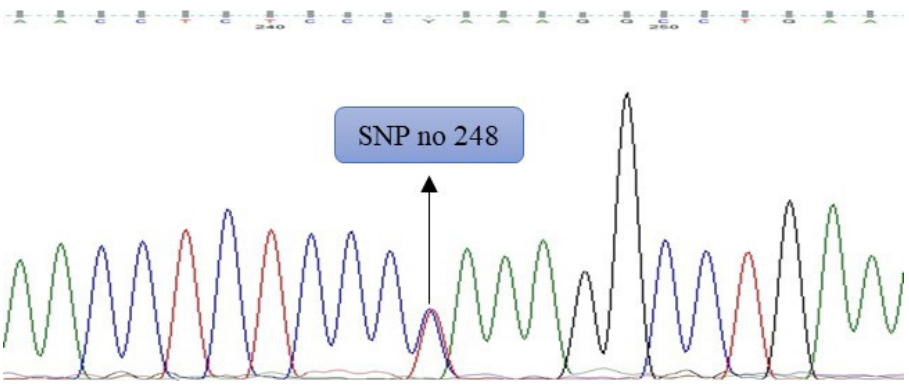

Figure 1. The P248P SNP in the BMP15 exon 2 region.

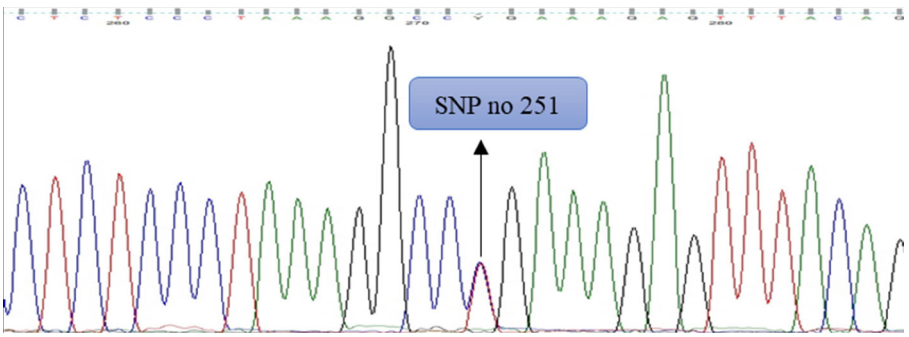

Figure 2. The L251P SNP in the BMP15 exon 2 region.

Table 2. Information on BMP15 exon region SNPs, observed heterozygosity (Ho), expected heterozygosity (He), p values from the Hardy-Weinberg (HW) equation, and minor allele frequencies (MAF).

Breeds	n	Genomic position	Codon	Amino acid exchange	Ho	He	HW p	MAF	Alleles
Çine Çaparı	19	X:56595196	ccT/ccC	P248P	0.00	0.00	1.00	0.00	T:T
		X:56595188	cTg/cCg	L251P	0.11	0.10	1.00	0.05	T:C
Gökçeada	17	X:56595196	ccT/ccC	P248P	0.00	0.00	1.00	0.00	T:T
		X:56595188	cTg/cCg	L251P	0.00	0.00	1.00	0.00	T:T
Awassi	26	X:56595196	ccT/ccC	P248P	0.00	0.00	1.00	0.00	T:T
		X:56595188	cTg/cCg	L251P	0.00	0.07	0.04	0.04	T:C
Karakacan	19	X:56595196	ccT/ccC	P248P	0.05	0.05	1.00	0.03	T:C
		X:56595188	cTg/cCg	L251P	0.32	0.27	1.00	0.16	T:C
Kıvırcık	19	X:56595196	ccT/ccC	P248P	0.11	0.10	1.00	0.05	T:C
		X:56595188	cTg/cCg	L251P	0.00	0.00	1.00	0.00	T:T
Karacabey Merino	20	X:56595196	ccT/ccC	P248P	0.10	0.10	1.00	0.05	T:C
		X:56595188	cTg/cCg	L251P	0.05	0.05	1.00	0.03	T:C
Chios	140	X:56595196	ccT/ccC	P248P	0.00	0.00	1.00	0.00	T:T
		X:56595188	cTg/cCg	L251P	0.06	0.08	0.37	0.04	T:C

*Genomic position information of SNPs is given according to Sheep_(Oar_rambouiller_v1.0). (https://www.ensembl.org/Ovis_aries_rambouillet/Transcript/Exons?db=core;g=ENSOARG00020012408;r=X:56594565-56601245;t=ENSOART00020018955)

SNP numbered 326, resulting from A→G transition, did not lead to an amino acid change, and glutamate (E) amino acid residue remained the same. As a result of the G→A transition at position 332, a missense mutation occurred, converting valine (V) to isoleucine (I). Heterozygosity frequencies of E326E and V332I were found to be exactly the same—0.08, 0.13, and 0.46 in Kıvırcık, Karacabey Merino, and Chios breeds, respectively. As a result of this study, it was determined that the heterozygous frequencies of E326E and V332I SNPs were found to be the same across the breeds. Consequently, it was concluded that these two SNPs were related to each other (Figure 3). Furthermore, it was observed that the frequencies of the SNPs were significantly higher in the Chios breed, known for its higher multiple birth rate compared to other breeds. As presented in Table 4, six different haplotypes were constructed in the GDF9 gene region. While five haplotypes were detected in the Kıvırcık and Karacabey Merino breeds, six haplotypes were determined in the Chios breed. Additionally, during the structuring of haplotypes in the Haploview program, the correlation coefficient, indicating the relationships between the obtained SNPs, was also calculated. In the case of total correlation, $r^2 = 1$, the full correlation was shown in black. For $0 < r^2 < 1$, it was shown in light grey, and for $r^2 = 0$, it was shown in white. Our study found that the E326E and V332I SNPs were fully correlated with each other, while the E241K SNP was not correlated with other SNPs (Figure 4).

4. Discussion

Studies have reported that genes such as BMP15 and GDF9 are associated with fecundity in some sheep breeds, where fertility is expressed as the number of lambs produced in a year, the number of lambing per year, and the number of offspring per litter. Reproductive traits typically exhibit low to moderate heritability. Therefore, traditional breeding methods relying on phenotypic data are time-consuming. Selection and breeding studies, utilizing molecular techniques and classical breeding approaches, play a crucial role in enhancing genetic improvements for reproductive efficiency [2]. Over one lambing per year has been reported in sheep breeds such as Fin, Romanov, and Dorset Horn. In the native sheep breeds of Türkiye, the Chios breed was recorded to have high fertility due to twinning [9]. Although it exhibits multiple birth characteristics, no association studies were found at the BMP15 and GDF9 gene levels in the Chios breed.

Bone morphogenetic protein 15 (BMP15) or FecX gene is considered one of the major genes on the X chromosome, consisting of two exons and one intron region. It is 5.6

Table 3. Genomic position information for SNPs in the GDF9 gene region, observed heterozygosity (Ho), expected heterozygosity (He), p values from the Hardy-Weinberg (HW) equation, and minor allele frequencies (MAF).

Breeds	n	Genomic position	Codon variant	Amino acid exchange	Ho	He	HW p	MAF	Alleles
Kıvırcık	48	5:46547268	cGc/cAc	R87H	0.396	0.405	0.67	0.28	G:A
		5:46545932	ctA/ctG	L159L	0.417	0.497	1.00	0.46	G:A
		5:46545688	Gaa/Aaa	E241K	0.292	0.432	0.53	0.32	G:A
		5:46545431	gaA/gaG	E326E	0.083	0.097	1.00	0.05	A:G
		5:46545414	Gtt/Att	V332I	0.083	0.097	1.00	0.05	G:A
Karacabey Merino	128	5:46547268	cGc/cAc	R87H	0.186	0.195	1.00	0.11	G:A
		5:46545932	ctA/ctG	L159L	0.453	0.500	1.00	0.49	A:G
		5:46545688	Gaa/Aaa	E241K	0.148	0.149	0.90	0.08	G:A
		5:46545431	gaA/gaG	E326E	0.125	0.127	1.00	0.07	A:G
		5:46545414	Gtt/Att	V332I	0.125	0.127	1.00	0.07	G:A
Chios	134	5:46547268	cGc/cAc	R87H	0.111	0.242	0.00	0.14	G:A
		5:46545932	ctA/ctG	L159L	0.485	0.492	0.85	0.47	G:A
		5:46545688	Gaa/Aaa	E241K	0.223	0.264	0.14	0.16	G:A
		5:46545431	gaA/gaG	E326E	0.455	0.422	0.51	0.30	A:G
		5:46545414	Gtt/Att	V332I	0.455	0.422	0.51	0.30	G:A

*The genomic position information of SNPs is given according to Sheep_(Oar_rambouillet_v1.0). (https://www.ensembl.org/Ovis_aries_rambouillet/Transcript/Exons?db=core;g=ENSOARG00020021050;r=5:46544645-46547585;t=ENSOART00020032535)

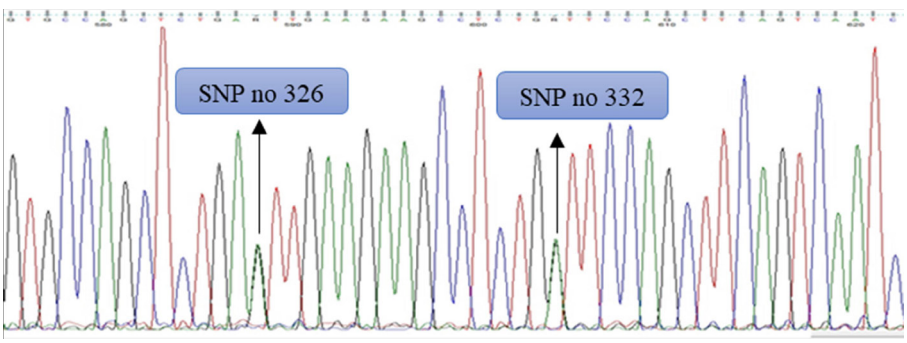

Figure 3. The E326E and V332I SNP in the GDF9 exon 2 region.

kb in size, encoding 393 amino acids, affecting fertility, and is expressed in oocytes. This gene region is also known as the most polymorphic locus among the genes affecting fertility in sheep [2,4]. In one of the previous studies conducted on Cele Black sheep, a three-bp (CTT) deletion was detected in the BMP15 gene exon 1 [10]. This three-base deletion, reported in the BMP15 gene exon 1 region, was also detected in Ramlıç and Dağlıç sheep from Türkiye, and this mutation was indicated to be associated with multiple births [11]. However, the three-base deletion

Table 4. The haplotypes detected in Türkiye native sheep breeds.

Breeds	GDF9 haplotype	Frequency
	R87H / L159L / E241K / E326E / V332I	
Kıvırcık	G/A/G/G/A	0.05
	A/A/A/A/G	0.28
	G/A/A/A/G	0.04
	G/A/G/A/G	0.09
	G/G/G/A/G	0.54
Karacabey Merino	G/A/G/G/A	0.07
	A/A/A/A/G	0.08
	G/G/G/A/G	0.49
	A/A/G/A/G	0.02
	G/A/G/A/G	0.35
Chios	G/A/G/G/A	0.29
	A/A/A/A/G	0.13
	G/A/A/A/G	0.03
	G/A/G/A/G	0.01
	A/G/G/A/G	0.01
	G/G/G/A/G	0.51

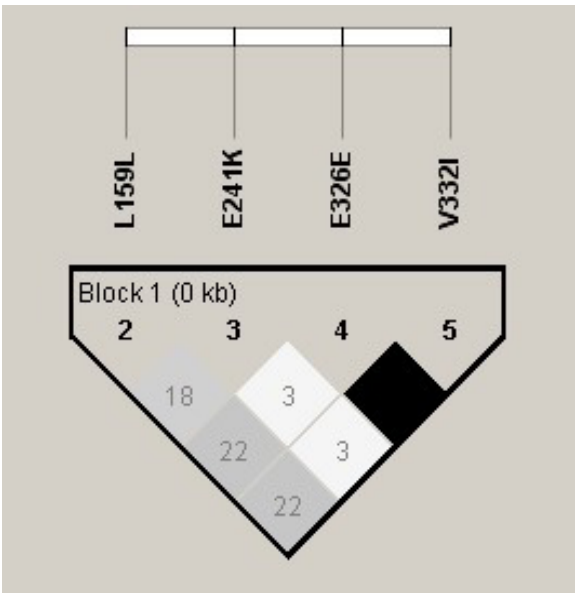

Figure 4. The results of the relationships between the obtained SNPs in the Haploview program. Black color indicates high r^2 value.

was not observed in all the studied breeds in our study. The L251P SNP in the BMP15 gene, located in exon 2 and previously detected by Di et al. [12] and Niu et al. [10], was also found in this study. These two studies reported that heterozygous sheep carrying this SNP have larger litter sizes compared to homozygous sheep, suggesting that this SNP may affect fertility in the Cele Black breed [12]. In our study, the heterozygosity rates of the L251P SNP were found to be 0.32, 0.11, 0.06, and 0.05 in Karakacan, Çine Çaparı, Chios, and Karacabey Merino breeds, respectively. The Karakacan breed showed the highest frequency of this SNP, while the Chios breed exhibited a relatively low frequency. Therefore, based on the data obtained in our study, we could not conclude that this SNP region was a fertility-related region in sheep breeds. In another study, a new SNP was reported as FecXG in the BMP15 gene in Chios, Gökçeada, Kıvırcık, and Awassi breeds. However, these SNPs were not found in this study [13]. In other studies conducted on native sheep breeds in Türkiye in the BMP15 gene region, the FecXI (Inverdale) mutation could not be detected in the Awassi breed [14], and the Pırlak sheep breed was found to be monomorphic in the same region [11]. Additionally, no polymorphism was observed in the FecB (Booroola), BMP15 (FecXI and FecXG), and FecXB in the Chios breed [15,16].

The growth differentiation factor 9 (GDF9) gene is a gene region consisting of one intron and two exons, with a size of 2.5 kb and the capacity to encode 453 amino acids. GDF9, reported to influence ovulation and birth rates in sheep, is located on the 5th chromosome [4]. Similar to BMP15, the autosomal GDF9 gene was also reported to have a significant effect on controlling follicular growth through its influence on granulosa cell function [3,5]. To date, 29 different SNPs have been reported in the GDF9 gene, and these SNPs were found to be associated with an increase in the ovulation rate in sheep [5]. In a study, it was reported that heterozygous sheep carrying the E241K SNP, found in Egyptian sheep breeds, exhibited an increase in lambing and twinning [17]. However, no association was found for the R87H, E241K, and V332I SNPs reported by Roy et al. [18]. The R87H SNP was found to be associated with abdominal size in a study by Hossain et al. [19], and in another study [20], the local sheep breed of Türkiye, the Of sheep, was found to be associated with multiple births. In another study, Daglic and Ramlic breeds (Daglic × Rambouillet crossbreed) from Türkiye were characterized in the GDF9 gene region [11]. Thirteen SNPs were detected in the GDF9 gene, including R87H, L159L, E241K, and V332I which were also identified in our study. Çelikeloğlu et al. [11] did not report any association with multiple births in their research. Another study reported that E326E and V332I SNPs, which were significantly related to litter size, had higher litter sizes in heterozygous sheep than in homozygous sheep at both loci [12]. In many studies, it was reported that the lamb yield and the multiple birth rate were found to be higher in the Chios breed than in other Türkiye native breeds [21]. In this study, similar to [12], the frequency of heterozygosity was higher in Chios sheep, known for their multiple births, compared to other breeds. Heterozygosity frequencies in SNP regions E326E and V332I were determined as 0.08, 0.13, and 0.46 in Kıvırcık, Karacabey Merino, and Chios breeds, respectively. The frequencies of E326E and V332I SNPs were found to be identical, with an r^2 value of 1 in the Haploview program. Therefore, it was concluded that the two SNPs were related to each other. In the previous studies, no correlation was reported in relation to the Chios breed, known for multiple births. In this study, it was determined for the first time that the frequencies of these two SNPs (E326E and V332I) were significantly higher in the Chios breed, which exhibits a higher rate of multiple births compared to other breeds.

As a result, the higher birth rate of the Chios breed may be in relation to the SNPs, E326E and V332I in the GDF9 exon 2 region. However, further research is needed in different flocks, considering both the number of ewes under rams and the number of lambs born. It is recommended to conduct association studies to identify SNPs in a larger number of animals and collect data on fertility, number of lambs born, and twinning rate.

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Conflict of interest
The authors declared that there is no conflict of interest.

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